MASTER

UR-3490-1776

Renal Effects of Inhaled Carbon Tetrachloride and
the Influence of Inhaled Isopropanol - A Comparison
with the Hepatic Effects

by

Ellen C. Henry

Submitted in Partial Fulfillment
of the
Requirements for the Degree

MASTER OF SCIENCE

Supervised by

Dr. Frank A. Smith

Department of Radiation Biology and Biophysics
University of Rochester
School of Medicine and Dentistry
Rochester, New York

1979

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED

DISCLAIMER

This report was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency Thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

DISCLAIMER

Portions of this document may be illegible in electronic image products. Images are produced from the best available original document.

MASTER

Renal Effects of Inhaled Carbon Tetrachloride and
the Influence of Inhaled Isopropanol - A Comparison
with the Hepatic Effects

by

Ellen C. Henry

Submitted in Partial Fulfillment
of the
Requirements for the Degree.

MASTER OF SCIENCE

Supervised by

Dr. Frank A. Smith

DISCLAIMER

This book was prepared as an account of work sponsored by an agency of the United States Government. Neither the United States Government nor any agency thereof, nor any of their employees, makes any warranty, express or implied, or assumes any legal liability or responsibility for the accuracy, completeness, or usefulness of any information, apparatus, product, or process disclosed, or represents that its use would not infringe privately owned rights. Reference herein to any specific commercial product, process, or service by trade name, trademark, manufacturer, or otherwise, does not necessarily constitute or imply its endorsement, recommendation, or favoring by the United States Government or any agency thereof. The views and opinions of authors expressed herein do not necessarily state or reflect those of the United States Government or any agency thereof.

Department of Radiation Biology and Biophysics
University of Rochester
School of Medicine and Dentistry
Rochester, New York

1979

DISTRIBUTION OF THIS DOCUMENT IS UNLIMITED *eb*

ACKNOWLEDGEMENTS

I especially wish to thank Dr. Frank A. Smith, not only for his willing guidance and advice which made my stay an enjoyable learning experience, but particularly for his sincere dedication to the whole educational process.

I would also like to express my appreciation to:

Dr. Richard Mavis for his helpful advice and for the use of laboratory space and equipment.

Dr. Raymond Baggs for his assistance in histological examination and interpretation.

Dr. Ronald Wood for the use of his IR Spectrophotometer - an indispensable instrument for this project.

Mr. Leonard Leach who always has a solution for inhalation chamber problems.

TABLE OF CONTENTS

	<u>Page</u>
Title Page	i
Acknowledgements	ii
Table of Contents	iii
List of Figures	iv
List of Tables	vi
INTRODUCTION	1
METHODS	6
RESULTS	17
DISCUSSION	47
SUMMARY AND CONCLUSION	77
BIBLIOGRAPHY	81

List of Figures

<u>Figure</u>	<u>Page</u>
1 Recordings of infrared analysis of chamber exhaust air during exposures	18
2 Malondialdehyde production and G-6-Pase activity in liver microsomes	19
3 Malondialdehyde production and G-6-Pase activity in kidney microsomes	21
4 Glucose-6-phosphatase activity in liver and kidney microsomes of rats exposed to CCl ₄	22
5 Photomicrograph of liver of rat exposed to CCl ₄ at 400 ppm	26
6 Photomicrograph of liver of rat exposed to CCl ₄ at 1200 ppm	27
7 Glucose-6-phosphatase activity in liver and kidney microsomes of rats after a 4-h exposure to 400 ppm CCl ₄	29
8 Photomicrograph of rat kidney 6h after a 4-h exposure to 400 ppm CCl ₄	34
9 Liver G-6-Pase activity in rats exposed to isopropanol and CCl ₄	35
10 Kidney G-6-Pase activity in rats exposed to isopropanol and CCl ₄	37

List of Figures (continued)

<u>Figure</u>		<u>Page</u>
11	Hepatic triglyceride concentrations in rats exposed to isopropanol and CCl_4	39
12	Photomicrograph of rat liver after exposure to isopropanol and CCl_4	41
13	Photomicrographs of mouse liver after exposure to CCl_4 at 400 ppm	45
14	Photomicrographs of mouse kidney after exposure to isopropanol and CCl_4	46

List of Tables

<u>Table</u>		<u>Page</u>
1	BUN and hepatic TGs in rats 12h after 4-h exposure to CCl ₄	24
2	TG concentrations in liver and kidney of rats exposed to CCl ₄ at 400 ppm for 4h	30
3	BUN concentrations in rats exposed to CCl ₄ at 400 ppm for 4h	32
4	BUN concentrations in rats exposed to isopropanol and CCl ₄	38
5	Response of mice to inhaled CCl ₄ and isopropanol	43

INTRODUCTION

The concept of interaction of toxic agents is a fundamental one with important implications in several circumstances. Occupational exposures for example seldom involve a single compound but more normally a mixture of substances, but TLV's are based on toxicity of and exposure to each compound alone. Similarly, domestic use of poisons is generally in uncontrolled, variable, and largely unpredictable environments which may alter the safety that is indicated on the containers. Synergistic and antagonistic effects are frequently encountered between drugs prescribed to be taken concurrently, thus altering their efficacy and/or toxic side effects. On the other hand, in studying the mechanism of action of a given compound, administration of a second toxic agent (to alter metabolic paths in a known fashion) may be a useful research tool, allowing comparison of patterns of toxicity following exposure to the compound alone with those seen in the combination exposure. Obviously every conceivable combination of toxic substances cannot be fully studied for possible interactions. Certain patterns tend to be predictable from a knowledge of the metabolism of the compounds in question, although others are quite unpredictable. In any case, the possibility, or rather probability, that the known toxicity of compound X will be altered in certain circumstances of concurrent exposure to other compounds should be considered in assessing the hazard from use of compound X.

This work involves one pair of potentially interacting agents, the common solvents, carbon tetrachloride and isopropyl alcohol. This combination has been encountered industrially and has also been studied as an adjunct of the extensive toxicological research on carbon tetrachloride. The study grew out of a preliminary observation that isopropanol appeared to enhance renal damage from CCl_4 in mice, in addition to enhancing liver damage, which is well-documented. The renal lesion from CCl_4 is poorly defined in the literature although its importance is apparent in clinical reports of CCl_4 poisonings. Indeed our isopropanol/ CCl_4 combination may have elicited a "new" lesion, since there was no evidence of renal damage from CCl_4 alone under the conditions used. Consequently, much of the present study turned out to be an attempt to characterize the renal response in comparison with that in liver following inhalation of CCl_4 alone. Such a base line must be established in order to examine the possibility of potentiation by isopropanol, and in order to compare it qualitatively and quantitatively with the liver lesion.

Historically, research interest in CCl_4 has centered on hepatotoxicity, which is characterized by fatty degeneration and centrilobular necrosis (as reviewed by Smuckler, 1976). Carbon tetrachloride was found to require activation to precipitate its hepatotoxicity. It was hypothesized that liver mixed function oxidases cleaved the carbon-chlorine bond yielding reactive $\text{CCl}_3\cdot$ free radicals. These were pictured as initiating

peroxidation of lipids and hence destruction of cellular membranes, accounting for the loss of activity of membrane-bound enzymes, the disruption of protein synthesis and ribosomal complexes, and the release of soluble enzymes into the blood from the damaged (necrosed) cells, as reviewed by Recknagel and Glende (1973).

Several mechanisms of CCl_4 interaction with kidney tissue can be postulated. CCl_4 reaching the kidney might react via the same mechanism as in liver, but cause generally less acute damage because less CCl_4 reaches kidney than liver, especially by the usual oral or i.p. exposures used in laboratory studies, and because kidney levels of cytochrome P-450, needed to activate CCl_4 , are much lower (Zenser et al. 1978; Villarruel et al. 1977). Conversely, kidney dysfunction or failure could be partially or completely an effect secondary to initial liver damage. On the other hand, CCl_4 might be acting directly in kidney, but via a completely different mechanism to that hypothesized for liver.

The lack of data in the literature on renal effects of CCl_4 (e.g. its site of action) made the choice of suitable parameters of kidney damage difficult. A brief survey of the literature on in vivo assessment of kidney damage led to the conclusion that no biochemical parameter was markedly and consistently more sensitive or specific than histological examination of tissue sections. It was decided to compare the effects in liver and kidney using glucose-6-phosphatase activity as a marker of endoplasmic reticulum

membrane integrity. Other studies have demonstrated the dependence of activity of this enzyme on normal membrane configuration and phospholipid content (Glende et al. 1976; Duttera et al. 1968) as well as the ability of CCl_4 to cause a loss of activity of this and other membrane-bound enzymes in the liver (e.g. Reynolds, 1963). Glucose-6-phosphatase is also present in the kidney at a comparable specific activity, and since it was found to lose activity when kidney microsomal lipid underwent peroxidation, it is here assumed to have the same requirements of membrane integrity. Thus, the use of this parameter is not to imply that this enzyme is the critical one in expression of a toxic effect: it is used merely as a marker and as a means of quantitatively comparing liver and kidney responses and as a test of the hypothesis that CCl_4 may also induce lipid peroxidation in kidney tissue. It is also a quantitative means of testing for the occurrence of potentiation of toxic effects by isopropanol.

In addition, blood urea nitrogen and protein excretion were measured, both of which are fairly non-specific but common indicators of renal functional deficit. Urinary glucose is a sensitive indicator of proximal tubular inability to reabsorb glucose; this was measured in some experiments. Histological assessment of liver and kidney was also included for comparison with biochemical quantitation.

The inhalation route of exposure was used in spite of the inherent difficulty of defining actual dose received by the

animals, because it seemed more appropriate in considering most human exposures than the usual i.p. injection or oral intubation. Also, the possibility implied by the clinical literature that renal failure may more frequently be the cause of or contribute to death from CCl_4 when exposure is by inhalation than oral made inhalation the route of choice for this study.

METHODS

Animals

Male Sprague Dawley rats (Blue Spruce Farm, Altamont, N.Y. 12209) of body weight 250-350 g were used after allowing at least one week for acclimation after their arrival from the breeder. Food and water were available ad libitum at all times except during the exposures. A few experiments were repeated using male ICR mice (Blue Spruce) of 25-35 g.

In some experiments, urine was collected in order to determine urinary protein and glucose excretion. In these cases, rats were housed individually in stainless steel metabolism cages (Lab Products Inc., Rochelle Park, N.J. 07662). Water consumption was recorded. Urine was collected (under mineral oil, with a crystal of thymol) before and after exposure so that each animal acted as its own control. Volume was recorded (calculated from weight change of cup and urine specific gravity) and samples saved and refrigerated or frozen in plastic tubes for protein and glucose determination.

Exposure Technique

Animals were exposed to the solvent vapors in a 28-litre glass battery jar fitted with a wooden front and glass door (Leach, 1963). Air was bubbled at a measured flow rate (Dwyer Rotameter; Dwyer Instruments Inc., Michigan City, Ind.) through the solvent in

a fritted glass bubbler to generate solvent vapor. Before entering the chamber, the resultant vapor-laden air was diluted with room air metered through another rotameter. Flow rates of diluting air, depending on the desired concentration, were generally about 20-23 l/min for CCl_4 exposures and 13-14 l/min for isopropanol exposures; bubbling air was 0.3-0.5 l/min.

During exposures, solvent concentration in the chamber was monitored continuously with a Miran I Infrared Analyzer (Wilks Scientific) equipped with a strip chart recorder. Cell path length was 1 m; slit width was 1 mm for isopropanol, 2 mm for CCl_4 .

From the outlet port at the base of the wooden front, chamber air was exhausted through tygon tubing (with filter inserted to trap any dust, fur, or other airborne material) into the cell of the IR Spectrophotometer. Before animals were put into the chamber, it was allowed to equilibrate and the concentration adjusted to the required level by altering air flow rates.

Solvent concentration is recorded as absorbance or percent transmittance at a given wavelength. To obtain the equivalent airborne concentrations (ppm), the machine is calibrated using a kit consisting of a bellows pump and teflon connecting tubes which attach to the cell to make a closed system of known volume into which known volumes of solvent can be injected. As successive aliquots are injected and circulated through the cell by the pump, the recorded transmittance represents cumulatively higher concentrations. The appropriate aliquot size (A) for each compound

is calculated using the formula:

$$A = \text{mol. wt.} \times \text{vol. of calibration chamber} \times \text{step size (ppm)} \times \\ 0.0000409 / \text{density}$$

The constant 0.0000409 takes account of conversion factors and gas law constants; the volume of the calibration chamber on this instrument was 0.39 litres.

The wavelength for analysis should ideally be chosen to be specific for the compound to avoid absorbance by interfering compounds, and to be narrow so that the same wavelength can be isolated with certainty each time. In this study, the wavelengths chosen from the respective IR profiles were 12.6 μm for CCl_4 and 3.36 μm for isopropanol, which are distinct from peaks for water, CO_2 and NH_3 .

Sacrifice Procedure

At sacrifice, animals were overdosed with sodium pentobarbital. Blood was withdrawn from the heart with a heparinized syringe, the cells spun down, and plasmas stored frozen in plastic capped tubes. Approximately 3.5-4 g of liver (from the same lobes in each animal) were quickly removed, weighed, and immediately immersed in ice cold sucrose buffer (0.25 M sucrose, 0.002 M Na_2EDTA) for the preparation of microsomes. Another 0.50 g sample of liver was removed, chopped into approximately 2-mm chunks, and placed into a test tube with 10 ml of 2:1 chloroform:methanol for extraction of triglycerides. These tubes were stoppered, shaken, and refrigerated until analysis.

Similarly, kidneys were quickly removed and a 0.50 g sample reserved as described above if required for triglyceride analysis; the remainder was weighed, and immersed in the ice cold sucrose buffer.

In the later experiments using mice, procedures were essentially the same except that tissues from 2-3 animals were pooled and homogenized in KCl/Tris buffer (0.15 M KCl, 5 mM Tris maleate, 1 mM Na₂EDTA). Plasmas from 2 mice were pooled for the blood urea nitrogen determinations.

Histological Procedures

Slices (2-3 mm thick) from both tissues were fixed in 10 percent neutral buffered formalin. Since exposures were by inhalation, the lungs from 2 animals in each group of 5 rats or 10 mice were, in most experiments, removed and fixed inflated with formalin. A cross-section was later sliced from one of the large lobes for mounting and staining.

All tissue sections were stained with hematoxylin and eosin and examined by light microscopy. Some liver sections were also stained with Oil Red O for verification of the presence of excess fat. Severity of any pathological lesions was ranked qualitatively on an arbitrary scale of 0 to ++++.

Preparation of Liver and Kidney Microsomes

The liver and kidney samples in buffer were minced with scissors and rinsed with buffer to remove most of the blood. Each was homogenized in glass with about 5 passes of the teflon pestle, and the homogenate transferred to Nalgene centrifuge tubes. These were spun at 16,000 g (12000 rpm) for 20 minutes (0°C) to remove cell debris, nuclei, mitochondria, etc. The supernatant was decanted into capped polycarbonate tubes and spun for 60 minutes at 100,000 g (33,000 rpm, Beckmann Ultracentrifuge L3-50; 0°C). The supernatant from these tubes was discarded and the pellet was resuspended in buffer (with 5-6 passes of a teflon pestle in a glass homogenizer). Resuspended pellets were diluted with buffer to yield protein concentrations of 2-5 mg/ml. These preparations are assumed to contain smooth and rough endoplasmic reticulum membranes restructured into microsomes by the homogenization procedure, though some mitochondria and some plasma membrane fragments are probably also present; no analysis of the purity of this preparation was attempted. The microsomal glucose-6-phosphatase activity¹ was assayed on the day of sacrifice, or the microsomal suspension was stored overnight in liquid nitrogen for assay the next day. Storage in this manner was found to cause no loss of activity.

1. Referred to hereafter as G-6-Pase activity.

Analytical Procedures

(a) G-6-Pase activity

The activity of this enzyme was assayed by slight modification of the method of Recknagel and Lombardi (1961). The final incubation tubes contained: 30 μ moles Tris maleate buffer at pH 6.6, 60 μ moles G-6-P, and about 0.5 - 1.0 mg microsomal protein in a total volume of 1.5 ml. Blanks, containing the same constituents, were left on ice. After a 20-minute incubation at 30°C, the reaction was stopped by putting the tubes on ice; 12 drops of concentrated sulfuric acid, then 12 drops of 70 percent perchloric acid were added to precipitate protein. After pelleting of precipitated protein, the supernatant was analyzed for phosphate by the method of Fiske and Subbarow (Bray, 1968) in which phosphomolybdate is reduced by 1-amino-2-naphthol-4-sulfonic acid to a blue chromogen (read at 660 nm).

Enzyme activity is expressed as μ moles of PO_4 released per mg microsomal protein per hour. The quantity of G-6-P used was determined to be a saturating concentration and the rate of phosphate production was linear during the incubation, and proportional to the amount of protein added.

(b) Blood urea nitrogen²

BUN was determined according to the method of Chaney and Marbach (1962). Diluted plasma is incubated with urease to release

2. Referred to hereafter as BUN

ammonia which reacts with the color reagents to give a blue chromogen, indophenol, absorbing at 625 nm. An enzyme blank is included for each plasma sample so that absorbance due to free ammonia can be subtracted from the total absorbance obtained in the presence of urease. The difference represents that ammonia attributable to hydrolysis of urea present in the blood. Since urea is freely diffusable, the urea concentration in plasma is assumed equal to that in whole blood.

(c) Triglycerides

The method of Biggs et al. (1975) was adapted to analyze concentrations of triglycerides in chloroform:methanol extracts of liver and kidney tissues. Aliquots of the 10 ml of extract were evaporated to dryness at 50-60°C by bubbling nitrogen through the solution. Since standards (triolein, Oxford Lipid Standards, Oxford Laboratories, Foster City, CA 94404) were made up in isopropanol, 0.5 ml of isopropanol was added to each evaporated sample before addition of the remaining reagents. The triglycerides were then extracted into heptane and reacted with NaOCH_3 at 60°C to release glycerol which is oxidized to release formaldehyde. The formaldehyde reacts at 60°C with acetyl acetone to yield the yellow chromogen 3,5-diacetyl-1,4-dihydrolutidine, absorbing at 410 nm.

(d) Protein

Microsomal and urinary protein concentrations were determined by a modification of the Lowry et al. (1951) method (Schlatz, 1971) using bovine serum albumin as a standard.

(e) Glucose

The enzymatic assay for glucose (Lowry and Passoneau, 1972) was used for urinary glucose. Hexokinase is used to produce glucose-6-phosphate, which is oxidized by G-6-P dehydrogenase. The latter reaction requires reduction of NADP to NADPH which is measured spectrophotometrically (340 nm).

In vitro experiments

The absolute dependence of loss of liver G-6-Pase on lipid peroxidation or loss of lipid components by other means has been well-documented (e.g. Glende et al. 1976; Duttera et al. 1968), but this correlation has not been verified in other tissues. Therefore, the validity of using loss of G-6-Pase activity as an indicator of membrane disruption (by lipid peroxidation) in kidney as well as in liver was tested in vitro.

Microsomes were prepared from both kidney and liver tissue of rats. In ice cold buffer (0.15 M KCl, 5 mM Tris maleate, 1 mM Na₂EDTA at pH 7.4) tissues were minced, rinsed and homogenized as described above. In order to obtain a cleaner microsomal suspension (lesser mitochondrial contamination), centrifugations

were performed as follows: 15 min. at 300 g, 10 min. at 1600 g, 10 min. at 30,000 g and 60 min. at 100,000 g. The pellets from the final spin were washed several times with KCl-Tris buffer without EDTA, then stirred up, resuspended in the EDTA-free buffer, and repelleted at 100,000 g for 60 min. The pellets were resuspended in EDTA-free buffer, the protein contents determined, and the suspensions stored in liquid nitrogen.

These microsomes were incubated (37°C) under conditions that would either allow or inhibit lipid peroxidation. After various intervals of incubation, the extent of lipid peroxidation and the activity of G-6-Pase were simultaneously determined. In a final volume of 1 ml, 0.6 - 0.8 mg protein was incubated in 40 mM Tris maleate (pH 6.6), 1 mM ascorbate, 5 μM FeSO_4 , and with or without 1 mM EDTA to prevent peroxidation. Aliquots of 100 μl were removed and added to tubes containing TCA and thiobarbituric acid to measure malondialdehyde (MDA) as described by Kornbrust and Mavis (1979). Glucose-6-phosphatase was assayed in the remaining suspension as described above. Because ascorbate also reduces the phosphomolybdate complex, its presence in the assay medium interfered with the Fiske-Subbarow method of phosphate determination which was used in the other experiments. Therefore the method of Chen et al. (1956) was adapted in which an excess of ascorbate is added with ammonium molybdate, and the blue chromogen is measured at 820 nm.

Protocols

All in vivo experiments involved single acute exposures of 4 hours duration. Control animals were exposed in the same manner in each case but with only air flowing through the chamber at the same flow rate as for treatment groups.

In order to establish a "baseline" of response, rats were exposed in groups of 5 to CCl_4 alone at concentrations of 10, 50, 100, 400, 800, and 1200 ppm. Liver and kidney G-6-Pase, liver triglycerides, and BUN were assayed, and liver, kidney, and lung histology examined. Sacrifice was at 12 h postexposure since liver G-6-Pase has been reported to be about maximally depressed by 12 h following oral or i.p. dosing (Klaassen and Plaa, 1969). At the higher concentrations, urinary protein and glucose were also measured.

Initially, a clear response was observed at 400 ppm, so this concentration was fixed for subsequent experiments. To define the time course of the observed effects and their repair, groups of 5 rats were sacrificed at 6, 12, 18, 24, or 48 hours or 6 days postexposure to 400 ppm CCl_4 . The same parameters as above were examined.

Protocols for interaction experiments were based on literature reports of potentiation of intraperitoneally (i.p.) administered CCl_4 by oral doses of isopropanol, in which maximal enhancement of hepatotoxicity as defined by several parameters was observed when the isopropanol dose preceded the CCl_4 by 18-24 hours (Cornish

and Adefuin, 1967; Traiger and Plaa, 1971). Therefore, exposure regimes for treatment (i) and control (ii and iii) groups were as follows:

EXPOSURE - INTERVAL - EXPOSURE - INTERVAL

- i) i-PrOH 4h - 18h - CCl₄ 4h - 6, 12, or 24h - Sacrifice
- ii) i-PrOH 4h - 18h - air 4h - 6, 12, or 24h - Sacrifice
- iii) air 4h - 18h - air 4h - 12h - Sacrifice

A few of these protocols were repeated using groups of 10 mice, in an attempt to reproduce the response seen in the pilot study in terms of triglyceride accumulation, elevated BUN, and histological changes. Solvent concentrations were chosen to enable direct interspecies comparison (400 ppm CCl₄, 1000 ppm isopropanol). Sacrifice was at 12 or 24 h following exposure.

RESULTS

I. Chamber Concentrations

Continuous monitoring of exhausted chamber air by IR spectrophotometry enabled exposure concentrations from day to day to be maintained constant, thus eliminating one source of variability in response among treated groups of animals. Any concentration changes during an exposure could immediately be corrected by altering air flow rates to regain the desired solvent concentration. Generally this was not necessary: solvent concentration in the chamber remained steady as indicated by transmittance holding within a range of 1-2 percent. Typical records from 4-hour exposures of rats to isopropanol and to CCl_4 are shown in Figure 1.

II. In vitro tests to validate changes in renal G-6-Pase activity as indicator of renal lipoperoxidation.

Figure 2 illustrates the progressive loss of G-6-Pase activity as rat liver microsomal lipid undergoes peroxidation. Enzyme activity is down to 10 percent of control by 8 minutes, at which time malondialdehyde production is only 60-70 percent of maximal. EDTA in the incubation medium chelates the Fe^{++} and essentially completely prevents MDA production, i.e., lipid peroxidation; accordingly, G-6-Pase activity is maintained. These results support those reported by Glende et al. (1976).

Figure 1

Recordings of infrared analysis of chamber exhaust air during exposures.

Figure 2

Malondialdehyde production and glucose-6-phosphatase activity in peroxidizing and non-peroxidizing rat liver microsomes.

Microsomes were incubated (37°C) in the presence of FeSO_4 and ascorbate with ($\circ$) and without ($\bullet$) EDTA.

A comparable correlation was observed in rat kidney microsomes, as illustrated in Figure 3. As seen in liver, G-6-Pase activity is down to about 10 percent within 8 minutes, when peroxidation is not yet maximal. EDTA completely prevents both MDA production (lipid peroxidation) and loss of G-6-Pase activity. Thus, it appears that the loss of renal and hepatic G-6-Pase activities are comparable parameters that can be used as indicators of peroxidative destruction of kidney and liver cellular membranes.

III. Baseline response in rats to carbon tetrachloride alone

In the first series of exposures, CCl_4 was used alone at concentrations of 0 to 1200 ppm (0 - 7560 mg/m^3). Figure 4 shows liver and kidney microsomal G-6-Pase specific activity. Animal to animal variability was high so no significant loss of activity in liver was observed until 400 ppm (2520 mg/m^3), and a further loss was seen at each successive higher concentration. In kidney, 400 ppm resulted in significant depression of activity in one experiment (hence the choice of 400 ppm for further exposures), although this was not the case when this exposure was repeated in another series of experiments. There appeared to be a minor loss of activity at 400 and 800 ppm which became statistically significant at 1200 ppm. However, the lack of clear dose-dependency in this response and also the variability of control activity between experiments preclude a conclusion of biological significance except perhaps at the highest dose. Even

Figure 3

Malondialdehyde production and glucose-6-phosphatase activity in peroxidizing and non-peroxidizing rat kidney microsomes.

Incubation conditions as in Fig. 2

Figure 4

Glucose-6-phosphatase activity in liver and kidney microsomes of rats exposed to CCl_4 . Activity was measured 12 hours after a 4-h exposure to the indicated concentrations of CCl_4 . Bars and lines represent the mean $\pm$ SEM for groups of 5 animals.

* Significantly different from controls at $p < 0.05$

at this level, the loss of activity in kidney tissue was less than 20 percent of control compared to a loss of 55-60 percent of control activity in liver at this concentration.

Table 1 lists the corresponding liver triglyceride and blood urea nitrogen values. Liver triglyceride concentration increased in a dose-dependent fashion and was statistically significantly higher than controls at 100 ppm and higher. The reason for the non-significance at 1200 ppm is uncertain. It may be attributable to the presence of extensive inflammatory cell infiltration at this concentration; these cells would contribute to the tissue weight but would be lower in lipid, tending to dilute any fatty infiltration. Or perhaps the extensive necrotic tissue had lost some of its accumulated lipid. It was realized, in light of literature reports, that triglyceride accumulation may not be maximally developed by 12 hours postexposure depending on actual dose of CCl_4 . However, it is known to begin soon after exposure, and so there should be dose-dependency even at this time point (as was observed). If a later sacrifice had been chosen, liver triglyceride concentrations might well have been significantly elevated at a lower concentration of carbon tetrachloride.

Control values of BUN were extremely variable so no significant changes were seen (Table 1). Renal functional deficit as measured by elevated BUN may take longer to develop than the 12-hour postexposure period adopted. However, even a trend towards BUN elevation is not evident.

Table 1 Blood urea nitrogen and hepatic triglyceride concentrations in rats 12 h after a 4-h exposure to CCl₄

CCl ₄ ppm	BUN mg% ±SEM		LIVER TRIGLYCERIDES mg/g ±SEM	
0	16.8 ± 1.1	(10)	12.4 ± 1.0	(10)
10	18.0 ± 0.58	(5)	12.8 ± 0.76	(5)
50	17.8 ± 0.54	(5)	12.4 ± 0.54	(5)
100	15.6 ± 0.49	(5)	16.1 ± 1.3	(5)*
400	15.7 ± 1.2	(10)	18.1 ± 1.2	(10)*
800	15.3 ± 0.45	(10)	22.1 ± 2.2	(5)*
1200	15.2 ± 0.48	(5)	14.5 ± 1.2	(5)

* Significantly different from controls at $p < 0.05$

Numbers in parentheses are numbers of animals.

At 1200 ppm, the 12-h postexposure urine collection may have shown an approximate doubling of glucose concentrations (and increased total glucose excretion) compared to a 12-h preexposure collection during the same time of day for the same animals (data not shown). No increased protein excretion or changes in urine volume were found at any concentration of CCl_4 .

The appearance of liver sections from rats exposed to 10 and 50 ppm CCl_4 was no different from sections from control animals, upon microscopic examination. At 100 ppm, there were occasional cells showing hydropic change and pyknotic nuclei, but no lipid accumulation was visible. Distinct fat accumulation was evident in liver sections stained with Oil Red O in rats exposed to 400 ppm or above. The lipid droplets were generally larger and the red stain more widespread at higher doses, though the histological response was as variable as the biochemical parameters. Balloon or foam cells with pyknotic nuclei in the centrilobular zone were numerous at and above 400 ppm, their extent generally dose-dependent (Figure 5). Although there were small centers of inflammation in livers of all dose groups, extensive inflammatory infiltration at the higher dose levels was clearly in response to cell damage and, especially at 1200 ppm, partially obliterated tissue structure (Figure 6). Kidney sections, on the other hand, showed no distinct lesion. Nonspecific effects such as tubule cell vacuolation (minor hydropic change) appeared not be dose-related, and are difficult to distinguish from artifacts of fixing and staining.

Figure 5

Photomicrograph of liver of rat exposed to CCl_4 at 400 ppm, showing the centrilobular distribution of pyknosis and hydropic change in the hepatocytes (C). Portal areas (P) are normal. Sacrifice was at 12 h after a 4-h exposure. H and E. x 48.

Figure 6

Photomicrograph of liver of rat exposed to CCl_4 at 1200 ppm.

Sacrifice was at 12 h after a 4-h exposure. Centrilobular necrosis affects a larger area, and there is extensive inflammatory response. H and E. x 48.

IV. Time course of response to CCl₄ alone

In order to follow the course of damage and repair through time, groups of 5 rats were sacrificed at various intervals from 6 hours to 6 days after a 4-h exposure to CCl₄ at 400 ppm. Figure 7 shows the G-6-Pase activity observed at each time. Liver enzyme activity was down to 45 percent of control by 6 hours, and at later times appeared to be recovering. By 24 hours it had returned to control values. In kidney microsomes on the other hand, there was a statistically significant loss of activity at 6 hours postexposure, but by 12 hours, it had returned to control levels. As mentioned above, at the 12-hour time point in this experiment, unlike that in Part III, kidney microsomal G-6-Pase activity was equal to control.

Triglyceride levels listed in Table 2 show that fatty accumulation in the liver was suggested even by 6 hours, but was not statistically significant until 12 hours, since this parameter was so variable among individuals. Peak accumulation appeared to be at 18 hours. In this experiment, renal triglyceride levels were also measured, partially as another parameter of comparison with the hepatic lesion, and also because lipid accumulation in kidneys of cats and mice following CCl₄ exposure had been reported previously (Wong and DiStefano, 1966; Estler et al. 1973) although at very much higher CCl₄ concentrations. Measured triglyceride concentrations in the kidney were much lower than in liver, and generally showed even more variability. No statistically significant changes were found here.

Figure 7

Glucose-6-phosphatase activity in liver and kidney microsomes of rats after a 4-hour exposure to 400 ppm CCl_4 . Each point represents the mean $\pm$ SEM of a group of 5 animals.

* Significantly different from controls at $p < 0.05$

Table 2 Triglyceride concentrations in liver and kidney of rats exposed to CCl₄ at 400 ppm for 4 hours.

	mg/g tissue ±SEM (n = 5)	
Time post-exposure	Liver	Kidney
6 h	16.4 ± 1.2	3.9 ± 0.69
12	17.8 ± 0.79*	3.8 ± 0.12
18	21.8 ± 2.3*	3.9 ± 0.14
24	17.1 ± 0.98	5.3 ± 0.74
48	16.5 ± 2.2	4.4 ± 0.46
6 d	11.7 ± 2.1	
Control	12.8 ± 2.1	5.8 ± 0.93

* Significantly different from controls at $p < 0.05$

Blood urea concentrations are shown in Table 3. In this experiment, control BUN was much lower than had been seen earlier, though the standard error was large. A slight elevation of BUN was observed at less than 24 hours postexposure, and appeared to correlate with the transient depression of G-6-Pase. Although this was statistically significant at 6 and 12 hours, such a small increase is not unequivocal evidence of renal dysfunction which had repaired itself by 18-24 hours.

The histological assessment of liver sections stained with H and E and Oil Red O generally paralleled these biochemical trends through time. Some pyknosis and balloon cells were apparent in centrilobular zones even by 6 hours, but were maximal at 12 hours. Thus, extensive peroxidation is detectable before maximal development of histological damage (necrosis). By 48 hours (and 6 days) there was no visible difference from controls. Oil Red O-stainable lipid was present in very fine droplets (mainly in central zones) at 6 h; at later times after exposure, the droplets were larger and more widespread throughout the section. The fatty infiltration peaked at 24 hours, at which time some droplets visible in H and E-stained sections may have been lipid. Inflammatory response to the centrilobular damage was most apparent at 12, 18 and 24 hours; 6 hours was insufficient for extensive damage and consequent immigration of inflammatory cells, while by 48 h recovery was nearly complete.

Table 3 Blood urea nitrogen concentrations in rats exposed to CCl_4 at 400 ppm for 4 hours.

Time post-exposure	mg/100 ml plasma	SEM
6 h	$20.2 \pm 0.67^*$	
12	$17.0 \pm 0.71^*$	(4)
18	16.1 ± 0.60	
24	13.8 ± 0.22	
48	12.9 ± 0.51	
6 d	13.2 ± 0.67	
Control	13.9 ± 1.2	

* Significantly different from controls at $p < 0.05$.

There was no very distinctive histologic response in kidney, although at 6h postexposure there was some tubular dilation particularly in the cortex, and apical vacuolation. Some eosinophilic fibrillar material was also present in occasional tubules of the papilla (Figure 8). Slight vacuolation of cortical tubule cells may have persisted at least until 48 h, although as remarked earlier these effects are difficult to distinguish from artifacts of fixing and staining.

V. Interaction Experiments

For these experiments, in which animals were preexposed to isopropanol vapor, a CCl_4 concentration of 400 ppm was chosen. This was a level at which a definite response was seen, at least in liver, but further G-6-Pase loss would still be possible if indeed potentiation did occur. Isopropanol concentrations of 1000 or 1500 ppm were used since this was the concentration range used in our preliminary tests with mice in which potentiation had been observed.

The glucose-6-phosphatase activities measured in rat liver microsomes are shown in Fig. 9. Control activity was comparable to previous controls; isopropanol/air controls may even have shown enhanced activity (at the 6 h sacrifice time and at 1500 ppm at 24 h). When animals were exposed to both solvents, hepatic G-6-Pase activity at 6 hours was significantly lower than controls exposed to CCl_4 alone but not appreciably different from that

Figure 8

Photomicrograph of rat kidney 6 h after a 4-h exposure to CCl_4 at 400 ppm. Fibrillar material is visible (arrows) in the lumen of some cortical tubules (also in papilla). H and E. x 240.

Figure 9

Liver glucose-6-phosphatase activity in rats exposed to isopropanol and CCl_4 .

iP - isopropanol

CCl_4 - 400 ppm

iP + CCl_4 - isopropanol 4h - 18h - CCl_4 4h

Lines and bars represent mean $\pm$ SEM for groups of 5 rats.

* Significantly different at $p < 0.05$ compared to air-exposed controls.

** Significantly different at $p < 0.05$ compared to appropriate CCl_4 -exposed group.

seen with CCl_4 alone at 12 hours. Whereas at 24 hours following CCl_4 alone, G-6-Pase activity seemed to have recovered to control levels (Figure 7), the livers of animals preexposed to either level of isopropanol before CCl_4 still showed depressed activity at 24 hours.

No loss of renal glucose-6-phosphatase activity was observed in any of these combination exposures (Figure 10).

No changes in BUN were significant (Table 4), but there was some accumulation of liver triglycerides in rats (Figure 11). Relative to air-exposed controls and isopropanol/air controls, the combination exposure groups had significantly elevated triglycerides except at the 12 h sacrifice time. At 1500 ppm, isopropanol preexposure significantly potentiated lipid accumulation measured 24 hours after CCl_4 exposure. Compared to exposure to CCl_4 alone, hepatic triglyceride concentration was increased about 40 percent by isopropanol pretreatment. At 1000 ppm however, isopropanol preexposure did not enhance triglyceride accumulation at 6, 12, or 24 h compared to the CCl_4 exposure alone. The reason for a lower triglyceride concentration 12 h after iPrOH/CCl_4 than after CCl_4 alone is uncertain. The more severely necrotic hepatocytes may have lost some of their accumulated lipid, or since the tissue sections taken for histology appeared hemorrhagic, blood remaining in the tissue may have contributed to the weight but not the lipid content measured.

Figure 10

Kidney glucose-6-phosphatase activity in rats exposed to isopropanol and CCl_4 .

iP - isopropanol

CCl_4 - 400 ppm

iP + CCl_4 - isopropanol 4h - 18h - CCl_4 4 h

Lines and bars represent mean $\pm$ SEM for groups of 5 rats.

* Significant at $p < 0.05$ compared to air-exposed controls.

Table 4 Blood urea nitrogen concentrations in rats exposed to isopropanol and CCl₄.

Exposure	Time of Sacrifice	mg% $\pm$ SEM (n=5)
iPrOH 1000 ppm	6h	9.6 $\pm$ 0.34
CCl ₄ 400 ppm	"	14.9 $\pm$ 0.98
iPrOH/CCl ₄	"	14.5 $\pm$ 0.73
iPrOH 1000 ppm	12h	16.2 $\pm$ 0.55
CCl ₄ 400 ppm	"	16.1 $\pm$ 0.91
iPrOH/CCl ₄	"	15.6 $\pm$ 0.35
CCl ₄ 400 ppm	24h	13.8 $\pm$ 0.22
iPrOH(1000 ppm)/CCl ₄	"	13.9 $\pm$ 0.48
iPrOH 1500 ppm	24h	13.4 $\pm$ 1.0
CCl ₄ 400 ppm	"	13.8 $\pm$ 0.22
iPrOH/CCl ₄	"	13.9 $\pm$ 0.90

Figure 11

Hepatic triglyceride concentrations in rats exposed to isopropanol and CCl_4 .

iP - isopropanol

CCl_4 - 400 ppm

iP + CCl_4 - isopropanol 4h - 18h - CCl_4 4h

Lines and bars represent mean $\pm$ SEM for groups of 5 rats.

* Significant at $p < 0.05$ compared to air-exposed controls

** Significant at $p < 0.05$ compared to appropriate CCl_4 -exposed group.

Histological examination of the livers from these experiments, unlike the biochemical parameters, suggested marked potentiation of response to CCl_4 in rats following isopropanol preexposure. Inhalation of isopropanol alone caused no necrosis or fatty change; there may have been a marginal increase in Oil Red O-stainable lipid but the droplets were very fine and dispersed throughout the section. The histological response to carbon tetrachloride alone at 400 ppm was quite variable between experiments even though exposure conditions were identical. But even compared to the "worst-case" CCl_4 controls, the appearance of liver sections following isopropanol and CCl_4 suggested much more severe damage (Figure 12). The extent of the affected areas around central veins was much larger (covering greater than 50 percent of section area) although the area did not vary markedly with time of sacrifice. Balloon or foam cells, with pyknotic nuclei, were more numerous, and ringed a larger area of cells of increased eosinophilia and in which large droplets of lipid were visible even with H and E staining. In addition, even by the 6-h sacrifice (also at 12 and 24 h), there was some inflammatory response in the affected areas. In general, the liver sections from the isopropanol preexposed animals which had then inhaled CCl_4 at 400 ppm resembled those from exposures to much greater concentrations of CCl_4 alone (e.g. 1200 ppm). Renal histology was unaffected by isopropanol preexposure.

Figure 12

Photomicrograph of rat liver after exposure to isopropanol (1000 ppm, 4h) 18 h before CCl_4 (400 ppm, 4h) showing more extensive centrilobular necrosis (cf. Fig. 5). Portal triads (P) are normal. Sacrifice was at 12 h postexposure. H and E. x 48.

VI. Comparison with mice

The results from the few experiments using mice suggest that this species is more sensitive than is the rat to CCl_4 inhaled at the same concentration. The G-6-Pase activities (Table 5) indicate that maximal loss of liver activity in mice, as in rats, inhaling 400 ppm CCl_4 alone occurred before 24 h; however, in mice it had not recovered completely within 24 h (cf. Figure 7). Preexposure to 1000 ppm isopropanol appeared to have no enhancing effect on liver G-6-Pase depression. G-6-Pase activity in the mouse kidney (Table 5) was extremely variable (large standard errors). Nevertheless, at 12 h postexposure, activity appeared to be reduced; this loss was not enhanced by isopropanol. No other changes were statistically significant.

BUNs (Table 5) were also indicative of greater sensitivity in the mouse. At 12 h postexposure to CCl_4 alone and to isopropanol/ CCl_4 , BUN was actually significantly lower than control, probably reflecting reduced urea synthesis by the liver without significant renal dysfunction. By 24 h, significant BUN elevation was observed, presumably as some renal functional impairment developed. Because the response was so variable among individuals, the suggested enhancement by isopropanol was not statistically significant.

Liver triglyceride accumulation (Table 5) was also more severe in mice than in rats. TG concentration was significantly elevated (by a factor of 2 - 3) by 12 h after CCl_4 alone, and by

Table 5 Response of mice to inhaled CCl₄ and isopropanol.

Exposure	Time of Sacrifice	G-6-Pase Activity Liver μmoles P/mg-h	Kidney	BUN mg%	LIVER TG mg/g
Controls-air	24h	17.4 ± .72 (7)	17.7 ± 1.8 (5)	16.9 ± .51 (8)	11.8 ± .76 (7)
10CC ppm iPrOH/air	24h	14.1 ± .31 (4)*	15.6 ± 1.1 (4)	18.2 ± 1.6 (4)	8.7 ± .41 (5)*
4CC ppm CCl ₄	12h	7.8 ± .63 (4)*	12.1 ± 1.0 (4)*	12.7 ± .96 (5)*	33.8 ± 6.0 (5)* 28.1 ± 2.3 (4) ^a
"	24h	11.7 ± .73 (4)*	21.2 ± .50 (4)	22.8 ± 1.4 (5)*	66.2 ± 8.0 (5)*
iPrOH/CCl ₄	12h	8.0 ± .53 (4)* **	13.5 ± 1.5 (4)	14.2 ± .39 (5)*	27.0 ± 2.7 (5)*
"	24h	11.4 ± .31 (4)* **	19.2 ± 1.7 (4)	27.5 ± 5.5 (5)*	43.6 ± 6.4 (5)*

* Significant at p < 0.05 compared to air control

** Significant at p < 0.05 compared to iPrOH control

Numbers in parentheses are number of animals.

^a Mean and SEM calculated eliminating one outlying sample.

24h had again doubled. Isopropanol preexposure at 1000 ppm, however, did not enhance this response.

The histological response in mice generally paralleled these biochemical effects. CCl_4 alone at 400 ppm (Figure 13) induced widespread centrilobular hepatic necrosis by 12 h, characterized by increased eosinophilia, pyknosis and some karyolysis, but few balloon cells in contrast to the numerous balloon cells and only slight eosinophilia in rats (cf. Figure 5). Fatty infiltration was also visible. This lesion persisted at 24 h in mice; indeed, the even more marked eosinophilia indicated extensive cell death, confirming the biochemical difference from the pattern seen in rats. Isopropanol preexposure did not significantly enhance the extent of the hepatic necrosis. Some (but not all) of the kidneys from mice exposed to isopropanol/ CCl_4 had granular, very darkly eosinophilic material in the tubules in the cortex, medulla, and papilla. The tubular epithelium was flattened, and in places, some apical cytoplasm was being sloughed into the lumen (Figure 14). This lesion was also present in groups exposed to CCl_4 alone and sacrificed at 12 h (but not 24 h) postexposure.

Figure 13

Photomicrographs of mouse liver after exposure to isopropanol and CCl_4 . A. Sacrifice at 12 h showing centrilobular eosinophilia. C - central vein; P - portal triad. B. Sacrifice at 24 h, when necrosis is more severe. H and E. x 48.

A

B

Figure 14

Photomicrographs of mouse kidney after exposure to isopropanol and CCl_4 . A. Papilla, showing granular material (large arrows) in tubules. Small arrow indicates erythrocytes for comparison. B. Cortex. Granular material is present in some tubules. Tubular dilation (D) and slight apical vacuolation (arrow) are also visible. H and E. x 120.

A

B

DISCUSSION

Kidney

Loss of glucose-6-phosphatase activity following exposure to CCl_4 is an indirect but an in vivo indicator of metabolism of the CCl_4 by the cytochrome P-450 - mixed function oxidases (i.e. activation) and subsequent membrane lipid peroxidation, and has been extensively documented in studies of CCl_4 hepatotoxicity (e.g. Reynolds and Yee 1968; Glende et al. 1976). Kidney tissue contains G-6-Pase of comparable specific activity, and when kidney microsomes were incubated with reduced iron and ascorbate, loss of this activity paralleled MDA generation just as was observed in liver microsomes (Figures 2,3). This implies that renal G-6-Pase indeed has the comparable requirements for membrane structural integrity, although a complete study of its particular lipid requirements has not been done as it has for the liver enzyme. Kidney lipids, at least in vitro, are also peroxidizable, although the maximal MDA production achieved in the incubation medium and conditions chosen here was only about 55 percent of that in liver. Both lost 95-99 percent of control G-6-Pase activity. This is reasonable since kidney has been found to contain less polyunsaturated fatty acids than liver (Kornbrust and Mavis, unpublished observations). Thus, assaying G-6-Pase appears to be a valid means of comparison of renal and hepatic responses to CCl_4 .

Kidney tissue also contains cytochrome P-450, although specific activities and content are about 1/5th to 1/10th those in liver (Zenser et al. 1978; Villarruel et al. 1977; Kornbrust and Mavis 1979; Muller et al. 1973). Particular drug metabolizing enzymes are species dependent and organ dependent in terms of activity, patterns of inducibility, etc. (e.g. Hook et al. 1975) but generally, renal P-450 - mixed function oxidases appear to be comparable to those in liver. Renal P-450 activities can be induced by various xenobiotic agents which are also active in liver, such as PBBs (McCormack et al. 1978), TCDD (Hook et al. 1975; Fowler et al. 1977) and xylene (Savolainen et al. 1978). McMurtry et al. (1978) hypothesized that the renal (and hepatic) toxicity (proximal tubular necrosis) of acetaminophen depended on its metabolism by renal P-450 to an active product. They observed less necrosis, less covalent binding in vivo and in vitro, and less depression of glutathione levels when the rats were pretreated with the P-450 inhibitor, CoCl_2 . Thus, it is reasonable to consider the possibility that CCl_4 might also be metabolized in the kidney by P-450 and its renal damage be mediated by lipid peroxidation comparable to that hypothesized in liver. The subsequent cellular events leading eventually to renal failure if exposure is severe, are not known, but activation of the CCl_4 in the kidney causing peroxidative damage is one possible mechanism of initial injury.

The experiments described here involving exposure to CCl_4 alone, however, do not substantiate this hypothesis of renal

toxicity. They do not prove that CCl_4 cannot stimulate lipid peroxidation in vivo in the kidney, but, at least using this protocol, the response indicating peroxidation in liver is severe before it is detectable in the kidney. At 400 ppm and above, liver endoplasmic reticulum apparently suffers increasing degrees of lipid peroxidation while kidney membranes remain unaffected. Since G-6-Pase is an indicator of the initial lipoperoxidative event (as opposed to later consequences of the lipoperoxidation), sacrifices were generally performed at relatively short intervals after exposure. Renal failure or insufficiency as defined by functional parameters such as anuria or proteinuria was not actually established by the experimental regimen during the period of observation before sacrifice (clinically, these symptoms tend to appear only several days after exposure). Therefore, it is not certain whether renal toxicity would indeed be "clinically" manifest at later times after exposures to the highest concentrations used here, even though renal G-6-Pase is not significantly depressed. It was assumed that, if CCl_4 were indeed metabolized and therefore were initiating peroxidation in the kidney, then loss of G-6-Pase activity should be a more sensitive indicator of the effect than any consequent clinical symptoms appearing later.

Some other recent studies have produced evidence that renal P-450 - mixed function oxidases may be important mediators of the nephrotoxicity of CCl_4 . Dietary intake of PBBs was found to

stimulate microsomal enzymes (biphenyl hydroxylases and aryl hydrocarbon hydroxylase) in both liver and kidney of rats while in vivo and in vitro parameters of renal function were unaffected (McCormack et al. 1978). This treatment enhanced the nephrotoxicity of several solvents including CCl_4 (0.03-2 mg/kg, p.o.) as measured by PAH accumulation by kidney slices and relative kidney weight (Kluwe et al., 1979), and potentiated the renal (and hepatic) toxicity of CHCl_3 , another nephrotoxic solvent requiring metabolism (Kluwe and Hook 1978). Activation of C^{14}Cl_4 as measured by C^{14} binding to microsomal lipids was observed in kidney and other tissues to an extent proportional to their respective contents of P-450 (Villarruel et al. 1977). The greater content of P-450 in kidney cortex than medulla (Villarruel et al. 1977) also seems to correlate with the proximal site of morphological changes that have been observed and are discussed later. Villarruel et al. (1978) also mention their observation of CCl_4 -induced destruction of P-450 in kidney cortex.

The implication from these studies is that renal P-450 - mixed function oxidases can metabolize CCl_4 . Since kidney microsomes are readily peroxidizable in vitro (Figure 3) and undergo comparable morphological alterations as do peroxidizing liver microsomes (Arstila et al. 1972), it might be expected that activation of CCl_4 in kidney, as in liver, could precipitate lipid peroxidation and hence membrane destruction. However, this has not been conclusively demonstrated in vivo. Villarruel et al.

(1977) detected lipid peroxidation only in livers of CCl_4 -treated rats; Srinivasan and Recknagel (1973) detected no conjugated dienes in kidneys of rats fed 5 ml/kg CCl_4 ; and we were unable to verify loss of G-6-Pase activity in kidney. Also, when CCl_4 was added to kidney microsomes in vitro, Kornbrust and Mavis (1979) were unable to detect enhanced lipid peroxidation (MDA production).

Several explanations for these apparent contradictions may be postulated though it is presently unknown which is (are) the most appropriate. The intact, in vivo kidney may contain a compound (e.g. an antioxidant) not present in liver and lost during preparation of microsomes, which inhibits generation of lipid peroxides and other reactive species. Thus, although the renal P-450 system might be capable of producing the $\text{CCl}_3^\bullet$ free radical which is thought to be the initiator of peroxidation, further propagation would be inhibited. Alternatively, membrane lipid peroxidation may indeed occur in kidney as in liver, but to a much lesser degree so as to be undetectable with present methodology. Renal P-450 activities are considerably lower than hepatic, and therefore the rate of generation of the initiating reactive metabolite must be much slower. Whereas in liver the propagation of membrane destruction initiated by the metabolite soon overwhelms cellular repair mechanisms, the concentration of reactive (toxic) metabolite produced in kidney at any time may be sufficiently less that damage is at least partially reparable. Present techniques of assaying in vivo peroxidative damage may be

too insensitive to detect such low levels. When renal microsomal enzymes were induced by other xenobiotics such as PBBs, the enhanced nephrotoxicity of CCl_4 (Kluwe et al., 1979) may thus have been attributable to an enhancement of the rate of production of the toxic metabolite ($\text{CCl}_3\cdot$), although these authors presented no direct evidence of renal metabolism of CCl_4 or renal peroxidation. It is also possible that direct interaction of CCl_4 in kidney is normally relatively unimportant, at least in rats, only because the dose reaching the kidney is much less than that reaching liver. In this case, simply delivering a larger dose to the kidney should produce detectable peroxidation. Further experiments are necessary to verify this. For example, infusion of CCl_4 directly via the renal artery would avoid secondary effects from excessive liver damage and any immediate renal effects could be assumed to be caused by direct interaction of CCl_4 with kidney tissue.

Only two other studies using G-6-Pase as a marker for renal damage were found. Activities in mouse liver and kidney were assayed 21 h after an oral dose of 2 ml CCl_4 /kg (Cawthorne et al. 1971). Although this is a much greater dose than used in our experiments, liver activity was reported to be only about 30 percent depressed compared to the maximal 50-60 percent in our inhalation experiments. In males (but not females) a 21 percent loss of activity in kidney homogenates was reported, although this was not statistically significant with the small groups used.

Striker et al. (1968) reported no changes in renal G-6-Pase of rats intubated with 2.5 ml CCl_4 /kg until 36 h when it increased and when cellular repair was occurring (but since standard error was > 25 percent, this was actually nonsignificant).

Both the numbers presented by these authors and our experience with this enzyme demonstrate great variability in measured G-6-Pase activity, a large part of which may be assumed attributable to individual animal variability. In the kidney, different activities have been found in different portions of the nephron (Schmidt and Guder 1976) and since P-450 content varies across the hepatic lobule (Gooding et al. 1978), probably a similar heterogeneous pattern exists in liver G-6-Pase. Activities assayed in whole tissue homogenate or microsomes would include contributions from all these various tissue "types". Therefore, from animal to animal and day to day, the inevitable variability in proportions of the tissue types obtained for homogenization could contribute substantially to the large range in measured values. The final reported activity also depends on two separate assays quantitated spectrophotometrically (inorganic phosphate and microsomal protein) each with its associated experimental error. Of particular significance may be the variability in measured protein concentration of the microsomal suspensions due to centrifugation conditions - small differences in time or speed of a spin result in more or less extraneous protein being pelleted, thus distorting the calculated specific enzyme activity (per mg protein). All

these factors reduce the sensitivity of the assay, by causing large variance both within groups and from day to day.

These analytical factors probably account for the variable response to the same exposure conditions in different experiments, such as the loss of renal G-6-Pase at 6 h (Figure 7) which was not reproduced (Figure 10). Loss of activity in the liver occurred consistently, supporting literature observations of this effect of CCl_4 . However, even though some changes in the kidney activity were statistically significant, their apparent inconsistent occurrence suggests that they were not due to the solvent exposure.

The lack of elevation of BUN reflects the lack of renal functional deficit by the time of sacrifice in these experiments. It was realized that this is not a particularly specific parameter, but it is a common tool of clinical chemistry, and elevated BUN has often been reported in cases of human poisoning by CCl_4 (e.g. Woods 1946; Guild et al. 1958; Markham 1967). Since urea is produced only by the liver, reduced urea synthesis resulting from liver damage may mask renal dysfunction until it becomes severe, therefore making it an insensitive measure of subtle changes. Significance of elevation of BUN by CCl_4 exposure thus indicates very marked renal deficit. Proteinuria is also a non-specific indicator of glomerular or proximal tubular dysfunction, although excess protein could also spill into the urine in the absence of renal damage if serum protein levels were elevated, for example by liver necrosis. Albuminuria has been reported in human cases of

CCl_4 exposure (e.g. Woods 1946; Guild et al. 1958). Since only a minor elevation in rat urinary protein was seen at our highest dose level, this cannot be conclusively interpreted as a demonstration of renal damage (liver damage was severe in those animals).

Thus, although we did not clearly characterize a renal lesion from CCl_4 using the rat as a model, it is interesting to note that, in this species, marked liver toxicity is manifest soon after exposure to these concentrations, while the same parameters in kidney remain relatively unaffected. The results reported here thus suggest that renal dysfunction from CCl_4 may be an effect secondary to liver damage or at least is elicited via a different mechanism. It must be pointed out that at least 2 experimental conditions were chosen to maximize the probability of occurrence and detectability of a direct renal response, namely (i) dose range and (ii) route of exposure.

(i) The doses encountered in our study are considerably lower than in most others. To get a rough estimate of a possible range of the expected dose absorbed by a rat inhaling CCl_4 for 4 h, one can use a minute volume of 0.1 l/min (Altman and Dittmer 1974).

Assuming complete absorption from the lungs (obviously an overestimate), 400 ppm is equivalent to approximately 0.15 ml/kg-bw. Using the estimate by McCollister et al. (1951) of 30 percent absorption of vapor from the lungs in monkeys, the equivalent dose is 0.05 ml/kg. This range can be compared to the i.p. LD50 of 2-3 ml/kg in rats and mice and to the 0.5 - 2.5 ml/kg

range generally employed, in which liver necrosis must be so severe as to overshadow any direct renal effect by secondary overloading of the kidney tubules by materials released into the blood from necrosed liver cells.

(ii) Secondly, exposure by inhalation was chosen partially because it seemed to be more realistic when considering human exposures to solvents. Since the absorbed dose following inhalation of the vapor is unknown, and would vary with the animals' activity during exposure (Astrand 1975) and extent of dermal absorption, as well as with the inherent biological variability in absorptive capacity of the lung, this route does introduce a major source of uncertainty and variability into the results. However, a compound absorbed from the lungs would initially undergo more general distribution to the body tissues compared to that absorbed from the GI tract, all of which initially goes to the liver via the portal circulation. Therefore, tissues other than liver may be exposed to much greater concentrations of the compound following inhalation than following oral or i.p. dosing. Gas chromatographic analysis of tissue CCl_4 concentrations appears to support this conjecture: CCl_4 detected in kidney equalled or exceeded that in liver of rats immediately after inhalation at 4000 or 7500 ppm (Dambrauskas and Cornish 1970) while the reverse was true following oral or i.p. dosing (Fowler 1969, using rabbits; Maling et al. 1974, using rats). Species differences, dose-dependence of distribution, or problems of measuring C^{14} rather than parent compound may account for

apparently higher concentration in liver than kidney of a monkey immediately after inhaling 50 ppm $C^{14}Cl_4$ (McCollister et al. 1951). Thus, the inhalation route in this study was assumed to maximize the potential for direct renal toxicity. However, using the protocols described here, a distinct renal response could not be defined in rats, at least in terms of the chosen parameters.

Although a complete study of the dose-response and the time course of response to CCl_4 in mice was not attempted here, the elevated BUN at 24 h, loss of G-6-Pase at 12 h, and more marked histological renal damage are suggestive of a species difference in CCl_4 nephrotoxicity, as will be discussed further below. In mice, renal G-6-Pase activity returned to control levels by 24 h, whereas the more functional parameter (BUN) was significantly elevated only by 24 h. Thus, if loss of G-6-Pase here is actually representative of a direct peroxidative effect of CCl_4 in mouse kidney, ER membranes have been repaired by 24 h, while functional deficit is only then detectable. It is not known whether the possible peroxidative damage is actually the initiator of this later renal dysfunction as it is assumed to be in the liver. If activation by P-450 does mediate the CCl_4 nephrotoxicity, it might be expected that mouse kidney P-450 levels exceed those in rat kidney; although Diaz-Gomez et al. (1975) found no such correlation between hepatotoxicity and liver P-450 among various species, this has not been examined in kidneys. Litterst et al. (1975) measured liver, kidney and lung P-450 content and activities

of several drug metabolizing enzymes in 5 laboratory animal species. Mouse kidney enzyme activities and P-450 content relative to those in liver generally exceeded the corresponding activities in the rat. However, it is probable that interstrain variability would be comparable to these interspecific differences, so that these observations cannot be assumed to be quantitatively generally applicable.

Review of the laboratory studies reported in the literature gives no clearer a picture of the possible mechanisms or biochemistry associated with the nephrotoxicity of CCl_4 . Plaa and Larson (1965) and Klaassen and Plaa (1966) dosed mice (i.p.) with CCl_4 at 1.5-4 ml/kg and saw marginal elevation of urinary protein concentration, but no changes in urinary glucose (dipstick only) or in excretion of phenolsulfonephthalein, nor any morphological alterations. At 2-4 ml/kg there was also some lethality. Using these parameters, CHCl_3 and 1,1,2-trichloroethane produced marked renal dysfunction. After daily i.p. dosing of rats, Perassi and Martin (1973) found elevated (160 percent) protein excretion. Immunochemical analysis suggested that the excess urinary proteins were serum-like proteins, only some of which were normally present in rat urine. Tissue-like proteins were decreased in urine of CCl_4 -treated rats. The authors did not report whether or not serum proteins from treated rats differed from control serum proteins; this might account for qualitative changes in urinary proteins, without renal

dysfunction. In this study (Perassi and Martin, loc. cit.), no histological changes were observed in the kidney by light microscopy; the appearance of the liver after these chronic treatments was not reported. Cornish and Ryan (1964) measured free urinary amino acids in rats inhaling CCl_4 . At 9000 ppm, they did observe aminoaciduria, but the qualitative pattern of the excreted amino acids corresponded to elevated serum amino acids rather than renal inability to reabsorb amino acids.

Two studies reported elevation of renal triglyceride concentrations. Estler et al. (1973) reported elevated serum non-esterified fatty acids and renal triglycerides 6-12 h after mice were injected with 3 ml CCl_4 /kg. Relative kidney weight and water content were also increased. The antilipolytic drug β -pyridylcarbinol prevented the lipid changes but had no effect on the renal weight and water content changes. While severe fatty infiltration and necrosis were seen in liver, the kidneys of these animals showed only slight tubular swelling and filamentous material (reported to be protein) in the lumen. Triglyceride changes were not visible histochemically. Elevation of renal cortical lipid was also reported within 15 minutes in cats inhaling CCl_4 at 10,000 ppm (Wong and DiStefano 1966). Lipid content remained high for 3 h after a 4-h exposure but declined to less than controls by 24 h. Increased liver lipid on the other hand was not detectable until 3 h after the 4-h exposure. This suggested that liver and kidney effects are independent, though the mechanism

of the renal fatty change was not further investigated. It neither rules out nor verifies a direct activation of CCl_4 by renal P-450.

The fact that we used much lower concentrations of CCl_4 probably accounts for our failure to detect renal triglyceride accumulation, although species differences in response to halogenated hydrocarbons may also be important. At least in the mouse (Estler et al. 1973) these lipid effects appeared not to be attributable to a direct kidney effect since they were prevented when serum free fatty acid levels were normalized by β -pyridylcarbinol. Fatty acids are extracted from blood and used as one of many sources of energy by the kidney; but it is in the liver where a large turnover (synthesis and breakdown) of fatty acids and complex lipids occurs according to the requirements of the whole body. Hence, while disruption of fat metabolism in the liver is severely damaging, it seems unlikely that renal fatty change could be as significant a mechanism of toxicity.

Striker et al. (1968) also suggested that the renal lesion in rats was independent of liver damage, on the basis of their observation by electron microscopy that proximal tubular mitochondrial swelling (and depressed succinate dehydrogenase activity) was the earliest (2 h) abnormality observed after intubation of 2.5 ml/kg, whereas ER disruption is generally reported to be the first change in liver. They did observe membranous aggregates in kidney at 2 h, which were most numerous at 24-48 h; these appeared to be smooth and rough membranes,

suggestive of kidney ER involvement supporting the hypothesis of membrane destruction as seen in liver. Cell edema developed by about 36 h. These authors did not actually examine the livers of the rats for direct comparison.

Using light microscopy only, Lundh (1964) reported early but rather non-specific proximal tubule changes in rats given a subcutaneous dose of 0.4 or 4 ml/kg. Swelling and vacuolation of cells was maximal at 16-24 h, compared to 36-48 h for maximal liver damage, and healed faster than did the liver. Some of these observations resembled ours, which by light microscopy at least, are very difficult to distinguish from effects of fixing, mounting, and staining.

On the basis of in vitro (kidney slice) tests of organic ion accumulation, Watrous and Plaa (1972a,b) reported nephrotoxicity of several chlorinated hydrocarbons, including CCl_4 , administered i.p., orally, or subcutaneously. However, the interpretation of these results is difficult due to the extreme variability of this method - control values of slice:medium ratios varied from day to day by over 200 percent (as did ratios in treated groups) and decreases in treated groups showed little dose-response relationship. This was also true for more potent nephrotoxic agents such as CHCl_3 .

In spite of the uncertainty of CCl_4 nephrotoxicity in laboratory animals, human poisonings with CCl_4 , both fatal and nonfatal, have certainly involved renal impairment (e.g., Woods

1946; Guild et al. 1958; New et al. 1962; Markham 1967). The clinical literature naturally describes primarily hospitalized cases so the doses of CCl_4 have usually been very high (but unquantitated) and frequently a history of alcoholism or recent intake of alcohol has been noted. Initial symptoms in humans, regardless of exposure route, seem to be gastrointestinal disturbance - nausea, vomiting - and subjective headache, malaise, etc. Liver dysfunction is seen almost invariably. When oliguria or anuria develop, it is generally only after several days' delay, and clinical signs of renal failure include BUN elevation, albuminuria, electrolyte imbalance, etc. A period of diuresis precedes recovery. Microscopic examination of human kidneys is obviously limited to autopsy specimens from fatal cases, and therefore any abnormalities may be due to secondary complications from renal failure rather than a specific toxic effect of CCl_4 . Indeed, the clinical histological reports lack any obvious common pattern. Tubular necrosis has been reported, as well as casts, bile pigments, and other material in the tubules, tubular swelling and vacuolation. Several authors report proximal tubules to be the primary site, but abnormalities have not been limited to that portion.

Since the human data are from completely uncontrolled exposures, comparability with laboratory animal studies is limited. There may be a real species dependence in response to CCl_4 , but even this cannot be certain since laboratory studies

have seldom followed the long-term response to an acute exposure at high concentrations of CCl_4 or to chronic lower-level exposure (common human situations of exposure). Although no oliguria developed in the rats in our study during 6 days following CCl_4 inhalation, the concentration used (400 ppm) was lower than expected in most human incidents, and indeed did not elicit irreversible liver damage. The lag between exposure and occurrence of renal failure reported in the literature suggests a different progression of damage in kidney than in liver. If the critical event were lipoperoxidation initiated by the free radical metabolite of CCl_4 with a time-course of repair comparable to that in liver, the peroxidative damage should be repaired by the time renal dysfunction is expressed. The implication is thus that some extrarenal factors may be important, at least in humans. For example, severe renal response may be secondary to the developing hepatic dysfunction. Or, since CCl_4 is a CNS depressant, a central effect, say, reducing blood flow to the kidney and/or liver might be postulated. Sirota (1949) measured renal blood flow and clearances in 4 patients with oliguria following CCl_4 inhalation. Reduced blood flow (measured as reduced clearance of PAH, inulin, creatinine) was observed during oliguria, but it is not possible to distinguish whether this was a cause of the oliguria (i.e., CCl_4 acting on the vascular system, either directly or via CNS) or was a secondary response to some direct effect of CCl_4 on kidney tubule cells. However, Lautt and Plaa (1974) found no

significant change in blood flow in the intact cat liver within 4 hours following 1 ml CCl_4 /kg, during which time SGPT increased significantly, although there were trends towards reduced portal pressure, increased portal flow, and reduced hepatic arterial resistance. Also, hepatic vascular response to norepinephrine and carotid occlusion was reduced in CCl_4 -treated animals. But it appears that a direct interaction of CCl_4 in vascular tissues does not contribute to the development of hepatic (and by inference renal) damage by restricting blood flow leading to ischemia.

Liver

Since the liver response to carbon tetrachloride is extensively documented, a complete literature review and assessment of the opposing hypotheses of its mechanism of toxicity will not be attempted here. Liver response was included in our protocol to serve as a "positive control", and as a basis of comparison with the literature and with the response measured simultaneously in the kidney.

The parameters assayed here demonstrate that the liver response did provide an adequate positive control. Our liver microsomal preparations produced MDA and lost G-6-Pase activity when incubated with reduced iron and ascorbate as has been reported by many authors (e.g. Wills 1971; Glende et al. 1976; Benedetti et al. 1979). The observed patterns of response in hepatic G-6-Pase, triglycerides, and histologic appearance are also in general

agreement with those in the literature. Divergence from other results can be attributed to the difference in administered dose of CCl_4 . The time course of depression of G-6-Pase activity was followed only at one dose (400 ppm), from which maximal depression occurred by 6 h (the earliest time of sacrifice). By 12 h and later it was recovering, and had returned to control levels by 24 h. When a dose calculated to be $0.75 \times \text{LD}_{50}$ was injected i.p. (1.4 ml/kg), Klaassen and Plaa (1969) observed depression of G-6-Pase to be maximal by 6-12 h and to persist for at least 36 h (last time of sacrifice). Most of those rats would have suffered irreversible liver damage, whereas the 4-h exposure to 400 ppm in this study appeared to have no lasting toxic effects. More prolonged loss of activity would likely have been observed if the dose of 1200 ppm had been chosen for following the time course of response.

Loss of G-6-Pase at 12 h was dose-related, the lowest exposure concentration to cause detectable loss being 400 ppm (estimated absorbed dose 0.05-0.15 ml/kg). This effective dose range is consistent with the histochemical detection by Reynolds and Yee (1968) of centrilobular loss of G-6-Pase by 1 h after 0.0025 ml/kg (oral) and observable necrosis at 24 h after 0.025 ml/kg. Klaassen and Plaa (1969) were unable to detect significant loss at 12 h from doses less than 0.3 ml/kg (i.p.). In their series of papers discussed later dealing with potentiation of CCl_4 hepatotoxicity, Traiger and Plaa reported nonsignificant or marginal losses of

G-6-Pase at 24 h after 0.1 ml/kg. However, if an earlier time of sacrifice had been chosen for defining the dose response of this parameter, a lower dose would likely have been effective.

Liver triglyceride accumulation after the 4-h exposure to 400 ppm peaked by 18 h after which it declined to control levels by 48 h. This time course can also be expected to be dose-dependent, as shown by data from Ugazio et al. (1973): after 0.06 or 0.25 ml/kg (oral), liver lipids peaked at 12-24 h then declined, while 1 ml/kg caused continued accumulation until 48 h. The elevation at 12 h in our experiments was significant at 100 ppm exposure concentration, which is comparable to other reported effective doses of 0.025 ml/kg (Korsrud et al. 1972), 0.03 ml/kg (Klaassen and Plaa 1969), 0.06 ml/kg (Ugazio et al. 1973), and very marked elevation from 0.1 ml/kg (Traiger and Plaa 1971, 1973).

Liver histology basically correlated with the biochemical measurements in terms of dose response and time-of-sacrifice effects. However, the biochemical assay of triglycerides tended to be more sensitive than histological assessment (especially with H and E staining only) while initial stages of necrosis (pyknotic nuclei, balloon cells) around central veins were discernible from doses at which G-6-Pase changes in microsomes from whole liver homogenates were not significant (e.g. 100 ppm, 12 h post exposure). It is interesting that Korsrud et al. (1972) also found that none of the 9 serum enzymes analyzed to detect CCl₄-induced liver damage was more sensitive than routine histological assessment.

Interaction

The clinical literature on carbon tetrachloride poisonings is replete with cases involving alcohol - indeed, enhancement of its toxicity in humans by ethanol consumption was recognized long before the mechanism of toxicity was understood. As knowledge of its mechanism expanded, interactions with many agents, both synergistic and antagonistic, were studied and provided further tests of hypotheses regarding the biochemistry of CCl_4 hepatotoxicity. Ethanol itself is a "classic" example of an "enhancer" of toxicity of many agents - drugs, other solvents, pollutants, etc. Academic studies have followed up that observation by comparing series of alcohols for interactions with known toxic agents. In the case of CCl_4 , isopropanol was found to be among the most potent potentiators of its hepatotoxicity in laboratory animals (Cornish and Adefuin 1967), and was the subject of some further investigation (as discussed below). Later, an industrial incident was reported in which several workers required hospitalization following exposure to CCl_4 vapor in an isopropanol packing plant (Folland et al. 1976). The authors concluded that the severity of the illnesses was suggestive of an enhanced response to CCl_4 vapor in individuals who had been exposed to isopropyl alcohol, thus corroborating the laboratory observations. It is of interest to note that symptoms reported from the industrial incident indicated that renal toxicity was significant and perhaps enhanced by isopropanol.

The literature on potentiation of CCl_4 by isopropanol (and other agents) in laboratory animals deals almost exclusively with hepatotoxicity. A high dose of the alcohol has generally been administered orally, and CCl_4 injected intraperitoneally. Traiger and Plaa (1971) observed that isopropanol exerted its maximum potentiation of CCl_4 -induced serum enzyme and hepatic triglyceride elevation, and hepatic G-6-Pase loss when administered to rats 18-24 h prior to 0.1 ml/kg CCl_4 . Delaying the elimination of isopropanol by administration of pyrazole, an inhibitor of alcohol dehydrogenase, reduced its potentiating capability, suggesting that metabolism of isopropanol (primarily oxidation to acetone) was necessary for enhancement of CCl_4 toxicity (Plaa and Traiger 1973). Indeed, pretreatment with acetone itself was also found to potentiate CCl_4 , although the time course of this capability has not been examined. On the other hand, aminotriazole, an inhibitor of hepatic catalase and microsomal P-450 and drug metabolizing enzyme activity, reduced the enhancement of CCl_4 hepatotoxicity by isopropanol and acetone without affecting the kinetics of blood alcohol or acetone. Aminotriazole administration also reduced the isopropanol-induced stimulation of aniline hydroxylase activity (Traiger and Plaa, 1973).

From the results summarized above, it may be hypothesized that this potentiation is a function of the metabolite, acetone, rather than the alcohol per se. The same relationship was found with

2-butanol, another alcohol which potentiates CCl_4 hepatotoxicity: its metabolite 2-butanone is also capable of a similar potentiation (Traiger and Bruckner 1976).

Given the lag time before maximal enhancement and also the fact that CCl_4 requires activation to exert its toxicity, it seems likely that isopropanol (and/or acetone) must be altering the hepatocellular endoplasmic reticulum so that CCl_4 metabolism is enhanced. However, the literature on microsomal effects of isopropanol and acetone is somewhat inconclusive. Some studies report enhancement of microsomal enzyme activities only when the compound is added to an in vitro system at unrealistic (e.g. exceeding 1 M) concentrations. The effects on different microsomal enzymes used to assay P-450 activities are also variable, presumably reflecting the existence of multiple forms of P-450 (Mailman et al. 1977). However, to generalize, it appears that acetone and isopropanol administration can cause enhanced activity of some microsomal enzymes such as aniline hydroxylase, aminopyrine demethylase, and DMN-N-demethylase measured at various times from 16-48 h after dosing (Powis 1975; Sipes et al. 1973; Traiger and Plaa 1973). Generally, no increase in P-450 content (nmoles/mg) was observed. Cote et al. (1974) observed (electron microscopically) liver endoplasmic reticulum proliferation at 42 h in rats given isopropanol, corroborating the biochemical assays. Pretreatment with isopropanol has also been observed to enhance CCl_4 activation as measured by C^{14} binding to liver protein

(Sipes et al. 1973) and diene conjugation of liver microsomal lipids (Maling et al. 1975), as well as to increase ethane expiration (an in vivo measure of lipid peroxidation) following CCl_4 treatment (Lindstrom and Anders 1978).

Since isopropanol appears to be metabolized extensively by alcohol dehydrogenase, it seems appropriate to consult the much more extensive ethanol literature. A microsomal ethanol oxidizing system (MEOS) containing P-450 has been documented, and may be the mediator in the enhancement of activities of P-450 and drug metabolizing enzymes and ER proliferation seen from chronic ethanol intake (reviewed by Lieber 1973). Isopropanol oxidation by MEOS has not generally been observed (Orme-Johnson and Ziegler 1965), although it has been studied only cursorily. However, the enhancement of some drug-metabolizing activities by isopropanol and acetone suggests that there may indeed be some microsomal metabolism of isopropanol, however minor it may be in the oxidation of the total dose of the alcohol.

Since none of the studies examined kidneys, it was of particular interest that in our preliminary inhalation exposures of mice to isopropanol followed by CCl_4 , in addition to the expected potentiation of triglyceride accumulation and liver necrosis, a marked renal lesion not seen following CCl_4 alone was observed histologically and BUN was significantly elevated. The same lesion in kidney (as well as potentiation of liver damage) was observed with acetone substituted for isopropanol. However, this

histopathological lesion was not reproduced in rats exposed to isopropanol and CCl_4 . In agreement with the histology, neither was there any loss of renal G-6-Pase activity, or BUN elevation in rats. Distinct species differences in hepatic response to CCl_4 have been found (Diaz-Gomez et al. 1975). For example, the mouse was found to be more susceptible than the rat, hamster, and guinea pig to liver necrosis, though CCl_4 concentration in liver was lowest in that species. Mice have been reported to be more susceptible to nephrotoxicity of chloroform, though sensitivity was strain-dependent (Kluwe and Hook 1978). This may account for our divergent results with rats.

The utilization of renal G-6-Pase activity to quantitate possible enhanced nephrotoxicity of CCl_4 by isopropanol was predicated on the assumption discussed above that CCl_4 acts directly in kidney, as in liver, to precipitate lipid peroxidation and that isopropanol might enhance the extent of peroxidation by enhancing renal metabolism of CCl_4 . Even though CCl_4 alone appeared not to significantly depress G-6-Pase, if renal CCl_4 metabolism were indeed aggravated by isopropanol pretreatment, loss of activity might then become detectable. However, this was not observed. This may corroborate the conclusion above that CCl_4 probably does not cause its renal damage through direct metabolism in kidney. Or perhaps isopropanol pretreatment does not cause the changes in kidney that occur in liver to sensitize it to CCl_4 toxicity. Or it may be that a larger dose of isopropanol is

necessary for detection of significant potentiation. In other studies, an oral dose of 2.5 ml/kg (about half the LD50) has been used, though there has been no report of the dose-response relation of the isopropanol potentiation phenomenon.

Losses of hepatic G-6-Pase activity that were observed here in rats were really only suggestive of a potentiated response. Although enhancement was statistically significant at 6 and 24 h post CCl_4 exposure, it was not at 12 h (Figure 9). Hepatic triglyceride accumulation was significantly enhanced only at 24 h and only when isopropanol was at 1500 ppm (not 1000 ppm). Even so, quantitative responses found here never exceeded those from the larger concentrations of CCl_4 alone, whereas Traiger and Plaa (1971) reported that following pretreatment with isopropanol (2.5 ml/kg), 0.1 ml/kg CCl_4 elicited greater toxic effects (including loss of G-6-Pase at 24 h sacrifice) than a 10-fold greater dose of CCl_4 alone. This difference may result from the concentrations of isopropanol used here (1000 - 1500 ppm) being just on the threshold for potentiating capability in the rat, since the 1500 ppm level seemed to give more certainly enhanced response in the hepatic parameters measured. Applying the same assumptions used for calculating absorbed dose of CCl_4 , a 4-h exposure to isopropanol at 1500 ppm is equivalent to approximately 0.15-0.45 ml/kg. It is possible that isopropanol can exert a potentiating effect at doses much smaller than the 2.5 ml/kg frequently chosen.

The explanation for the significantly enhanced loss of G-6-Pase at 6 and 24 h but not at 12 h is not clear. If isopropanol preexposure sensitized the liver so that initial damage was more extensive (enhanced loss at 6 h), it is reasonable that complete repair of damage would be slower (activity was still depressed at 24 h, whereas it was back to control levels following CCl_4 alone). However, depressed activity would also be expected at 12 h, which was not observed here. Part of this apparent anomaly may stem from different sensitivities among different groups of test animals: when interaction experiments were performed, control exposures to CCl_4 alone were not repeated except for the 6 h sacrifice. Thus, comparisons of quantitative response are being made among animals from shipments received several weeks to months apart. That their sensitivities are variable is illustrated by the 6 h sacrifice groups, where the hepatic response to CCl_4 alone was less severe and the original renal response was not seen at all when this exposure was repeated.

Although the biochemical parameters did not unequivocally demonstrate marked potentiation in rats, microscopic assessment of liver sections revealed more definite differences in response following isopropanol preexposure. At 6, 12, and 24 h, the extent of the affected area around the central veins in isopropanol preexposed groups was larger than after exposures to CCl_4 alone. These areas of variably increased eosinophilia and with visible lipid droplets but little evidence of pyknosis, were ringed by

large balloon or foam cells with pyknotic nuclei. Within these centrilobular areas were inflammatory centers with evidence of phagocytosis of necrosed hepatocytes. Kidney sections from exposed rats never showed very specific or distinctive changes, as expected from the lack of biochemical changes. Although a dose-response relation was not clearly discernible, some of the exposed animals did have sections of the cortex in which tubule cells appeared vacuolated. But there was no frank necrosis or fatty change detectable.

The mouse was clearly more susceptible than the rat to the toxicity of inhaled CCl_4 . A dose-response curve was not developed here, but at least part of the difference can be assumed to result from the difference in absorbed dose of CCl_4 due to the greater relative respiration rate in the mouse. Whereas the estimated dose absorbed from the lungs of rats inhaling CCl_4 at 400 ppm was calculated above to be about 0.05-0.15 ml/kg, the comparable calculation for mice (using a minute volume of 0.025 l/min) gives 0.11-0.34 ml/kg, assuming the same absorptive capacities in both species. Similarly, a greater degree of potentiation might be anticipated on the basis of an absorbed dose of 0.68 ml/kg in the mouse inhaling 1000 ppm isopropanol compared to 0.33 ml/kg in the rat. However, this cannot be the only factor, since Diaz-Gomez *et al.* (1975) found a similar species variation in hepatotoxicity when animals were dosed i.p. at the same levels per kg-bw. Species differences (among mouse, rat, guinea pig, chicken,

hamster) did not seem to correlate with liver levels of P-450, NADPH reductase, or diene conjugation. In spite of their greater sensitivity to CCl_4 , however, mice did not show any greater potentiation of response by preexposure to isopropanol. As concluded above, the isopropanol concentration may have been too low to cause significantly enhanced activation of CCl_4 .

The renal histologic lesion observed in the pilot exposures of mice to isopropanol/ CCl_4 was reproduced here in mice (although not appearing in all individuals) exposed to CCl_4 alone (12 h sacrifice) as well as to isopropanol/ CCl_4 (12 and 24h sacrifice). That it was not detected in the pilot exposure to CCl_4 alone was probably because sacrifice was previously done only at 24 h and because the CCl_4 concentration was slightly lower. Considering exposures to CCl_4 alone, both renal G-6-Pase depression and the histological lesion were observed only at 12 h. However, following isopropanol/ CCl_4 , the histologic response was seen at both 12 and 24 h, while G-6-Pase was depressed only at 12 h. Thus, histological and G-6-Pase responses precede the functional response (BUN).

In light of the severe liver necrosis in mice, the renal pathology cannot be interpreted as evidence of direct interaction of CCl_4 in kidney, for the same arguments as presented above for rats. Necrotic hepatocytes would be dumping a large excess of protein and other cellular material into the bloodstream; this extra load in the glomerular filtrate can be expected to affect

tubular reabsorptive processes. In rats the liver damage was less severe and generally only minor hydropic change was detectable in kidney, while in mice the more marked liver damage could account for the apparent more marked renal response. Indeed, the renal tubular dilation and accumulation of material in the tubules in both species occurred when the toxic response in liver (as measured by loss of G-6-Pase) was maximal.

SUMMARY AND CONCLUSION

In the clinical literature there are numerous cases of renal involvement in CCl_4 poisoning and its possible potentiation by alcohols. But no good animal model has been developed and the mechanism of the renal damage is unknown. Microsomal G-6-Pase was used as a marker for a possible direct effect elicited in the rat kidney in comparison with that in the liver. It was verified that kidney microsomes peroxidize (produce MDA) as readily as liver when incubated with iron and ascorbate, and concurrently lose G-6-Pase. When adult male rats were exposed to CCl_4 vapor for 4 h and sacrificed at 12 h, loss of hepatic G-6-Pase was significant and dose-related at and above 400 ppm, and hepatic TGs were significantly elevated at and above 100 ppm. Centrilobular pyknosis, hydropic change, and presence of visible lipid droplets were also dose-related paralleling these biochemical parameters. However, no changes in renal histology or BUN were detected and only a marginal loss of renal G-6-Pase was observed at the highest dose (1200 ppm).

Serial sacrifice of rats after a 4-h exposure to 400 ppm CCl_4 indicated that loss of hepatic G-6-Pase was maximal (to 45 percent of control level) by 6 h postexposure and it recovered to control levels by 24 h, while TG accumulation peaked at 18 h. Hepatocellular hydropic change and pyknosis were maximal at about 12-18 h, and histology appeared normal at least by 48 h. Renal

G-6-Pase may have shown a slight and transient loss at 6 h; no renal functional deficit was noted (elevated BUN, proteinuria, glucosuria). The only renal histological change was slight tubular dilation and cellular vacuolation at 6 h.

A large oral dose of isopropanol has been reported to markedly potentiate CCl_4 hepatotoxicity presumably by enhancing its metabolism, and results of our pilot exposures of mice to both solvents by inhalation were suggestive of an enhanced renal lesion as well as hepatic. Preexposure of rats to isopropanol at 1000 ppm (TLV) or 1500 ppm slightly enhanced the loss of hepatic G-6-Pase and the accumulation of TG (at 1500 ppm only) following 400 ppm CCl_4 , while liver histology suggested much more severe centrilobular necrosis and fatty infiltration in the isopropanol-preexposed rats. However, renal G-6-Pase, functional parameters and histology were unaffected in these animals.

A few experiments repeated using mice showed this species to be more sensitive than the rat to CCl_4 -induced hepatic necrosis and fatty accumulation, which was also reflected quantitatively in loss of G-6-Pase at 12 and 24 h. In contrast to the lack of renal effects in rats, renal tubular dilation, vacuolation of tubule cells, sloughing of apical cytoplasm and resultant presence of granular and fibrillar material in the tubules were observed in mice 12 h (not 24 h) after CCl_4 exposure at 400 ppm. Isopropanol preexposure did not significantly affect the quantitative parameters, although histological damage appeared slightly more

severe; the renal lesion was still observable at 24 h in the isopropanol-preexposed group. In mice, BUN was significantly elevated by 24 h but isopropanol preexposure did not enhance this.

Considering the overall picture presented by these experiments, direct interaction of CCl_4 in the kidney leading to a toxic response via the peroxidative mechanism proposed for its hepatotoxicity probably does not occur in rats and mice. Although kidney does contain lipid peroxidizable in vitro, renal metabolism of CCl_4 initiating membrane lipid peroxidation as monitored by loss G-6-Pase activity cannot be detected in vivo following exposures which cause severe peroxidation in liver. Like liver, kidney tissue contains P-450 activity which is inducible and other data in the literature indicate its possible importance in mediating renal toxicity of some agents. However, the results of our experiments are more consistent with the hypothesis that renal effects of CCl_4 are secondary to the more severe response in liver. We observed only occasional and transient G-6-Pase depression in the kidney, and some histological abnormalities. The histological changes were fairly nonspecific and may be observed when tubule reabsorptive processes are overloaded, e.g. by spillage of tissue proteins from damaged organs such as liver. The quantitative loss in G-6-Pase might also be attributable to the presence in renal tissue and blood of excess extraneous protein (spillage from liver) which could pellet with the microsomes and dilute the calculated specific activity. No increase in protein

yield of tissue homogenates was detectable in these treated groups, but there was high variance within groups as well. In both species, any losses of renal activity were observed to coincide with the maximal liver damage.

It is unknown whether the animal models are appropriate for extrapolating to human exposures, but species differences observed here appear to be quantitative rather than qualitative. At least in rats and mice, it must be concluded that direct activation of CCl_4 by renal P-450 to precipitate lipoperoxidation does not occur or is not an important mechanism at exposure levels eliciting severe liver damage. Although marked potentiation by a large oral dose of isopropanol has been documented, the more moderate exposures by inhalation here did not appear to be adequate to consistently enhance the measured responses.

BIBLIOGRAPHY

- Altman, P.L. and Dittmer, D.S. (eds.) 1974. Biology Data Book (2nd ed.), Volume III. Federation of American Societies for Experimental Biology, Bethesda, Maryland.
- Arstila, A.V., Smith, M.A. and Trump, B.F. 1972. Microsomal lipid peroxidation: morphological characterization. *Science* 175:530-533.
- Astrand, I. 1975. Uptake of solvents in the blood and tissues of man. *Scand. J. Work Envir. Hlth.* 1:199-218.
- Benedetti, A., Casini, A.F., Ferrali, M. and Comporti, M. 1979. Effects of diffusible products of peroxidation of rat liver microsomal lipids. *Biochem. J.* 180:303-312.
- Biggs, H.G., Erikson, J.M., and Moorehead, W.R. 1975. A manual colorimetric assay of triglycerides in serum. *Clin. Chem.* 21:437-441.
- Bray's Clinical Laboratory Methods, VII Edition, Bayer, J.D., P.G. Ackerman, G. Toro (eds.). St. Louis: C.V. Mosby Co. (1968).
- Cawthorne, M.A., Palmer, E.D., Bunyan, J. and Green, J. 1971. In vivo effects of carbon tetrachloride and chloroform on liver and kidney glucose-6-phosphatase in mice. *Biochem. Pharmacol.* 20:494-496.
- Chaney, A.L. and Marbach, E.P. 1962. Modified reagents for determination of urea and ammonia. *Clin. Chem.* 8:130-132.
- Chen, P.S., Toribara, T.Y. and Warner, H. 1956. Microdetermination of phosphorus. *Anal. Chem.* 28:1756-1758.
- Cornish, H.H. and Adefuin, J. 1967. Potentiation of carbon tetrachloride toxicity by aliphatic alcohols. *Arch. Environ. Health* 14:447-449.
- Cornish, H.H. and Ryan, R.C. 1964. A study of carbon tetrachloride VI. Aminoaciduria in response to carbon tetrachloride inhalation. *Tox. Appl. Pharm.* 6:96-102.
- Cote, M.G., Traiger, G.J. and Plaa, G.L. 1974. Effect of isopropanol-induced potentiation of carbon tetrachloride on rat hepatic ultrastructure. *Tox. Appl. Pharm.* 30:14-25.

- Dambrauskas, T. and Cornish, H.H. 1970. Effect of pretreatment of rats with carbon tetrachloride on tolerance development. *Tox. Appl. Pharm.* 17:83-97.
- Diaz-Gomez, M.I., DeCastro, C.R., D'Acosta, N., DeFenos, O.M., De Ferreyra, E.C. and Castro, J.A. 1975. Species differences in carbon tetrachloride-induced hepatotoxicity: the role of CCl_4 activation and of lipid peroxidation. *Tox. Appl. Pharm.* 34:102-114.
- Duttera, S.M., Byrne, W.L., and Ganoza, M.C. 1968. Studies on the phospholipid requirement of glucose 6-phosphatase. *J. Biol. Chem.* 243:2216-2228.
- Estler, C.-J., Schmidt, B. and Pesch, H.-J. 1973. Investigation of the accumulation of fat in the kidneys of mice poisoned with carbon tetrachloride. *Arch. Toxikol.* 30:103-110.
- Folland, D.S., Schaffner, W., Ginn, H.E. Crofford, O.B., and McMurray, D.R. 1976. Carbon tetrachloride toxicity potentiated by isopropyl alcohol. *J.A.M.A.* 236:1853-1856.
- Fowler, B.A., Hook, G.E.R. and Lucier, G.W. 1977. Tetrachlorodibenzo-p-dioxin induction of renal microsomal enzyme systems: ultrastructural effects on pars recta (S_3) proximal tubule cells of the rat kidney. *J. Pharm. Exp. Ther.* 203:712-721.
- Fowler, J.S.L. 1969. Carbon tetrachloride metabolism in the rat. *Br. J. Pharmacol.* 37:733-737.
- Glende, E.A., Hruszkewycz, A.M. and Recknagel, R.O. 1976. Critical role of lipid peroxidation in carbon tetrachloride induced loss of aminopyrine demethylase, cytochrome P-450 and glucose-6-phosphatase. *Biochem. Pharmacol.* 25:2163-2170.
- Gooding, P.E., J. Chayen, B. Sawyer, and T.F. Slater. 1978. Cytochrome P-450 distribution in rat liver and the effect of sodium phenobarbitone administration. *Chem.-Biol. Interactions* 20:299-310.
- Guild, W.R., Young, J.V. and Merrill, J.P. 1958. Anuria due to carbon tetrachloride intoxication. *Annals Internal Med.* 48:1221-1227.

- Hook, G.E.R., Haseman, J.K., and Lucier, G.W. 1975. Induction and suppression of hepatic and extrahepatic microsomal foreign-compound-metabolizing enzyme systems by 2,3,7,8-tetrachlorodibenzo-p-dioxin. *Chem-Biol. Interactions* 10:199-214.
- Klaassen, C.D. and Plaa, G.L. 1966. Relative effects of various chlorinated hydrocarbons on liver and kidney function in mice. *Tox. Appl. Pharm.* 9:139-151.
- Klaassen, C.D. and Plaa, G.L. 1969. Comparison of the biochemical alterations elicited in livers from rats treated with carbon tetrachloride, chloroform, 1,1,2-trichloroethane and 1,1,1-trichloroethane. *Biochem. Pharmacol.* 18:2019-2027.
- Kluwe, W.M. and Hook, J.B. 1978. Polybrominated biphenyl-induced potentiation of chloroform toxicity. *Tox. Appl. Pharm.* 45:861-869.
- Kluwe, W.M., Herrmann, C.L. and Hook, J.B. 1979. Effects of dietary polychlorinated biphenyls and polybrominated biphenyls on the renal and hepatic toxicities of several chlorinated hydrocarbon solvents in mice. *J. Tox. Envir. Hlth.* 5:605-615.
- Kornbrust, D.J. and Mavis, R.D. 1979. Microsomal lipid peroxidation: I. Characterization of the role of iron and NADPH; II. Stimulation by carbon tetrachloride. Manuscript submitted.
- Korsrud, G.O., Grice, H.C. and McLaughlan, J.M. 1972. Sensitivity of several serum enzymes in detecting carbon tetrachloride-induced liver damage in rats. *Tox. Appl. Pharm.* 22:474-483.
- Lautt, W.W. and Plaa, G.L. 1974. Hemodynamic effects of CCl_4 in the intact liver of the cat. *Can. J. Physiol. Pharmacol.* 52:727-735.
- Leach, L.J. 1963. A laboratory test chamber for studying air-borne material. Univ. Rochester Atomic Energy Project Report UR-629.
- Lieber, C.S. 1973. Hepatic and metabolic effects of alcohol (1966-1973). *Gastroenterology* 65:821-845.
- Lindstrom, T.D. and Anders, M.W. 1978. Effect of agents known to alter carbon tetrachloride hepatotoxicity and cytochrome P-450 levels on carbon tetrachloride-stimulated lipid peroxidation and ethane expiration in the intact rat. *Biochem. Pharmacol.* 27:563-567.

- Litterst, C.L., Mimnaugh, E.G., Reagan, R.L. and Gram, T.E. 1975. Comparison of in vitro drug metabolism by lung, liver and kidney of several common laboratory species. *Drug Metab. Dispos.* 3:259-265.
- Lowry, O.H. and Passoneau, J.V. 1972. *A Flexible System of Enzymatic Analysis.* Academic Press, New York.
- Lowry, O.H., Rosebrough, N.J., Farr, A.L., and Randall, R.J. 1951. Protein measurement with the Folin phenol reagent. *J. Biol. Chem.* 193:265-275.
- Lundh, H.A.B. 1964. Sequence comparison between kidney and liver lesions in the rat following carbon tetrachloride poisoning. *J. Occup. Med.* 6:123-128.
- Mailman, R.B., Edmundson, W., Muse, K., and Hodgson, E. 1977. Multiplicity of hepatic cytochrome P-450 in intact microsomes: effect of 3-methylcholanthrene induction. *Gen. Pharmac.* 8:281-284.
- Maling, H.M., Eichelbaum, F.M., Saul, W., Sipes, I.G., Brown, E.A.B., and Gillette, J.R. 1974. Nature of the protection against carbon tetrachloride-induced hepatotoxicity produced by pretreatment with Dibenamine [N-(2-chloroethyl) dibenzylamine]. *Biochem. Pharmacol.* 23:1479-1491.
- Maling, H.M., Stripp, B., Sipes, I.G., Highman, B., Saul, W., Williams, M.A. 1975. Enhanced hepatotoxicity of carbon tetrachloride, thioacetamide, and dimethylnitrosamine by pretreatment of rats with ethanol and some comparisons with potentiation by isopropanol. *Tox. Appl. Pharm.* 33:291-308.
- Markham, T.N. 1967. Renal failure due to carbon tetrachloride. *J. Occup. Med.* 9:16-17.
- McCollister, D.D., Beamer, W.H., Atchison, G.J. and Spencer, H.C. 1951. The absorption, distribution and elimination of radioactive carbon tetrachloride by monkeys upon exposure to low vapour concentrations. *J. Pharmacol. Exp. Ther.* 102:112-124.
- McCormack, K.M., Kluwe, W.M., Rickert, D.E., Sanger, V.L. and Hook, J.B. 1978. Renal and hepatic microsomal enzyme stimulation and renal function following three months of dietary exposure to polybrominated biphenyls. *Tox. Appl. Pharm.* 44:539-553.

- McMurtry, R.J., Snodgrass, W.R. and Mitchell, J.R., 1978. Renal necrosis, glutathione depletion, and covalent binding after acetaminophen. *Tox. Appl. Pharm.* 46:87-100.
- Muller, D., Forster, D., Dietze, H, Langenberg, R. and Klinger, W. 1973. The influence of age and barbitol treatment on the content of cytochrome P-450 and b₅ and on the activity of glucose-6-phosphatase in microsomes of the rat liver and kidney. *Biochem. Pharmacol.* 22:905-910.
- New, P.S., Lubash, G.D., Scherr, L., and Rubin, A.L. 1962. Acute renal failure associated with carbon tetrachloride intoxication. *J.A.M.A.* 181:197-200.
- Orme-Johnson, W.H. and Ziegler, D.M. 1965. Alcohol mixed function oxidase activity of mammalian liver microsomes. *Biochem. Biophys. Res. Commun.* 21:78-82.
- Perassi, R. and Martin, A. 1973. Urinary proteins of the rat during CCl₄ poisoning. *Pathol. Biol.* 21:569-572.
- Plaa, G.L. and Larson, R.E. 1965. Relative nephrotoxic properties of chlorinated methane, ethane, and ethylene derivatives in mice. *Tox. Appl. Pharm.* 7:37-44.
- Plaa, G.L. and Traiger, G.J. 1973. Mechanism of potentiation of CCl₄-induced hepatotoxicity. *Pharmacology and the Future of Man. Proc. 5th Int. Congr. Pharmacology, San Francisco, vol. 2, 100-113 (Karger, Basel, 1973).*
- Powis, G. 1975. Effect of a single oral dose of methanol, ethanol and propan-2-ol on the hepatic microsomal metabolism of foreign compounds in the rat. *Biochem J.* 148:269-277.
- Recknagel, R.O. and Glende, E.A., 1973. Carbon tetrachloride hepatotoxicity: an example of lethal cleavage. *CRC Critical Reviews in Toxicology* 2:263-297.
- Recknagel, R.O. and Lombardi, B. 1961. Studies of biochemical changes in subcellular particles of rat liver and their relationship to a new hypothesis regarding the pathogenesis of carbon tetrachloride fat accumulation. *J. Biol. Chem.* 236:564-569.
- Reynolds, E.S. 1963. Liver parenchymal cell injury. I. Initial alterations of the cell following poisoning with carbon tetrachloride. *J. Cell Biol.* 19:139-157.

- Reynolds, E.S. and Yee, A.G. 1968. Liver parenchymal cell injury. VI. Significance of early glucose 6-phosphatase suppression and transient calcium influx following poisoning. *Lab. Invest* 19:273-281.
- Savolainen, H., Vainio, H., Helojoki, M. and Elovaara, E. 1978. Biochemical and toxicological effects of short-term, intermittent xylene inhalation exposure and combined ethanol intake. *Arch. Toxicol.* 41:195-205.
- Schlartz, L. 1971. Calcium and hormone interactions with isolated liver plasma membranes. Ph.D. Thesis, Univ. of Rochester, Rochester, N.Y.
- Schmidt, U. and Guder, W.G. 1976. Sites of enzyme activity along the nephron. *Kidney International* 9:233-242.
- Sipes, I.G., Stripp, B., Krishna, G., Maling, H.M., and Gillette, J.R. 1973. Enhanced hepatic microsomal activity by pretreatment of rats with acetone or isopropanol. *Proc. Soc. Exp. Biol. Med.* 142:237-240.
- Sirota, J.H. 1949. Carbon tetrachloride poisoning in man. The mechanism of renal failure and recovery. *J. Clin. Invest.* 28:1412-1422.
- Smuckler, E.A. 1976. Structural and functional changes in acute liver injury. *Environ. Hlth Perspect.* 15:13-25.
- Srinivasan, S. and Recknagel, R.O. 1973. Ultraviolet spectra of rat kidney lipids after carbon tetrachloride administration. *Exp. Mol. Pathol.* 18:214-218.
- Striker, G.E., Smuckler, E.A., Kohnen, P.W., Nagle, R.B. 1968. Structural and functional changes in rat kidney during CCl₄ intoxication. *Amer. J. Pathol.* 53:769-789.
- Traiger, G.J. and Bruckner, J.V. 1976. The participation of 2-butanone in 2-butanol-induced potentiation of carbon tetrachloride hepatotoxicity. *J. Pharm. Exp. Ther.* 196:493-500.
- Traiger, G.J. and Plaa, G.L. 1971. Differences in the potentiation of carbon tetrachloride in rats by ethanol and isopropanol pretreatment. *Tox. Appl. Pharm.* 20:105-112.
- Traiger, G.J. and Plaa, G.L. 1973. Effect of aminotriazole on isopropanol- and acetone-induced potentiation of CCl₄ hepatotoxicity. *Can. J. Physiol. Pharmacol.* 51:291-296.

- Ugazio, G., Koch, R.R., and Recknagel, R.O. 1973. Reversibility of liver damage in rats rendered resistant to carbon tetrachloride by prior carbon tetrachloride administration: bearing on the lipoperoxidation hypothesis. *Exp. Mol. Pathol.* 18:281-289.
- Villarruel, M. del C., Toranzo, E.G.D. de, and Castro, J.A. 1977. Carbon tetrachloride activation, lipid peroxidation, and the mixed function oxidase activity of various rat tissues. *Tox. Appl. Pharm.* 41:337-344.
- Villarruel, H.C., Toranzo, E.G.D. and Castro, J.A. 1978. Carbon tetrachloride activation and lipid peroxidation in different rat tissues: Its relation to mixed function oxygenase activity (abstract only). In: *Proc. 1st Internat. Congress on Toxicol.*, G.L. Plaa and W.A.M. Duncan (eds.) Academic Press, 1978.
- Watrous, W.M. and Plaa, G.L. 1972a. Effect of halogenated hydrocarbons on organic ion accumulation by renal cortical slices of rats and mice. *Tox. Appl. Pharm.* 22:528-543.
- Watrous, W.M. and Plaa, G.L. 1972b. The nephrotoxicity of single and multiple doses of aliphatic chlorinated hydrocarbon solvents in male mice. *Tox. Appl. Pharm.* 23:640-649.
- Wills, E.D. 1971. Effects of lipid peroxidation on membrane-bound enzymes of the endoplasmic reticulum. *Biochem. J.* 123:983-991.
- Wong, L.C.K. and DiStefano, V. 1966. Rapid accumulation of renal fat in cats after single inhalations of carbon tetrachloride. *Tox. Appl. Pharm.* 9:485-494.
- Woods, W.W. 1946. The changes in the kidneys in carbon tetrachloride poisoning, and their resemblance to those in the "Crush Syndrome". *J. Path. Bact.* 58:767-773.
- Zenser, T.V., Mattammel, M.B. and Davis, R.R. 1978. Differential distribution of the mixed-function oxidase activities in rabbit kidney. *J. Pharm. Exp. Ther.* 207:719-725.