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THE ROLE OF LIVER LIPID IN THE POTENTIATION OF CARBON TETRACHLORIDE TOXICITY

BY

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A dissertation submitted in partial fulfillment
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THE ROLE OF LIVER LIPID IN THE ALCOHOL POTENTIATION OF CARBON TETRACHLORIDE TOXICITY

By Raymond E. Bahor

The increase in hepatic lipid observed following the ingestion of ethanol has been implicated as a factor in the potentiation of toxicity of carbon tetrachloride. It has been suggested that increased amounts of liver lipid would favor the partition of the hydrocarbon into the liver which would result in increased tissue damage. This investigation was initiated to determine the relationship of lipid to the hepatotoxicity of carbon tetrachloride.

To determine whether the enhanced toxicity of carbon tetrachloride is manifested only in animals with elevated hepatic lipid levels, the effects of various doses of carbon tetrachloride were measured in rats with hepatic steatosis induced by high fat or choline deficient diets, ethionine, and orotic acid. In other studies, the toxic effects of carbon tetrachloride were investigated in ethanol treated rats whose hepatic steatosis was inhibited by adrenalectomy, cord section, or hypothermia.

The toxic effects of carbon tetrachloride were measured in various ways. Changes in serum levels of glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, isocitric dehydrogenase, and iron served as indirect indices of hepatic alteration.

Hepatic concentrations of pyridine nucleotides, total lipids, and carbon tetrachloride were determined following administration of carbon tetrachloride and/or ethanol. Morphological changes produced by various treatments were estimated from histopathological examination of various tissues.

Nine hours after ingestion of ethanol (5ml/kg) an increase in total hepatic lipid was observed which disappeared within twenty-four hours after dosing. Ingestion of ethanol eighteen hours prior to administration of various doses of carbon tetrachloride resulted in markedly elevated levels of serum enzyme activity and serum iron concentration. Carbon tetrachloride also caused more extensive morphological changes in the liver of animals pretreated with ethanol.

Morphological and serum enzyme changes produced by carbon tetrachloride were similar in control rats and those with hepatic steatosis induced by choline deficiency or orotic acid. Prior ingestion of ethanol rather than total lipidic changes appeared to be necessary for enhanced toxicity of carbon tetrachloride. Only animals pretreated with ethanol showed marked elevations in serum enzymes, iron or liver pyridine nucleotides following carbon tetrachloride administration. Increased blood or liver concentrations

of carbon tetrachloride could not be detected in animals treated with ethanol or the other steatogenic agents.

Hepatic lipid levels remained normal following ethanol ingestion by adrenalectomized or cord sectioned rats, but serum enzyme and iron levels were greatly increased after carbon tetrachloride administration to those animals. The concentration of carbon tetrachloride in blood and liver of intact, adrenalectomized or cord sectioned animals given the solvent was independent of prior treatment with ethanol or amount of hepatic lipid present. Morphological changes were more extensive in the animals treated with both carbon tetrachloride and ethanol than in those given carbon tetrachloride alone. Since no protective effect against the enhanced toxicity of carbon tetrachloride was observed when ethanol induced fatty liver was prevented, hepatic lipid accumulation cannot be presumed to be the causative mechanism of potentiation.

Sorbitol, which is metabolized similarly to ethanol, exacerbated the toxicity of carbon tetrachloride. Serum enzyme activities were elevated in animals given sorbitol eighteen hours prior to carbon tetrachloride exposure. Fatty accumulation of the liver was not produced following sorbitol ingestion. When total metabolic activity was reduced by hypothermia, liver damage did not occur following administration of carbon tetrachloride or ethanol and carbon tetrachloride.

In summary, enhanced toxicity of carbon tetrachloride was not observed when hepatic steatosis was induced by agents other than ethanol. Conversely when hepatic steatosis following ethanol ingestion was inhibited, enhanced toxicity of carbon tetrachloride still was observed. It was concluded that ethanol induced fatty change in the liver is not necessary for potentiation of toxicity of carbon tetrachloride.

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LIST OF ABBREVIATIONS

ATP	Adenosine triphosphate
CCl_4	Carbon Tetrachloride
ETOH	Ethanol
GOT	Glutamic oxaloacetic transaminase
GPT	Glutamic pyruvic transaminase
H & E	Hematoxylin and eosin
ICD	Isocitric dehydrogenase
MDH	Malic dehydrogenase
NAD	Nicotinamide adenine dinucleotide, oxidized form
$NADH_2$	Nicotinamide adenine dinucleotide, reduced form
NADP	Nicotinamide adenine dinucleotide phosphate, oxidized form
$NADPH_2$	Nicotinamide adenine dinucleotide phosphate, reduced form

I. INTRODUCTION

Since its preparation by Regnault in 1839, carbon tetrachloride has found diverse medical and industrial applications. Because of its wide use, the literature abounds in cases of accidental or intentional intoxication. With the recognition of the anesthetic properties of chloroform in 1847, carbon tetrachloride was similarly employed by Simpson in 1865 (66). As an anesthetic, carbon tetrachloride enjoyed some degree of popularity for the next ten years. However, its toxicity in this case was soon appreciated and it fell into disfavor. In this regard Nunneley, (122), one of the first to use carbon tetrachloride as an anesthetic wrote, "The boundary between insensibility and death appears to be so narrow as in practice not to be capable of regulation."

At the turn of the century, when fashion decreed that women should wear their hair long, carbon tetrachloride was used as a dry shampoo. After several accidents, with some fatalities, its use as a shampoo was discontinued. Because of its solvent properties for fats, waxes, rubber, oil, and greases, carbon tetrachloride has been widely used as a dry cleaner and industrial solvent. In 1915, Alice Hamilton (64) reported signs of intoxication occuring among rubber workers exposed to carbon tetrachloride. Since that time, frequent reports of toxicity resulting from the industrial use of carbon tetrachloride have appeared in the literature. In 1922, Starr (166) re-

ported 22 cases of poisoning resulting from the use of carbon tetrachloride in an adhesive. Davis in 1929 (35) reported additional cases of intoxication from the organic halide as used in the rubber industry, and extended his study to other industrial applications in 1934 (34). On the basis of many reports of industrial toxication and studies of toxicity in animals, the threshold limit value of carbon tetrachloride, as defined by the American Conference of Governmental Industrial Hygienists (174), has been lowered to 10 ppm.

In 1921, the effectiveness of carbon tetrachloride as an anthelminthic was reported by Hall (62, 63). This work was confirmed by Lake (90), and carbon tetrachloride has been used extensively in the Philippines (96), Brazil (161), Ceylon (38, 119) and the Fiji Islands (91) as a vermicidal agent against hookworm. In a report on its use in 50,000 cases in the Fiji Islands, a few deaths were reported which were regarded as hypersensitivity to the drug. In Brazil, toxic symptoms were observed in two patients who were alcoholics. Several reports of toxicity and deaths resulting from the anthelminthic use of carbon tetrachloride followed (73, 130), and extensive animal experimentation was undertaken to investigate the toxic effects of the chlorinated hydrocarbon. Although carbon tetrachloride has been replaced by tetrachloroethylene as an anthelminthic, interest in the physiological and pharmacological actions of the compound has continued.

Early studies by Lamson (92, 93, 94) et al., Gardner et al (54), Robbins (143) elucidated the toxic nature of carbon tetrachloride and described the characteristic pathological changes associated with intoxication. Carbon tetrachloride was shown to produce a diffuse central necrosis and fatty accumulation of the liver and lower nephron nephrosis of the kidney. Degenerative changes in adrenals and blood dyscrasias have been reported. The reports of toxicity of carbon tetrachloride have been summarized in reviews by von Oettingen (123), Lewis (101), Chenoweth and Hake (28), and others.

Although the nephrotoxic and hepatotoxic properties of carbon tetrachloride have been recognized for some time, the mechanism of this action has been studied only recently. One of the early investigations of the site of action of carbon tetrachloride was made by Judah and Christie (80). These workers and others (81) suggested that carbon tetrachloride caused mitochondrial disruption which resulted in fatty accumulation and death of the hepatocyte, through changes in permeability and fatty acid oxidation. Although this hypothesis has found some support, Calvert and Brody (26) have found that fatty accumulation preceded mitochondrial changes and these mitochondrial changes could be alleviated by the administration of ethylene-diamine-tetra-acetic acid. This compound, which is capable of chelating calcium, demonstrated a marked protective effect on mitochondrial integrity. Following administration of carbon tetrachloride, Reynolds et al (138, 140) observed in-

creased concentrations of mitochondrial calcium which was accompanied by a decrease in potassium levels. Electron microscopy revealed early changes in mitochondrial architecture, which may cause interference with transport mechanisms and metabolism of the cell.

To explain the lipid changes that occur following carbon tetrachloride administration, Recknagel and others (6, 133, 138, 152) have postulated another mechanism of toxic action. Since maximum concentration of carbon tetrachloride in the liver occurred with a concomitant increase in triglycerides and changes in the endoplasmic reticulum, these workers have proposed that interference with a triglyceride secreting mechanism is the primary lesion. Upon the administration of Triton, a non-ionic detergent which causes marked increases in serum triglyceride, and carbon tetrachloride, the secretion of neutral fat from the liver was inhibited. Rossi (146) has reported an inhibition of fatty acid activation following intoxication, and Robinson and Seakins (144) have demonstrated accumulation of hepatic lipid following inhibition of protein synthesis. Similarly, Smuckler et al (163, 164) observed an inhibition of C^{14}-glycine incorporation into protein of mitochondria, microsomes, and supernatant protein of rats poisoned with carbon tetrachloride. These effects were accompanied by changes in the endoplasmic reticulum.

The work of Brody and co-workers (19, 20, 27, 170) has revealed the mediation of the sympathetic nervous system in carbon

tetrachloride intoxication. The administration of adrenergic blocking agents prior to administration of carbon tetrachloride effectively prevented fatty accumulation of the liver. Adrenalectomy and cord section at the C-7 level were both effective in preventing this fatty change. In addition, increased urinary excretion and depleted adrenal gland levels of catecholamine were found following carbon tetrachloride intoxication. Thyroid hormones, which increase body temperature and the oxygen consumption of hepatocytes were found to exacerbate the toxicity of carbon tetrachloride. These observations suggested that carbon tetrachloride exerted its toxic effect by producing a massive adrenergic discharge and the resulting vasoconstriction induced anoxic parenchymal changes. Recently, Larson and Plaa (95) have extended this hypothesis. Cervical cordotomy, which induces hypothermia, prevents or delays necrotic and fatty changes following carbon tetrachloride intoxication. By inducing hypothermia in intact rats, these workers were able to demonstrate similar protective effects against carbon tetrachloride. The oxygen consumption of these hypothermic animals was reduced to 50% of the normal value. This reduction in the metabolic rate was suggested as the mechanism of protection against carbon tetrachloride toxicity.

Although the toxic manifestations of carbon tetrachloride intoxication have been well documented, the metabolism of carbon tetrachloride is not well understood. The greater portion of an administered

dose of carbon tetrachloride is excreted unchanged through the lungs. McCollister et al (113) have reported that half the activity of a dose of C^{14}-CCl_4 could be recovered in urine as urea, bicarbonate, carbon dioxide, and possibly a glucuronide (the latter has not been definitely identified). The hypothesis that carbon tetrachloride exerts its toxic action through one of its metabolites has been suggested by several workers. One of the early investigators, Graham (58), proposed that hydrochloric acid was formed by carbon tetrachloride metabolism, and that pathological changes were induced through the action of this product. This hypothesis could not be confirmed by later workers, but more recent investigation has suggested that metabolites may play a role in carbon tetrachloride poisoning. Butler (24) has shown that carbon tetrachloride was converted to chloroform both in vivo or with tissue homogenates. The author proposed that this conversion resulted from the cleavage of carbon tetrachloride with the formation of free radicals. The highly reactive free radicals could combine with proteins or other cellular constituents to produce a deleterious effect. Paul and Rubinstein (125) have confirmed the dehalogenation of carbon tetrachloride and suggested that the reaction is enzyme catalyzed. The dehalogenation was stimulated in rat liver slices by acetate and citrate, and inhibited by such metabolic poisons as iodoacetate, fluoride, arsenate, cyanide, or acid, alkali, or heat treatment of the tissue. Similarly, Reynold (139) observed that following dosage with 0.1 gm/kg

$C^{14}Cl$, 10% of the non-volatile hydrocarbon was recovered in the acid soluble fraction of rat liver, this amount increased to 30% following administration of 4 gms/kg $C^{14}Cl_4$. Concomitantly, C^{14} protein labeling decreased from 40% with the lower dose to 10% with the higher dose. No incorporation of C^{14}, derived from carbon tetrachloride, was observed in either nuclear DNA or nuclear or cytoplasmic RNA. With both large and small doses approximately 1 micromole of carbon derived from carbon tetrachloride was bound per gram liver within two hours after dosing. This observation suggests that liver parenchymal damage may be due in part to the formation of active free radicals which are capable of acting as alkylating agents, or to the direct reaction with carbon tetrachloride.

In numerous reports of clinical and experimental intoxication with carbon tetrachloride, ethanol has been implicated in the development of severe poisoning. As mentioned above, Pessoa (161) observed toxic symptoms involving the liver and kidney in two alcoholic patients treated for hookworm with carbon tetrachloride. Gardner et al. (54), and Lamson and Wing (92) demonstrated the increased hepatotoxicity of carbon tetrachloride when administered with alcohol. Gardner and associates found that liver damage and death rate produced by carbon tetrachloride was greater with comcomitant ethanol administration and increasing the dose of alcohol did not affect the apparent degree of hepatotoxicity. Robbins (143) has shown that gastrointestinal absorption of carbon tetrachloride was increased when given in con-

junction with ethanol. However, when the route of exposure to carbon tetrachloride is respiratory, this mechanism of increased intestinal absorption would not apply. In addition, ingestion of alcohol after carbon tetrachloride has also been shown to pre-dispose to serious injury, (79, 123). Guild and co-workers (60) described twenty cases in which ingestion of ethanol was implicated in severe poisoning by carbon tetrachloride. It was noted that severe intoxication could result from inhalation of carbon tetrachloride accompanied by ethanol ingestion. These workers suggested that carbon tetrachloride could react with ethanol to produce ethyl chloroformate which could be a renal toxin. New and associates (118) have presented numerous cases in which acute renal failure in carbon tetrachloride intoxication was associated with alcohol ingestion. Similarly, Joron and others (79) have reported severe intoxication from carbon tetrachloride inhalation when ingestion of ethanol occurred some time before the exposure. Several reports by Smetana (160), Moon (116), Cohen (29), Nicholson (120), and Jennings (77) have confirmed the potentiation of toxicity of carbon tetrachloride by ethanol.

Although many clinical and experimental observations have confirmed the potentiation of carbon tetrachloride toxicity by ethanol, the mechanism of this action has not been defined. The severe intoxication noted among chronic alcoholics lead Guild (60) to suggest that choline deficiency may be implicated in the potentiation of toxicity. Choline deficiency has been shown to produce a kidney lesion in young

rats (67, 59). Klatskin and Krehl (84) have shown that rats on a minimal protein diet (similar to alcoholics) exhibited an elevated choline requirement after chronic ingestion of alcohol. This increased choline requirement was attributed to the alcohol directly, and not related to total caloric intake. Rats fed an equivalent caloric dose of sucrose did not exhibit an elevated choline requirement. The addition of methione prevented the appearance of cirrhosis in rats given alcohol. It was suggested by New (118) that choline deficiency "May point to an important pathogenic factor in man" in the potentiation of toxicity. However, increased toxic response to carbon tetrachloride has been reported following a single acute dose of ethanol when chronic choline deficiency was not implicated. As mentioned above, Guild and associates (60) suggested that a toxic compound, ethylchloroformate, may form upon simultaneous ingestion of ethanol and carbon tetrachloride. These workers suggest that the severe toxic symptoms may be due to the effects of this compound. However, potentiation of carbon tetrachloride toxicity has been reported (79) when the ingested dose of ethanol had been largely metabolized and excreted prior to exposure. The in vivo oxidation of carbon tetrachloride to phosgene, and the nephro- or hepatotoxicity of ethylchloroformate have not been demonstrated.

Stewart and others (171) have demonstrated that ingestion of alcohol at the time of exposure to carbon tetrachloride vapors in-

creased the blood concentration of the organic halide in rabbits. These authors suggested that the high solubility of carbon tetrachloride in ethanol may account for its increased concentration found in blood when alcohol was present. Because of its high lipid solubility, it has been suggested by several authors (60, 118, 120) that the increased hepatotoxicity of carbon tetrachloride caused by ethanol may be related to hepatic lipid. Since the ingestion of ethanol results in the fatty accumulation of the liver, it has been suggested that a greater concentration of the lipid soluble carbon tetrachloride would be developed in this vulnerable organ. Analysis of tissue concentrations of carbon tetrachloride have not been reported to confirm this hypothesis.

Recently, Kutob and Plaa (89) have investigated the effect of acute ethanol intoxication on chloroform-induced liver damage in mice. Following acute intoxication with ethanol, an increase in liver lipid was demonstrated by these authors. Measurement of liver function by bromsulphalein retention and pentobarbital sleeping time showed increased liver impairment in animals treated with the organic halide. Since ethanol had been metabolized completely at the time of chloroform administration, no direct interaction between these agents was assumed. Since the increased hepatotoxicity was observed following a single acute dose of alcohol, this implies that the enchanced susceptibility was mediated by factors not related to nutritional complications resulting from chronic ethanol ingestion. These authors claimed that the elevated liver lipid level favored the partition of chloroform into this

organ and caused increased hepatotoxicity. These authors extended their observations on the potentiation of chloroform toxicity by ethanol to include carbon tetrachloride and suggest that a similar action occurs with this halogenated hydrocarbon.

PURPOSE OF THE INVESTIGATION

This investigation was initiated to study the role of hepatic lipids in the potentiation of carbon tetrachloride toxicity by ethanol. The objective of this study was to determine whether ethanol induced fatty accumulation of the liver was causally related to the enhanced toxicity of carbon tetrachloride or whether other factors or biological imbalances could be involved. The experiments, which were designed to clarify these relationships, may be divided into three categories:

1. The toxic response following carbon tetrachloride administration at various dosages was compared in normal and ethanol treated animals. On the basis of these studies, various parameters were chosen to measure the extent of the toxic response and the relationships were evaluated.

2. Lipid accumulation of the liver was induced in animals by agents other than ethanol, and the toxic response following carbon tetrachloride administration was observed.

3. Hepatic lipid levels were maintained at normal values following ethanol ingestion and the toxic response to carbon tetrachloride was measured.

II. METHODS

II-A -- ANALYSIS OF LIVER LIPID

For the determination of hepatic lipid concentrations, a modification of the method of Folch (48) was used.

PROCEDURE

Immediately after sacrifice of the animal, a 4-6 gm section of liver was excised, rinsed in distilled water, and dried with filter paper. The liver specimen was weighed accurately and frozen until analysis could be performed. At the time of analysis, samples were thawed and transferred to the macro vessel of a Virtis 45 homogenizer. Homogenization of tissue was made in a convenient volume of chloroform-methanol (3:1 v/v). The homogenate was transferred to a 250 ml erlenmeyer flask and the volume was adjusted to make a 1:20 homogenate in the chloroform-methanol solvent. The homogenate was boiled for ten minutes on a steam bath and filtered through a medium sintered glass filter. The filter cake was rinsed with several portions of hot solvent.

The filtrate was transferred to a 250 ml beaker and the filter cake reserved for dry weight determination. The filtrate was evaporated to dryness at room temperature in a dynamic flow hood.

Petroleum ether (b.p. 30-60°C) was added, and the residue dissolved with heating on a steam bath. Solution and trituration of the

residue was repeated 6-7 times and the petroleum ether fraction was filtered into a dry, tared 50 ml Erlenmeyer flask. Petroleum ether was evaporated with an air stream and samples placed in a vacuum oven. Samples were dried to constant weight over silica gel at 55°C in a vacuum oven. Weight of total lipid was determined by difference and calculation of percent lipid was made on the basis of wet weight of the liver sample, or the dry weight of the filter cake.

II-B -- DETERMINATION OF SERUM ENZYME ACTIVITY

INTRODUCTION

Early in the investigation of the enzymatic function of proteins, the diagnostic significance of serum enzyme activity was recognized. In 1908, Wolhgemuth, and shortly afterwards, Rona demonstrated the use of blood lipase and amylase determinations for diagnosis of pancreatic disease. With increased interest in the biochemical and biological significance of serum enzyme activity and with improved methods of determination, many reports of serum enzyme levels appeared in the literature. When the correlation of clinical changes with serum enzyme activity was recognized, a new stimulus was given to applied enzymology. Because of the sensitivity and specificity of enzyme reactions, many enzymes not normally found in plasma have been identified in serum following tissue damage. The appearance of these enzymes in the serum serves as an index of cellular damage. Injured cells, through necrosis or changes in permeability, release their enzymes by diffusion into the extracellular space. These enzymes are then distributed to the serum, inactivated and eliminated. The increase in serum enzyme activity observed following cellular damage is related to the extent of pathological changes, the rate of enzyme liberation and distribution, and the rate of elimination and inactivation of the protein. As a function of cellular damage, the determination of serum enzyme activity has found widespread clinical applications.

In 1955, Wroblewski (189) applied the measurement of serum glutamic-oxaloacetic transaminase levels to detect the liver lesion induced by carbon tetrachloride. It was observed that transaminase activity rose markedly following carbon tetrachloride intoxication. Reports by Cornish and Block (32) have shown the application of this enzyme determination to assess the relationship between extent of carbon tetrachloride exposure and hepatotoxicity. Other workers (47, 49, 74, 98, 186) have reported the application of transaminase determination as a liver function test. Following intoxication with carbon tetrachloride, Fleisher and Wakim (47) have reported that electrophoretic examination of the enzyme showed that it could be separated into two fractions (GOT I and GOT II) after carbon tetrachloride poisoning in the dog. Maximal concentration of GOT II, which accounted for 16% of the total activity was produced within 48 hours after administration of the hydrocarbon. No GOT II activity was observed in control animals. Similarly, electrophoretic examination of esterase by Cornish (31) demonstrated the appearance of three additional esterases following intoxication with carbon tetrachloride.

The interest in the correlation of serum enzyme activity with pathological changes induced by carbon tetrachloride has produced many reports in the literature. Because of its organ specificity, ornithine transcarbamylase, which is found exclusively in the liver, was measured by Reichard (136) after administration of carbon tetra-

chloride in the dog. Marked increases in the serum activity of this enzyme were observed following administration of the hydrocarbon. Several other enzymes have been reported in increased amounts in the serum following carbon tetrachloride administration. Among these enzymes are: alkaline and acid phosphatase (88), arginase (124), esterase (32), fumarase (56), lactic dehydrogenase, malic dehydrogenase (49), quinine oxidase (177),and xanthine oxidase (10, 32, 177, 178).

In this study, measurement of serum glutamic oxalacetic transaminase (S-GOT), glutamic pyruvic transaminase (S-GPT), and isocitric dehydrogenase (S-ICD) was used primarily to evaluate the extent of tissue injury. The clinical application of these serum enzyme measurements has been well established for the diagnosis of myocardial infarction and liver lesions. The examination of serum transaminase levels provides a clue to the severity of cellular damage. Although both GOT and GPT are present in liver, their localization within the cell is different. Glutamic pyruvic transaminase is freely soluble in the cytoplasm whereas 40% of the GOT activity is bound to cellular structures. Following cellular injury, GPT migrates rapidly into the serum to produce the characteristic elevation in serum activity. The kinetics of GOT release from the liver cell is influenced by its greater association with cellular membranes. Because of this difference in the rate of release, serum activity of S-GPT may be higher than that of S-GOT even though the

liver activity of the latter is about one third greater. The De Ritis ratio (activity GOT/GPT) may be less than unity in cases of parenchymatous jaundice in infectious hepatitis (142).

An elevation in serum isocitric dehydrogenase has been associated with acute hepatic injury by Wolfson et al. (187) and by Sterkel (169). The measurement of S-ICD has been widely applied clinically a) to demonstrate acute hepatic injury, b) to follow the course of viral hepatitis, c) to detect metastatic malignancy involving the liver, and d) in the differential diagnosis of intrahepatic versus extrahepatic obstructive jaundice (156).

PROCEDURE

Animals were sacrificed by decapitation and blood was collected in 15 ml centrifuge tubes. Samples were allowed to clot for thirty minutes in a cold room and centrifuged to separate serum. All serum samples were centrifuged again to remove any remaining erythrocytes. Only samples which were free of visible hemolysis were used for enzyme activity determinations.

DETERMINATION OF GLUTAMIC OXALACETIC TRANSAMINASE IN SERUM (156)

The enzyme glutamic oxalacetic transaminase catalyzes the following reaction (I):

$$HOOC\ CH_2\ CH\ NH_2\ COOH + \alpha\text{-}\ HOOC(CH_2)_2COCOOH \longrightarrow$$

Aspartic Acid — α- Keto Glutaric Acid

$$HOOC\ CH_2COCOOH + HOOC\ CH_2CH_2CHNH_2COOH$$

Oxalacetic Acid — Glutamic Acid

Since no readily measurable difference in optical density occurs by the removal or disappearance of reactants, reaction (I) is coupled with the following reaction (II).

$$\underset{\text{Oxalacetic Acid}}{\text{HOOC CH}_2\text{CO COOH}} + \beta\text{-NADH}_2 \xrightarrow{\text{MDH}} \underset{\text{Malic Acid}}{\text{HOOC CH}_2\text{CHOH COOH}} + \beta\text{-NAD}$$

Malic dehydrogenase (MDH) catalyzes the reduction of oxalacetic acid (produced in the first reaction) and results in the production of diphosphopyridine nucleotide. Since β-$NADH_2$ has a strong absorption band at 340 mμ, the rate of reaction can be followed readily by the rate of removal of this compound. Measurement of optical density was made at timed intervals employing a Beckman DU, or automatically recorded with the Gilford Biomedical spectrophotometer.

The reaction mixture was pipetted directly into the quartz curvette in the following order:

a) 0.2 ml β-NADH (1 mg/ml).

b) 0.5 ml 0.2M l-aspartic acid in phosphate buffer.

c) 0.1 ml malic dehydrogenase (in excess, containing at least 200 units) Sigma.

d) 1.8 ml distilled deionized water.

e) 0.2 ml serum or diluted serum (serum diluted with phosphate buffer pH 7.5 when activity was high).

The reaction mixture was allowed to come to thermal and chemical equilibrium for at least ten minutes. (Pyruvate in serum

reacts with some of the excess $NADH_2$ and is destroyed). Optical density at 340 mμ was recorded at this time and was at least 0.7 vs. water as a blank.

f) 0.2 ml 0.1M α-ketoglutarate was added and the solution mixed thoroughly.

The decrease in optical density at 340 mμ was measured at timed intervals to determine the rate of disappearance of β-$NADH_2$.

Activity of S-GOT was calculated from the change in optical density observed per minute from the following equation:

$$\text{S-GOT Activity} = \frac{\text{Decrease in O.D. per minute x 1000}}{\text{ml serum used x temp. coefficient x effective light path (1 cm)}}$$

Temperature coefficient factor was obtained from Sigma Technical Bulletin No. 410 (156). Phosphate buffer was prepared from:

5.90 gms K_2HPO_4, reagent anhydrous

0.95 gms KH_2PO_4, reagent anhydrous

Adjusted to pH 7.5 and diluted to 100 ml total volume.

Sigma biochemicals were used throughout this study.

DETERMINATION OF GLUTAMIC-PYRUVIC TRANSAMINASE IN SERUM

Glutamic pyruvic transaminase catalyzes the following reaction (I):

$$CH_3CHNH_2COOH + HOOC(CH_2)_2COCOOH \xrightarrow{GPT} CH_3COCOOH + HOOC\,CH_2CH_2CHNH_2COOH$$

Alanine — α-Keto Glutaric Acid — Pyruvic Acid — Glutamic Acid

Since no readily measurable change in optical density occurs in this reaction, a coupled reaction with lactic dehydrogenase serves as an indicator of the rate of reaction. Lactic dehydrogenase catalyzes the reaction:

$$CH_3COCOOH + \beta\text{-}NADH_2 \xrightarrow{LDH} CH_3CHOHCOOH + \beta\text{-}NAD$$

Pyruvic Acid — Lactic Acid

The pyruvic acid formed in reaction I is reduced to lactic acid with a stoichiometric decrease in β-$NADH_2$. The measurement of decrease in optical density with respect to time at 340 mμ allows the estimation of the rate of reaction I.

To perform this assay reagents were pipetted directly into the curvette in the following order:

a) 0.2 ml β-$NADH_2$, 1 mg/ml.

b) 0.5 ml 0.2 M L-alanine in phosphate buffer

c) 0.1 ml lactic dehydrogenase (Sigma, 8000 units per ml).

d) 1.8 ml deionized distilled water.

e) 0.2 ml serum or dilution of serum (serum diluted with phosphate buffer pH 7.5 when activity was high).

The components were well mixed and allowed to come to thermal and chemical equilibrium. Optical density at 340 mμ was recorded and was at least 0.7 vs. water as a blank.

f) 0.2 ml 0.1 M α-ketoglutaric acid added and the solution mixed thoroughly.

The decrease in optical density at 340 mμ was measured at timed intervals to determine the rate of reaction of β-$NADH_2$.

The activity of S-GPT was calculated from the change in optical density measured per minute employing the following equation:

$$\text{S-GPT Activity} = \frac{\text{Decrease in O.D. per minute} \times 1000}{\text{ml serum used} \times \text{temp. coefficient} \times \text{effective light path (1 cm)}}$$

The temperature coefficient factor was obtained from Sigma Technical Bulletin No. 410. Phosphate buffer was that described in determination of S-GOT (156).

DETERMINATION OF ISOCITRIC DEHYDROGENASE

The ubiquitous enzyme isocitric dehydrogenase catalyzes the following reaction:

$$(HOOC)CH_2CH(COOH)CHOH(COOH) + NADP \xrightarrow{ICD}$$

Isocitric Acid

$$HOOC\ CH_2\ CH(COOH)COCOOH + NADPH_2$$

Oxalosuccinic Acid

The rate of formation of $NADPH_2$ is proportional to the amount of enzyme activity. Since $NADPH_2$ has a strong absorption at 340 mμ, the rate of $NADPH_2$ formation can be determined by

measuring the rate of increase in optical density at this wavelength (157).

The reaction mixture was pipetted directly into a cuvette in the following order:

a) 1.00 ml 0.1 M Tris buffer, pH 7.5

b) 0.50 ml NADP solution, 0.68 mg/ml in 0.15 M sodium chloride

c) 0.50 ml 0.02 M manganese chloride in 0.15 M sodium chloride

d) 0.10 ml d-isocitric acid, 7.5 mg/ml

The reaction mixture was stirred thoroughly and allowed to reach thermal equilibrium within the spectrophotometer chamber.

e) 1 ml of a 1:10 dilutions of serum in 0.15 M NaCl

was added and contents mixed.

The optical density at 340 mμ was recorded at timed intervals and the increase in readings was recorded. The increase in optical density which was linear with respect to time was employed for calculation of S-ICD activity.

Wolfson and Williams-Ashman (187) have defined one unit of serum ICD activity as that amount which causes the formation of one millimicromole of $NADPH_2$ per milliliter of serum per hour at 25°C. This calculation of S-ICD activity was performed employing the following equation:

$$\frac{\text{O. D. min}^{-1} \times 60 \text{ min hr}^{-1}}{\text{ml serum used x temp correction x light path (1 cm) x 0.0020}}$$

where 0.0020 is the optical density of 1 millimicromole of $NADPH_2$ in 3.1 ml of solution. With a 1 cm curvette, at 25° C, the calculation simplifies to:

$$\text{S-ICD Activity/ml serum} = \text{O. D. min}^{-1} \times 30,000$$

DISCUSSION

The relationship of increased serum enzyme activity to tissue damage has been investigated by many workers in an attempt to assess the extent of pathological changes. The migration of enzymes not normally found in plasma into the serum can be accomplished by necrosis and lysis of tissues. However, such drastic morphological changes are not necessary for enzyme efflux into the serum. The cell membrane, under normal conditions, maintains a high concentration gradient of different enzymes between the cell and serum. Enzyme protein is synthesized and concentrated within the cell and the membrane serves as a barrier to the efflux of these components into the extracellular space.

Physiological changes, which can affect the pore size or charge of the cell membrane, may result in the efflux of enzymes into the serum. Under the force of the high concentration gradient, enzymes may diffuse into the serum when the membrane barrier is altered. Strenuous physical activity or passive congestion induces an increase in malic and lactic dehydrogenase (69). Such

anaerobiosis of skeletal muscle produces a reversible physiological increase in cellular permeability. Apparently after strenuous muscular activity, the production of ATP through glycolysis is sufficient to maintain muscular contraction at the expense of membrane permeability. Other investigators, Wu (190), Schade (148), and others have demonstrated the loss of cellular enzymes from isolated cells under anaerobic conditions. Similarly, the administration of such metabolic poisons as fluoracetate, nitrophenol, or iodoacetate produces an increase in serum levels of many enzymes (23). These studies demonstrated the relationship of metabolic control to cellular membrane permeability. Interference with energy yielding reactions may result in insufficient energy to maintain the membrane barrier.

An increased cellular permeability, resulting in increased serum enzyme efflux, may be produced without apparent morphological changes. Henley (69) and others have shown that isolated liver cells could liberate enzymes into the medium while retaining morphological integrity. The changes in permeability may be reversible without destruction of the cell, or irreversible and ultimately leading to lysis of the cell.

Factors, other than changes in permeability, affect the appearance of enzymes in the serum. The level of enzyme activity found in serum is also a function of its release from subcellular

structures, concentration within the cell, rate of inactivation in the serum or proteolytic half-life, and its rate of synthesis. Many investigations have been made to relate the level of serum enzyme activity found to the extent of tissue damage. A correlation between elevation in serum enzyme activity and the morphological extent of tissue damage produced by experimental means has been demonstrated by Wroblewski and co-workers. Dinman (37) and others have correlated hepato-structural changes following carbon tetrachloride intoxication with alterations in serum enzyme activity.

In the present study, serum enzyme levels were determined twenty hours after administration of carbon tetrachloride. By comparison of normal values to those observed in test animals, an evaluation of the extent of toxic tissue changes was obtained. Comparison of values at a fixed time interval (20 hours) would tend to diminish variations in measured enzyme response due to differences in release kinetics.

The term potentiation index has been introduced to furnish a simplified estimation of the alteration of serum enzyme levels. The potentiation index is defined as the ratio of the mean enzyme activity/ml serum found in the test animals compared to that of control animals. This ratio gives a comparison of the increase in serum activity observed in groups of treated animals with that of the control group.

II-C -- ANALYSIS OF SERUM IRON

INTRODUCTION

Reissmann and co-workers (137) have reported the relationship between serum iron concentration and chemotoxic necrosis induced by carbon tetrachloride. These authors were able to correlate the increase in serum iron levels with morphologic alteration of the hepatocytes. A close time relationship was observed between onset of necrosis and increase of serum iron concentration. Twenty four hours after the administration of carbon tetrachloride, increases in serum iron levels were found to correlate with the extent of necrosis produced by carbon tetrachloride. The authors concluded that "serum iron (levels) would provide a unique criterion of actual hepato-cellular necrosis, independent of the remaining liver function and dependent primarily on the volume of liver tissue undergoing disintegration."

PROCEDURE

Analysis of serum iron concentration was performed using a spectrographic technique. Aliquots, 0.5 ml of serum or standard iron solution were evaporated on Ultra Carbon #107 electrodes. The total sample was burned using an AC arc for 45 seconds. Emission patterns were photographed on Kodak SA #3 glass plates. Plates were developed in D-19 for three minutes and calibrated by the two line method. The spectrograph employed was the Bausch and Lomb 2.0 meter grating type with a slit width of 20μ.

The wavelength range examined was 2200-3500Å, second order. The iron emission lines at 3021 and 3024Å were used as analytical wavelengths. Line intensities at these wavelengths were measured using a National Spectrographic Laboratories densitometer. Concentration of serum iron was estimated by comparing the intensity of these lines to that of standard iron solutions. Six standard iron solutions in the range of concentration of serum iron levels were employed.

DISCUSSION

Although increases in serum iron concentration were correlated with production of necrosis, other factors may influence these results. Among these factors, hemolysis of erythrocytes would influence the concentration of serum iron. For this reason, only serum samples which were free of visible hemolysis were used in the analysis of serum iron levels.

Carbon tetrachloride in vitro has been shown (131) to cause hemolysis of erythrocytes. This hemolysis has also been reported in vivo by Takasaka (172) following severe carbon tetrachloride intoxication. An efflux of the enzyme xanthine oxidase into serum has been reported (32, 177) following carbon tetrachloride exposure. This enzyme contains riboflavin as a prosthetic group, and iron and molybdendum are also associated parts of the molecule. It is possible that the increased amount of this enzyme in the serum following carbon tetrachloride exposure may contribute part of the total iron found. It is of parenthetical interest to note that spectrographic examination of sera from animals with elevated serum iron levels showed the presence of molybdenum. No molybdenum was observed in serum when serum iron levels were within the normal range, only when iron was present in excess of 15 ppm was molybdenum detected. The concentration of molybdenum, however, was too low to accurately assess its concentration by the techniques used.

II-D -- LIVER PYRIDINE NUCLEOTIDES

INTRODUCTION

To prevent intoxication from carbon tetrachloride, Gallagher and co-workers (50-53) have found that compounds which stimulate pyridine nucleotide synthesis have proven effective in rats. It was claimed by these authors that the higher mitochondrial coenzyme levels produced, counteracted the loss of respiratory coenzymes caused by the action of carbon tetrachloride. Nicotinic acid was found to stimulate replacement of pyridine nucleotides lost from mitochondria after carbon tetrachloride exposure. The greatest degree of protection against the organic halide was found when nicotinic acid was given two or three days prior to the exposure. The direct precursor of nicotinic acid, dl-tryptophan, also demonstrated some protective effect against carbon tetrachloride intoxication but was less effective. Fiore-Donati (46) observed a similar protective effect using 5-hydroxytryptamine. This compound, it was suggested, probably exerts a sparing effect on tryptophan and effectively increases its concentration.

Following the ingestion of alcohol the level of $NADH_2$, formed as a metabolic by-product, is increased in the liver. Reboucas and Isselbacher (132) and Smith and Newman (162) have reported this increase in hepatic $NADH_2$ and have suggested

that increased fatty acid synthesis may be associated with elevated pyridine nucleotide levels. Although many investigations suggest that $NADPH_2$ is the primary hydrogen donor in fatty acid synthesis, $NADH_2$ is also involved.

Bloomfield and Bloch, (11) and Holloway and co-workers (71) have reported that $NADH_2$ is utilized in the elongation of fatty acids and in the formation of unsaturated fatty acids.

Because of the intimate association of pyridine nucleotides with protection against carbon tetrachloride toxicity and the development of hepatic steatosis, it was considered pertinent to examine nucleotide concentrations in this study. The method described by Gallagher (50) was employed for the analysis of pyridine nucleotides.

PROCEDURE

Animals were sacrificed by decapitation. The liver was excised rapidly, a section (approximately 1 gm) was weighed accurately and placed in a glass homogenizer containing ice cold 0.25 M sucrose. The volume of sucrose was adjusted to make a 5% homogenate (w/v), and tissue was homogenized immediately. Duplicate 0.5 ml samples of this homogenate were quickly transferred to 10 ml centrifuge tubes containing 4.5 ml of 5.56% trichloracetic acid at 0°C. The contents were mixed

thoroughly and incubated at room temperature for ten minutes. The tubes were then centrifuged for five minutes at 2000 rpm. The supernate was drawn off into a quartz cuvette and the optical density at 260 mμ recorded using a Beckman DU spectrophotometer. A blank of 5 percent trichloroacetic acid was used. A standard calibration curve (see appendix) was prepared by appropriate dilution of Sigma purified β-NAD in 5 percent trichloroacetic acid.

Optical density of alkaline extracts of liver homogenates was measured in the following manner. Immediately following excision of the liver a 1 gm portion was weighed accurately and quickly immersed in ice cold 0.25 M sucrose containing 0.1 M nicotinamide in a glass homogenizer. The volume was adjusted to produce a 2 percent homogenate in the solution of 0.25 M sucrose and 0.1 nicotinamide. Following homogenization, 9.0 ml of the homogenate were placed in a plastic centrifuge tube and 1.0 ml of ice cold 1 N sodium hydroxide added with mixing. Homogenates were extracted for 30 minutes, and then centrifuged at 35,000 g for 20 minutes in a refrigerated Beckman Model L ultracentrifuge. The supernatant solution was drawn off into a quartz cuvette and the optical density at 340 mμ measured with a Beckman DU spectrophotometer. As a blank, a solution of 9 ml of 0.25 M sucrose containing 0.1 M nicotinamide mixed with 1.0 ml 1 N sodium hy-

droxide was used. To prepare a standard calibration curve, Sigma purified β-$NADH_2$ was appropriately diluted with this blank solution and optical density at various concentrations measured. (see appendix).

Concentrations of pyridine nucleotides in acid and alkaline extracts were calculated from the calibration curves. Individual pyridine nucleotide concentrations were reported as μgm pyridine nucleotide per gram wet weight liver. The ratio of oxidized to reduced pyridine nucleotide values was calculated for each animal examined. Comparison of this ratio found in normal animals was compared to that found in variously treated animals.

DISCUSSION

This determination of pyridine nucleotides is based upon the direct extraction of the oxidized or reduced forms in acid or alkaline solutions. The measurement of the optical density at the absorption maxima (260 or 340 mμ) of these compounds , implies that the pyridine nucleotides are the only compounds in the extract which absorb strongly at these wavelengths. However, as noted by Feiglson (45), alkaline tissue extracts contain compounds which absorb light at 340 mμ other than reduced pyridine nucleotides. This contribution to the optical density measured would impose an additional error in

this determination. Auto-oxidation of pyridine nucleotides and variability of extraction similarly would tend to increase the error of this determination. In addition, the formation of sucrose decomposition products has been shown (104) to produce quenching effects in the optical measurement of pyridine nucleotide concentrations. In spite of the many errors inherent in this determination, this method has been applied widely by Gallagher and others (51, 52) for the determination of pyridine nucleotides. The method has the great advantage of simplicity, and large numbers of simultaneous determinations can be made.

The method of pyridine nucleotide assay described by Lindall and Lazarow (104), and confirmed by Gibb (55), has been shown to minimize the errors in analysis due to auto-oxidation. Although the measurement of pyridine nucleotides by the Gallagher method suffers from the inherent errors mentioned above, it is of interest to note that ratios of oxidized to reduced liver pyridine nucleotides determined by this technique were not statistically different from those reported by Lindall and Lazarow. The value of the NAD:$NADH_2$ ratio determined by Lindall and Lazarow in normal fed male rats was 2.58 ± 0.40. In animals fasted for twenty four hours this value decreased to 1.62 ± 0.13. This dependence of the nucleotide rate upon the state of nutrition of the animal should be considered in the interpretation of results. Following the administration of ethanol or carbon tetrachloride a marked loss of appetite was observed in animals. A decrease

in pyridine nucleotides observed following administration of these agents may be the result of both loss of appetite and the effect of the agent itself. A causal relationship between pyridine nucleotide alteration and the administered agent should not be assumed without consideration of other mediating factors.

II-E -- ANALYSIS OF CARBON TETRACHLORIDE

INTRODUCTION

The analysis of carbon tetrachloride in blood and tissue has been reported by several investigators. In 1945, Habgood and Powell (61) described an analysis of carbon tetrachloride in blood which involved steam distillation of the blood sample and collection of the hydrocarbon in iced toluene. Aliquots of the toluene were added to pyridine and sodium hydroxide, heated for 5 minutes at 100°C and cooled. The purplish-red color of the Fujiwara chromogen which is developed in the pyridine layer was measured photometrically and the concentration read from standard curves prepared in the same way. A similar technique for the determination of carbon tetrachloride was described by Fabre, Truhaut and Laham (41) in 1951. Alford (2), in 1947 described an apparatus whereby carbon tetrachloride was separated from blood by a combination of steam distillation and aspiration. The organic halide was decomposed to inorganic halide by passing the vapors through a heated platinum tube and absorbed in alkaline arsenious oxide solution. The halide was then determined by a standard Volhard technique.

Because of losses of carbon tetrachloride during the heating and steam distillation of tissue, and difficulties with frothing of the solution, Weber (182) suggested that the distillation be made in the cold at 0.5 mm Hg pressure. The amount of carbon tetrachloride distilled by this technique was measured volumetrically

in a graduated receiver. Modification of this technique to micro amounts of carbon tetrachloride proved unsatisfactory due to losses on the surfaces of the apparatus and incomplete removal from the tissue.

An infrared analysis of carbon tetrachloride in blood was described by Stewart et al. (171). The halogenated hydrocarbon was extracted from blood into carbon disulphide and the concentration determined by measurement of infrared absorption at specific wavelengths characteristic of carbon tetrachloride. The method, however, is unsuitable for tissue analysis because of the wide variety of interfering substances co-extracted from the sample. The presence of these interfering compounds prevent the use of gas chromatographic, infrared, or colorimetric methods of analysis.

Another method, utilizing the Conway microdiffusion technique, was described by Rechnagel and Litteria (134). In this method, a sample of liver was homogenized and allowed to come to equilibrium with toluene in the Conway diffusion cell. The carbon tetrachloride collected in the toluene was determined colorimetrically by development of the Fujiwara chromogen and comparison with standards. The major disadvantage of this method is the loss of the volatile hydrocarbon during the procedure. In a study of recovery, a mean loss of 48 percent of

known amounts of carbon tetrachloride was observed, by these authors, through the entire homogenization and microdiffusion procedure. Most of this loss occurred during the homogenization of the tissue.

To prevent this loss of carbon tetrachloride, this procedure was modified to obviate the need for homogenization. Samples of tissue to be analyzed were digested within the diffusion cell by the use of sulphuric acid, and the carbon tetrachloride released from the digestion mixture was collected in toluene. After diffusion equilibrium was attained, analysis of carbon tetrachloride was made by development of the Fujiwara chromogen and colorimetric comparison with standards. To facilitate handling of tissue and to accomodate the larger volumes than usual, a modified Conway diffusion cell was developed. Essentially the cell was a shallow, wide bottomed flask with ground glass stopper. A ring, fused to the bottom of the flask, served as a holder for the tube containing the toluene phase. This separate tube allows the entire toluene-carbon tetrachloride sample to be removed following microdiffusion.

PROCEDURE

Rats were sacrificed by decapitation and blood was collected in tared diffusion flasks. Both the flask, stopper, and toluene carrier tube were included in the tare weight. Flasks were quickly stoppered and a final weight recorded. The blood weight was

determined by difference. The liver was rapidly excised and approximately three grams weighed on a Mettler direct reading balance. This weighed section of liver was placed in a diffusion flask containing 5 ml of concentrated sulphuric acid, and the collection tube containing 0.5 ml of toluene. Flasks were stoppered securely and placed on a rotary shaker. All flasks were agitated on a rotary shaker to provide thorough mixing and care was exercised so that the toluene containing tubes were not contaminated by the tissue or blood phases. Flasks were shaken for 36 hours by which time diffusion equilibrium was attained.

The colorimetric determination of carbon tetrachloride in the toluene phase was performed by the development of the Fujiwara chromogen. The color reaction was carried out in 25 ml glass stoppered Pyrex Erlenmeyer flasks. The chromogen mixture was prepared by addition of reagents in the following order: 10 ml of pyridine, 3 ml of 35 percent w/w potassium hydroxide, 0.15 ml of acetone. Additions were made below the surface and flasks were kept stoppered between additions. The tubes containing the toluene phase were removed from the diffusion flasks, the outside was rinsed with a jet of distilled water, wiped dry, and the entire tube and its contents placed in the chromogen mixture. The flasks were sealed and shaken vigorously. The Fujiwara chromogen was developed by heating at 70°C for 40 minutes in a

water bath equipped with a shaker. The red color of the Fujiwara chromogen was developed in the upper pyridine phase. After incubation, the flasks were placed in an ice bath and the two phases allowed to separate. Two ml of the upper phase were added to 0.5 ml of 0.01 N sodium hydroxide in 1 cm Beckman cuvettes. The sodium hydroxide served to clear any residual cloudiness. The contents of the cuvettes were mixed thoroughly and the optical density was measured at 530 mμ with a Beckman DU or Gilford Multi Sample Absorbance Recorder.

Standard solutions of carbon tetrachloride were prepared by dilution of measured amounts in toluene. (See appendix). Additions, of known amounts of carbon tetrachloride to the chromogen mixture for the preparation of standard curves, were made with a micro pipette.

In the preparation of standards, the total toluene volume was brought to 0.5 ml to be comparable to the sample concentration.

Recovery studies were made by addition of known amounts of carbon tetrachloride standard solution to samples of blood and tissue. Mean recovery as determined by development of Fujiwara chromogen and photometric measurement was 94% (Range 87-104%). Addition of varying amounts of tissue did not affect recovery of known amounts of carbon tetrachloride. Both normal and fatty

livers were used in recovery studies and an increased liver lipid content did not adversely affect recovery. The total digestion of the liver by the strong acid probably serves to minimize hydrocarbon solubility in tissue components.

Analysis of carbon tetrachloride collected by the microdiffusion technique in toluene was also made using an Aerograph gas chromatograph. Comparison of elution areas of the sample with standard amounts of carbon tetrachloride showed 99% recovery of the hydrocarbon from liver. (See appendix). In spite of the advantages of gas chromatographic techniques for the analysis of carbon tetrachloride, the colorimetric method was chosen for routine analysis for several reasons. First, the large number of samples to be analyzed would require a long period of time, employing chromatographic procedures, and storage of samples during this period might result in loss of the volatile components. In addition, the length of time required for complete elution from the column of the large excess of organic solvent would be disadvantageous. The necessity for continual calibration during analysis would prove more time consuming than the simple colorimetric method described.

A rapid technique in handling tissue, from sacrifice of the animal to its placement in the diffusion cell, was developed

to prevent loss of the volatile hydrocarbon. It was found that significant losses did not occur if the operations were performed rapidly and tissue was not exposed to the atmosphere for more than thirty seconds. Since losses during this short handling period were not significant, tissues were used immediately upon excision. Duplicate samples of liver taken from the same animal were found to agree within 15%.

In the development of the Fujiwara chromogen, impurities in the reagents exert a marked effect on the production and stability of the color. The purification techniques described by Recknagel and Litteria (134) were followed. Pyridine was distilled from sodium hydroxide and only used when colorless. Toluene was extracted three times with one tenth volume of concentrated sulphuric acid then redistilled. Due to the variation in color produced by minute amounts of impurities or variation in temperature, a calibration curve was prepared for each analysis to diminish errors caused by small variations in the technique. (See appendix).

II-F -- EXPOSURE METHODS

Administration of carbon tetrachloride by inhalation was performed using a dynamic flow, stainless steel, exposure chamber. Carbon tetrachloride, purified reagent, was delivered continuously with a Modern Metalcraft Dual Syringe Feeder into an all glass evaporating apparatus. The vapors of the solvent were mixed with the air stream flowing into the exposure chamber. Vapor concentration was determined by the rate of delivery of the solvent and variation of flow rate of the chamber. Analysis of air concentrations of carbon tetrachloride were made using the Davis Micro Analyzer, photometric determination following collection on silica gel, and by infrared absorption using a Beckman ten meter gas cell. Vapor concentrations determined were within ten percent of the calculated values. A water seal was employed in the small exposure chamber to prevent absorption of organic vapors in sealant compounds. (Figure 1). The large stainless steel exposure chamber was fitted with non-absorbent plastic gaskets to prevent similar losses in organic vapors. In addition, this chamber was thermostatically controlled to allow maintenance of constant temperature during exposure.



Figure 1 -- Small stainless steel dynamic flow exposure chamber.

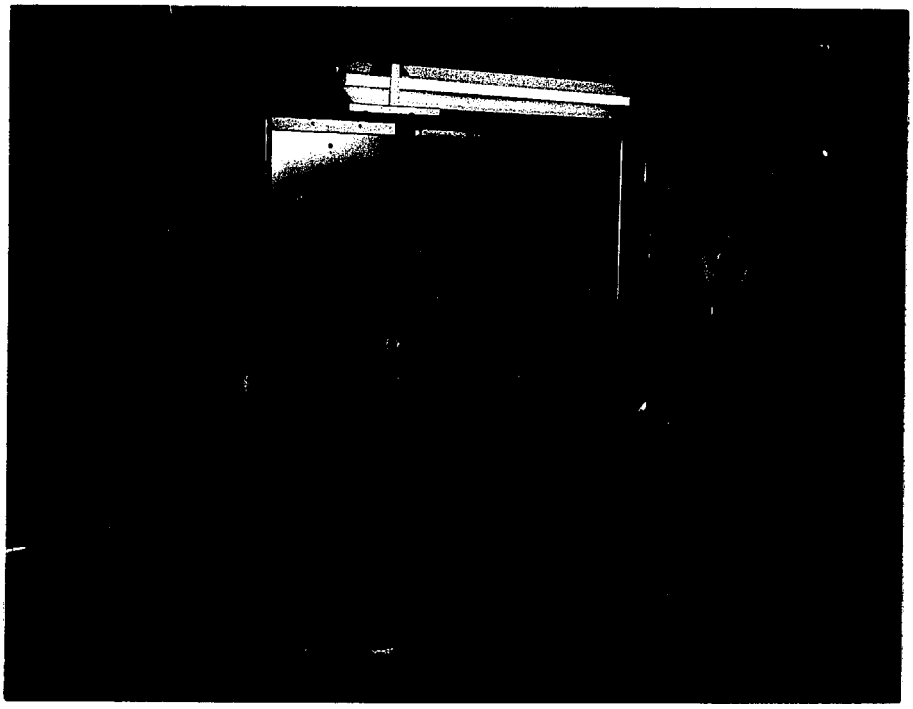


Figure 2 -- Large stainless steel exposure chamber with thermostatic control.

II-G -- STATISTICAL ANALYSIS

Results were reported as the mean value $\pm$ the standard error of the mean. In comparison of two means, the t test was employed. The difference between two means was considered statistically significant when p values less than 0.05 were obtained.

II-H -- HISTOPATHOLOGICAL EXAMINATION

Evaluation of tissue pathology was aided by reports received from the Department of Pathology at the University of Michigan. The author also wishes to acknowledge the assistance of Mary M. Marshall, M. D., and Vernon N. Dodson, M. D., Associate Professor of Industrial Health in evaluating histopathological changes observed.

III BIOLOGICAL RESPONSE FOLLOWING CARBON TETRACHLORIDE AND/OR ETHANOL ADMINISTRATION

III-A -- ACCUMULATION OF HEPATIC LIPID FOLLOWING ETHANOL INGESTION

INTRODUCTION

The development of hepatic steatosis following the ingestion of alcohol has been reported in animals as well as in the human species. To investigate the relationship between hepatic lipid levels and ethanol ingestion in the rat, the rate and extent of fatty accumulation of the liver was determined following the administration of a single acute dose of alcohol.

PROCEDURE

Male Sprague-Dawley rats, 200-225 gms., were given 5 ml/kg ethanol as a 50% solution by oral intubation. At 1, 3, 6, 9, 12, 15, 18, 24, 36, 48, and 72 hours following dosage, animals were sacrificed and liver samples reserved for analysis of total lipid as described in Section II. Comparison of lipid levels was made with similar ethanol untreated animals. Animals were maintained on Rockland Rat Chow and drinking water ad libitum throughout the period of study.

RESULTS

DETERMINATION OF LIVER LIPID LEVELS

A loss of appetite was noted which persisted for at least eight hours after the administration of ethanol. Nine hours after the administration of ethanol, an increase in total liver lipid was noted

TABLE 1

ACCUMULATION OF HEPATIC LIPID FOLLOWING INGESTION OF ETHANOL

Time After Ethanol Ingestion[a] (Hours)	Percent Total Liver Lipid (± S. E.)
0	4.19 ± 0.18
1	4.05 ± 0.22
3	4.25 ± 0.17
6	4.66 ± 0.20
9	5.84 ± 0.29[1]
12	5.06 ± 0.18[1]
15	4.61 ± 0.07
18	4.97 ± 0.28
24	4.39 ± 0.05
36	4.29 ± 0.25
48	4.12 ± 0.25
72	4.22 ± 0.09

At least six animals per group.

[a] 5 ml/kg ethanol, oral.

[1] $p < 0.05$ vs. normal.

(Table 1). Twenty four hours after ingestion of ethanol, liver lipid levels in alcohol treated animals were comparable to those found in control animals.

HISTOLOGICAL STUDIES

Eighteen hours following the ingestion of 5.0 ml ethanol, lipidic accumulation of the liver was present, as confirmed by the fat stained section illustrated in Figure 4. The extent of fatty accumulation of the hepatic parenchyma can be assessed by comparison with a similarly stained section from a normal rat (Figure 3). Examination of heart, liver, lung, kidney, pancreas, and adrenals showed no abnormalities following ingestion of this dose of ethanol.

DISCUSSION

The accumulation of hepatic lipid following an acute intoxicating dose of ethanol has been reported by several investigators. Although this increase of hepatic lipid has been confirmed, the mechanism of this action is not completely understood. Several mechanisms which would lead to accumulation of hepatic lipids have been suggested by various investigators. Among these mechanisms which may act singly or in combination are:

1) Increased mobilization of fatty acids from fat depots to the liver.
2) Interference with a hepatic triglyceride release mechanism.
3) Increased fatty acid synthesis in the liver.

Figure 3

Liver from a normal rat. Oil Red O, 145X.

Fat stain of the normal rat liver can be regarded as negative. Hepatic structure surrounding a central vein is intact and normal.

Figure 4

Liver from a rat which received 5 ml/kg ethanol eighteen hours prior to sacrifice. Oil Red O, 145X.

Lipidic accumulation in the hepatic parenchyma can be observed as red droplets. A central vein appears in the center of the picture and surrounding hepatic architecture is intact.

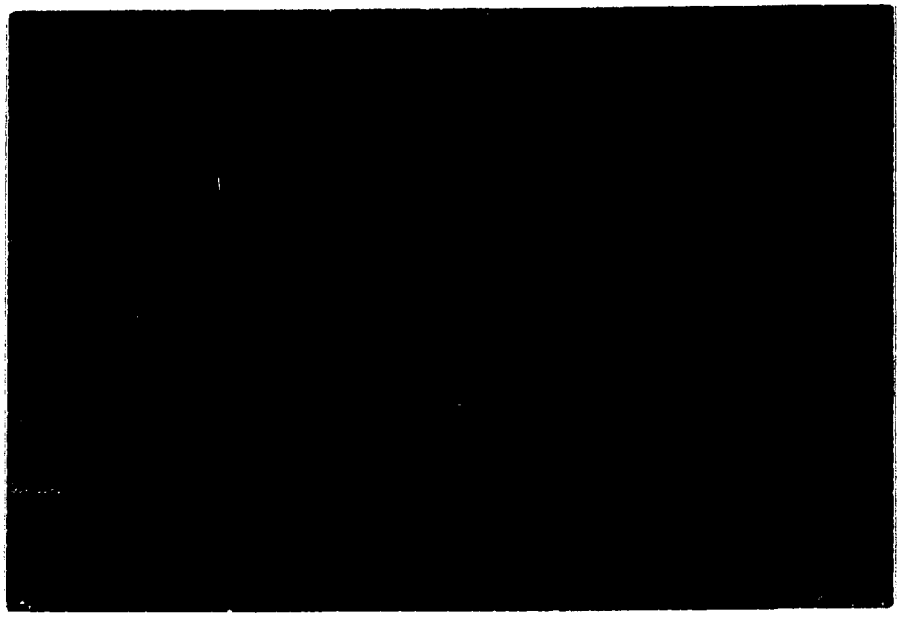


Figure 3

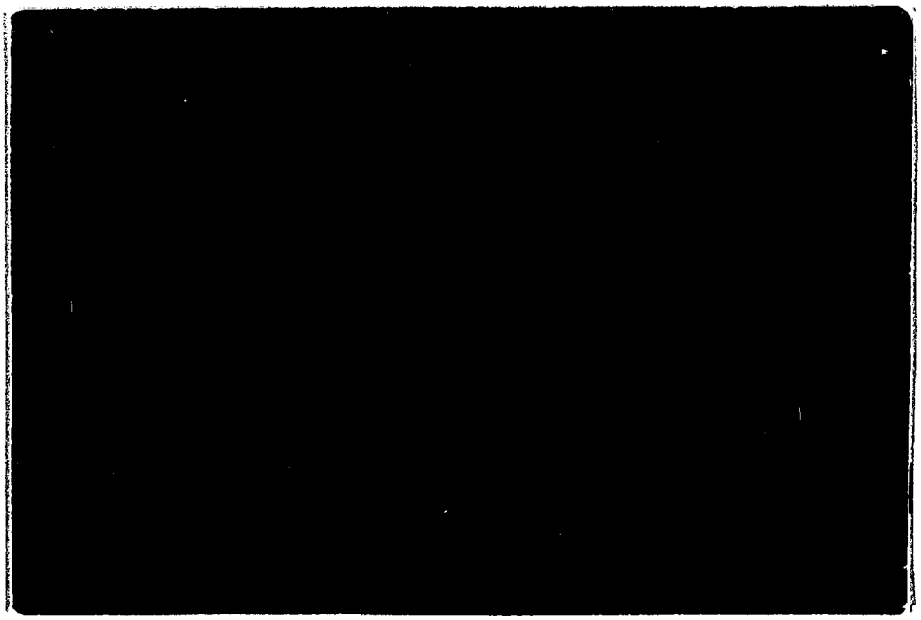


Figure 4

4) Increased synthesis of hepatic triglycerides.

5) Decreased fatty acid oxidation in the liver.

Among these five mechanisms, the mobilization of fatty acids from peripheral adipose depots is generally considered to play the major role in hepatic lipid accumulation following ethanol ingestion (16, 36, 72, 76). Adrenalectomy (16, 109), cordotomy (132), hypophysectomy (17, 132), and ganglionic blocking agents (15, 16) which interfere with mobilization of fatty acids from adipose tissue, are all effective in preventing the ethanol induced fatty liver. The mediation of the catecholamines is a major factor in the mobilization of fatty acids from adipose tissue. Wool (188) and more recently, Brodie and Maickel (15) have also demonstrated the involvement of the adrenal cortex in fatty acid mobilization. It was observed that administration of cortisone was capable of inducing a fatty liver in the adrenalectomized animal.

Brodie and others (16, 17, 72) have demonstrated the importance of fatty acid mobilization in the development of fatty liver by measuring the increase of hepatic linoleic acid. This essential fatty acid is derived from dietary sources and stored in adipose tissue. Following the administration of ethanol, these investigators observed an increase in the amount of linoleic acid in hepatic triglycerides. Since linoleic acid levels cannot be elevated by increased hepatic fatty acid synthesis, this evidence

suggests an increased mobilization from adipose tissue. In addition, an increase in plasma free fatty acids after ethanol administration has been measured by Schapiro (149), Mallov (110) and others (15). This observation lends support to the important role of increased mobilization in the production of fatty liver. DiLuzio and Poggi (36) have demonstrated that hepatic triglyceride is at least partially derived from adipose tissue following ethanol administration. Using the in vivo incubation of epididymal fat pads to label adipose triglycerides, as suggested by Stein and Stein (167), these authors observed an increase in labeled hepatic triglyceride following ethanol administration. By examination of specific activity of the labelled liver triglycerides an increased synthesis of hepatic triglyceride was also associated with the increased mobilization.

The second mechanism, the interference with hepatic triglyceride release, has already been mentioned as an important factor in the production of carbon tetrachloride induced fatty liver. Schapiro et al. (150) have demonstrated that high alcohol concentrations cause a depression of triglyceride secretion in the perfused rat liver. The accompanying uptake of fatty acids, however, was not diminished. As supporting evidence for this action, Jones and co-workers (78) have observed decreased serum lipoprotein levels after ingestion of alcohol. Triglycerides, which are secreted as

a lipoprotein complex, would accumulate in the hepatic parenchyma under such conditions.

The mechanism of increased fatty acid synthesis in the liver as a contributory factor in lipidic accumulation has been investigated by Lieber and Schmid (103). These authors suggested that the increased concentration of $NADH_2$ formed by ethanol metabolism may cause an increase in fatty acid synthesis. Reboucas and Isselbacher (132) also observed an increase of fatty acid synthesis in the ethanol induced fatty liver, but were able to distinguish this action from the processes leading to a fatty liver. After sorbitol and glucose administration, these authors observed an enhancement of fatty acid synthesis, but without the accompanying production of a fatty liver. Although increased fatty acid synthesis occurs following ethanol, this mechanism is probably not of major importance in the production of fatty liver.

The fourth mechanism, that of increased synthesis of hepatic triglycerides, has been investigated by Maling (108). In liver homogenates of alcohol treated rats, an increase in incorporation of fatty acid into triglycerides was observed. Concomitantly, a decrease in incorporation of labelled palmitate into phospholipid was observed. A similar study in a cell free system demonstrated that in a liver microsomal preparation from alcohol treated rats, an enhancement of labelled palmitate incorporation into

triglycerides occurred. However, alcohol does not appear to exert this effect directly upon the liver microsomes. The addition of alcohol to a liver microsomal preparation did not increase the triglyceride synthesis and the mechanism of this action is as yet uncertain. The addition of ethanol to liver slices resulted in a decrease in labelled palmitate oxidation as noted by Lieber and Schmid (103). These authors suggested that increased levels of $NADH_2$ formed during ethanol metabolism may inhibit oxidation in the tricarboxylic acid cycle. However, administration of glucose resulted in a similar inhibition of fatty acid oxidation but did not produce a fatty liver. Sorbitol, xylitol, and nicotinamide, which do not lead to hepatic steatosis, also produce a similar inhibition of fatty acid oxidation. Although this decrease in fatty acid oxidation is observed following ethanol administration, this mechanism may not be of prime importance to the development of the characteristic fatty liver. Mobilization of fatty acids from adipose tissue and increased production of hepatic triglycerides appear to be quantitatively more significant than the other factors mentioned.

III-B -- EFFECT OF ETHANOL INGESTION ON THE BIOLOGICAL RESPONSE TO CARBON TETRACHLORIDE

The parameters of toxicity described in the preceding section II were applied to animals treated with ethanol, carbon tetrachloride, or the combination to demonstrate the efficacy of these measures of toxicity. Comparison of the response in carbon tetrachloride treated animals was made to that observed in animals treated with both ethanol and carbon tetrachloride to determine the extent of the enhanced toxicity following treatment with the combination of agents.

PROCEDURE

To determine serum enzyme response to carbon tetrachloride, alone or in conjunction with ethanol, the enzyme activity was measured in animals given various doses of carbon tetrachloride. Animals were exposed for two or four hours to concentrations of 50, 100, 250, 500, 1000, 2000, 4000, 6000, or 10,000 ppm carbon tetrachloride. Animals which had been pre-treated with 5 ml/kg ethanol eighteen hours previously were similarly exposed. Twenty hours following exposure, animals were sacrificed and serum enzyme activity determined as described in Section II-B. Comparison of enzyme activity was made with that found in normal or ethanol treated animals.

Measurement of changes in serum enzyme activity was made when carbon tetrachloride was administered by intraperitoneal injection.

By this route, carbon tetrachloride was administered at the rate of 0.6 ml/kg. By the oral route, carbon tetrachloride was administered by intubation in a dose of 2.5 ml/kg in an equal volume of corn oil.

Measurements of liver pyridine nucleotides and serum iron concentrations were made as described in Section II D and C. Sections of liver were reserved for histopathological examination and analysis of total lipid.

RESULTS

SERUM ENZYME ACTIVITY

Following exposure to 50 ppm of carbon tetrachloride for two hours, serum (GOT), (GPT), and (ICD) activity was comparable to normal values in both ethanol treated and untreated animals (Table 2). Exposure to 100 or 250 ppm carbon tetrachloride for two hours did not produce elevated serum enzyme activity in ethanol untreated rats. However, rats given an oral dose of ethanol prior to exposure to these doses of carbon tetrachloride exhibited significantly elevated values of S-GOT ($p < 0.05$). This difference was associated with a potentiation index of 2.1 and 2.8 for ethanol treated animals exposed to 100 and 250 ppm carbon tetrachloride, respectively (Table 3). The difference in enzyme response between ethanol treated and untreated animals was still more striking following exposure to 1000 ppm carbon tetrachloride for two hours. Serum enzyme activity was not significantly altered in control rats exposed to this concentration of the organic halide. However, ethanol pre-treated animals

TABLE 2

SERUM ENZYME ACTIVITY FOLLOWING CARBON TETRACHLORIDE EXPOSURE

CCl_4 (ppm)	Ethanol (5 ml/kg)	Time of Exposure (Hours)	Serum Enzyme Activity S-GOT	S-GPT	S-ICD
0	-	0	248 ± 44	25 ± 4	455 ± 77
	+		266 ± 32	26 ± 3	491 ± 78
50	-	2	164 ± 8	25 ± 3	561 ± 210
	+		172 ± 12	22 ± 2	500 ± 77
100	-	2	220 ± 13	23 ± 2	642 ± 106
	+		516 ± 14[a]	43 ± 10	967 ± 410
250	-	2	228 ± 30	38 ± 4	480 ± 39
	+		692 ± 121[a]	164 ± 130	810 ± 300
1000	-	2	258 ± 30	40 ± 13	525 ± 64
	+		2200 ± 142[c]	195 ± 23[c]	1850 ± 150[c]
2000	-	2	3172 ± 428[b]	257 ± 22[b]	3788 ± 1278[b]
	+		5320 ± 432	320 ± 124	18000 ± 3055
4000	-	4	11000 ± 1598	1852 ± 243[b]	32190 ± 7581[b]
	+		22660 ± 1962[c]	1952 ± 198	40710 ± 6652[b]
10000	-	2	4225 ± 560	851 ± 131[b]	4229 ± 560[b]
	+		6158 ± 210[b]	1458 ± 114[b]	5964 ± 185[b]

At least six animals per group.

[a] $P < 0.05$ vs. CCl_4 exposed.

[b] $P < 0.05$ vs. normal.

[c] $P < 0.001$ vs. CCl_4 exposed.

TABLE 3

POTENTIATION INDEX FOLLOWING INHALATION OF CARBON TETRACHLORIDE

CCl_4 (ppm)	Ethanol (5 ml/kg)	Time of Exposure (Hours)	Potentiation Index SGOT	S-GPT	SICD
0	-	0	1.0	1.0	1.0
	+		1.0	1.0	1.1
50	-	2	0.7	1.0	1.2
	+		0.7	1.0	1.1
100	-	2	0.9	1.0	1.4
	+		2.1[a]	2.0	2.1
250	-	2	0.9	1.7	1.1
	+		2.8[a]	7.5	1.8
1000	-	2	1.0	1.8	1.2
	+		8.9[c]	8.9[c]	4.1[c]
2000	-	2	12.8[b]	11.7[b]	8.3[b]
	+		21.4	14.5	39.6
4000	-	4	44.3	84.2[b]	70.7
	+		91.4[c]	88.7	89.5[b]
10000	-	2	17.0	38.7	9.3
	+		24.8[b]	66.3[b]	13.1[b]

Ethanol administered as a 50% solution eighteen hours prior to carbon tetrachloride exposure.

At least six animals per group.

[a] $P < 0.05$ vs. CCl_4 exposed.

[b] $P < 0.05$ vs. normal.

[c] $P < 0.001$ vs. CCl_4 exposed.

exhibited values of 2200 ± 142, 195 ± 23, and 1850 ± 150 for S-GOT, S-GPT, and S-ICD following this dosage of carbon tetrachloride. This increase in serum enzyme activity represents potentiation indexes of 8.9, 8.9, and 4.1 for these enzymes. The differences in serum enzyme activity between ethanol treated and untreated animals were highly significant (p 0.001) following exposure to this concentration of carbon tetrachloride. This level of carbon tetrachloride exposure (1000 ppm) elicited essentially an all-or-none serum enzyme response dependent upon pre-treatment of the animals with ethanol.

With higher dosage of carbon tetrachloride, this sharp contrast in response was obscured by increased serum enzyme activity in animals given the organic halide alone. Although elevations in serum enzyme activity could be detected in the ethanol groups exposed to these higher concentrations of carbon tetrachloride, the differences in enzyme response were more readily demonstrated with lower concentrations of carbon tetrachloride.

LIVER PYRIDINE NUCLEOTIDE LEVELS

Two hours after intubation of rats with 5.0 ml/kg ethanol, the liver pyridine nucleotide ratio ($NAD_{260\ m\mu}/NADH_2 340\ m\mu$), as determined by the Gallagher method, was similar to the normal value (Table 4). Twenty hours later, the mean value of this ratio rose to 2.42 ± 0.24 but was not statistically different from the normal value at the 95% confidence level. Two hours and 20 hours after the intra-

TABLE 4

APPARENT LIVER PYRIDINE NUCLEOTIDE RATIOS

Treatment	2 Hours After Treatment		20 Hours After Treatment	
	Ratio (± SE)	% of Normal	Ratio (± SE)	% of Normal
E	1.90 ± 0.19	97	2.42 ± 0.24	123
C	2.09 ± 0.07	107	1.93 ± 0.07	98
EC	1.99 ± 0.10	102	1.08 ± 0.19 [a]	55
NORMAL	1.96 ± 0.04	100		

E -- 5 ml/kg ethanol administered orally.

C -- 0.6 ml/kg carbon tetrachloride, intraperitoneal.

EC - Carbon tetrachloride administered 18 hours after ethanol.

[a] $P < 0.05$ vs. normal.

peritoneal injection of 0.6 ml/kg carbon tetrachloride, no alteration in the pyridine nucleotide ratio was detected. Similarly, the combination of ethanol and carbon tetrachloride had no effect at 2 hours. However, twenty hours later, a significant decrease in this ratio was observed in animals which were treated with both ethanol and carbon tetrachloride. At this time, the mean value in these animals was 1.08 ± 0.19 which represented a decrease of 45% compared to the normal value.

SERUM IRON CONCENTRATION

Twenty hours after the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride, an increase in serum iron concentration was observed. The concentration of serum iron in the carbon tetrachloride treated animals was 16.7 ± 1.3 ppm compared to 10.0 ± 1.0 ppm for normal animals at this time, as shown in Table 5. Animals treated with 5 ml/kg ethanol eighteen hours prior to injection of this dose of carbon tetrachloride had a serum iron concentration of 21.0 ± 1.0 at this time.

As mentioned in the discussion of serum iron determination (Section II), molybdenum was also detected in serum of animals intoxicated with carbon tetrachloride. Molybdenum was not detected in control animals. However, the technique employed did not permit quantitative estimation of the amount of molybdenum present.

TABLE 5

SERUM IRON CHANGES FOLLOWING CARBON TETRACHLORIDE ADMINISTRATION

Treatment	Serum Iron Concentration (ppm + SE)	Extent of Hepatic Changes
EC	21.0 ± 1.0[a]	+++
C	16.7 ± 1.3	++
NORMAL	10.0 ± 1.0	0

EC -- 5.0 ml/kg ethanol administered orally eighteen hours prior to intraperitoneal injection of 0.6 ml/kg carbon tetrachloride.

C - 0.6 ml/kg carbon tetrachloride injected intraperitoneally.

[a] $P < 0.05$ vs. normal.

Animals sacrificed twenty hours after carbon tetrachloride administration.

CONCENTRATION OF CARBON TETRACHLORIDE IN BLOOD AND LIVER

Two hours after the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride, 0.273 $\pm$ 0.023 mg CCl_4/gm liver and 0.095 $\pm$ 0.017 mg CCl_4/gm blood were detected. (Table 6). Animals treated with ethanol eighteen hours prior to carbon tetrachloride injection had similar concentrations of carbon tetrachloride in blood and liver at this time.

Concentrations of carbon tetrachloride in blood and liver were also determined when the organic halide was administered by the oral route. When carbon tetrachloride was administered orally at the rate of 2.5 ml/kg in an equal volume of corn oil, 0.342 $\pm$ 0.084 mg CCl_4/gm liver and 0.030 $\pm$ 0.011 mg CCl_4/gm blood were detected two hours later. Animals which were given 5 ml/kg ethanol orally, eighteen hours prior to administration of this dose of carbon tetrachloride exhibited similar blood and liver concentrations of the organic halide. Two hours after carbon tetrachloride administration, liver and blood concentrations were 0.367 $\pm$ 0.125 mg/gm and 0.035 $\pm$ 0.019 mg/gm, respectively.

Blood and liver concentrations of carbon tetrachloride were similar in ethanol pre-treated animals and those which received carbon tetrachloride alone. The administration of ethanol eighteen hours prior to carbon tetrachloride dosage did not alter the concentration of the

chlorinated hydrocarbon in blood or liver.

Concentration of carbon tetrachloride in blood and liver was determined following inhalation of 4000 ppm for 4 hours. Both normal animals and those which had been pre-treated with ethanol eighteen hours previously were exposed at the same time in the chamber. Since an air lock was not available to remove animals individually after exactly four hours of exposure, animals were removed as a group. To minimize errors in analysis due to the rapid excretion of carbon tetrachloride following exposure, animals were serially sacrificed at timed intervals at the end of the exposure period. To determine the losses due to rapid exhalation of carbon tetrachloride, animals were sacrificed alternately from the normal and ethanol pre-treated exposed groups. Individual values of carbon tetrachloride concentrations found were plotted against time after exposure and the line of best fit was calculated. Although excretion of carbon tetrachloride following exposure can be defined as an exponential function, the rate observed during this short period of time can be expressed linearly by the relationship: $X = 0.0188 - \frac{Y}{770}$ where X is the concentration of carbon tetrachloride in mg/gm, and Y is the time after exposure in minutes. The mean concentration of carbon tetrachloride found in blood of normal and ethanol treated animals two hours following exposure was 0.012 ± 0.0006 and 0.011 ± 0.0006 mg/gm blood. The concentration of carbon tetrachloride in liver was very low

TABLE 6

CONCENTRATION OF CARBON TETRACHLORIDE IN BLOOD AND LIVER

Dose of Carbon Tetrachloride	Ethanol[a] 5 ml/kg	Concentration of Carbon Tetrachloride Liver mg/gm	Blood mg/gm
0.6 ml/kg ip	+	0.277 ± 0.047	0.103 ± 0.024[b]
	-	0.273 ± 0.023	0.095 ± 0.017[b]
2.5 ml/kg oral	+	0.367 ± 0.125	0.035 ± 0.019[b]
	-	0.342 ± 0.084	0.030 ± 0.011[b]
4000 ppm/4 hrs.	+	0.002	0.011 ± 0.001
Inhalation	-	0.002	0.012 ± 0.001

[a]Ethanol administered orally eighteen hours prior to carbon tetrachloride.

[b]All animals sacrificed two hours after carbon tetrachloride administration.

at this time and approached the minimum detection limit. Only 0.0021 mg CCl_4/gm liver was detected in both normal and ethanol pre-treated animals. At this level of detection, blank variation and non-linearity of colormetric techniques can add appreciable errors to the determination. It can be observed, however, that blood concentrations of carbon tetrachloride were independant of pre-treatment with ethanol.

HEPATIC LIPID LEVELS

Total liver lipids were determined in normal animals and those given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to carbon tetrachloride administration. The results of this lipid analysis are summarized in Table 7. Comparison of the individual lipid values was made to the amount of carbon tetrachloride found in the corresponding animal. No correlation between the amount of hepatic lipid and the quantity of carbon tetrachloride determined was observed.

HISTOPATHOLOGICAL STUDIES

Two hours following exposure to 2000 ppm carbon tetrachloride for four hours, a moderate lipidic infiltration in the liver could be detected as shown in Figure 5. No major disruption of hepatic parenchyma was evident at this time but early hydropic degenerative changes could be observed. Twenty hours following inhalation of this dose of carbon tetrachloride , the characteristic

TABLE 7

HEPATIC LIPID LEVELS FOLLOWING CARBON TETRACHLORIDE ADMINISTRATION

Dosage of Carbon Tetrachloride	Ethanol[a] (5 ml/kg, oral)	% Total Lipid (± S. E.)
0.6 ml/kg ip	+	6.93 ± 0.59
	-	5.10 ± 0.47
2.5 ml/kg oral	+	5.22 ± 0.12
	-	4.91 ± 0.12
4000 ppm/4 hrs.	+	6.12 ± 0.14
Inhalation	-	6.07 ± 0.16

[a]Ethanol administered eighteen hours prior to carbon tetrachloride.

hepatic lesion was produced, as shown in Figure 6. Acute inflammatory response with infiltration of neutrophilic leucocytes and degenerative fatty infiltration were noted. Focal necrosis of the central lobular hepatic cord cells was detected at this time. Similarly, animals which were treated with 5.0 ml/kg ethanol eighteen hours prior to this exposure showed moderate central lobular fatty infiltration of the liver two hours after exposure (Figure 7). The extent of fatty accumulation of the liver produced by alcohol ingestion can be assessed by examination of Figure 4. Twenty hours after inhalation of 2000 ppm carbon tetrachloride for four hours, the hepatic lesion observed was more severe in the alcohol treated animals than in the corresponding control group. Severe central lobular necrosis, acute inflammation with infiltration of polymorphonuclear leucocytes could be observed.

Administration of a lower dose of carbon tetrachloride, inhalation of 1000 ppm carbon tetrachloride for two hours, produced minimal histopathological changes in normal animals. As shown in Figure 9, only minimal focal fatty accumulation was observed. The hepatic architecture remained intact. Administration of 5.0 ml of ethanol to animals eighteen hours prior to this exposure produced more severe histopathological hepatic changes. Diffuse degenerative fatty infiltration with neutrophilic infiltrates were observed. Focal early central lobular necrosis could be detected, as shown in Figure 10.

Figure 5

Liver from a rat two hours after exposure to 2000 ppm of carbon tetrachloride for four hours. H & E, 145X.

Moderate degenerative fatty accumulation of central lobular cord cells can be observed. Early hydropic degenerative changes are present in the central area.

Figure 6

Liver from a rat twenty hours after exposure to 2000 ppm of carbon tetrachloride for four hours. H & E, 145X.

This is representative of the classic hepatic lesion induced by carbon tetrachloride. Acute inflammation with infiltration of neutrophilic leucocytes and degenerative fatty infiltration can be observed. Focal necrosis of central lobular hepatic cord cells is present.

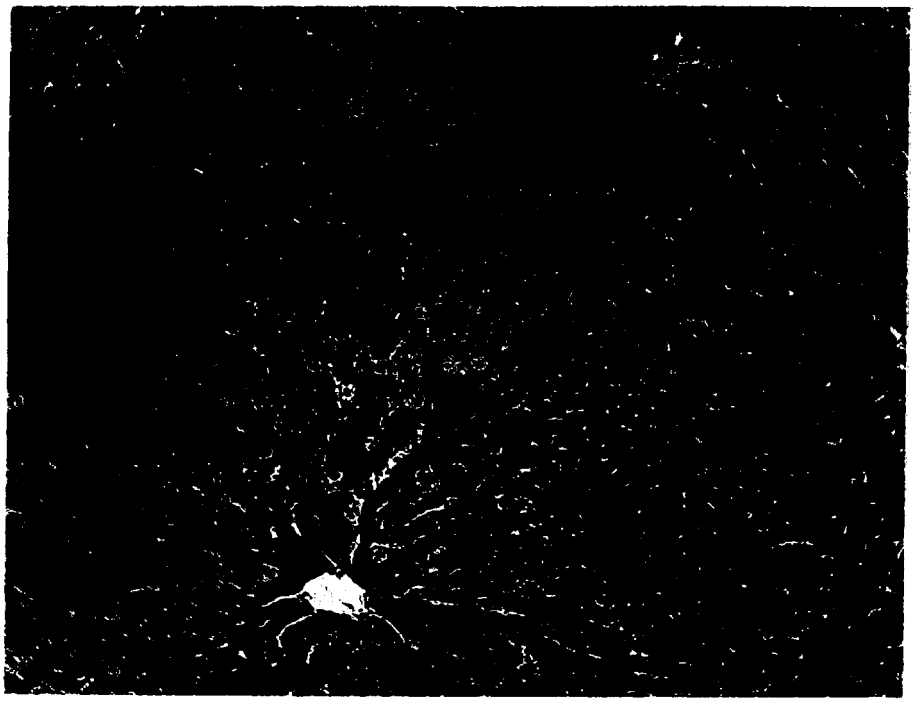


Figure 5

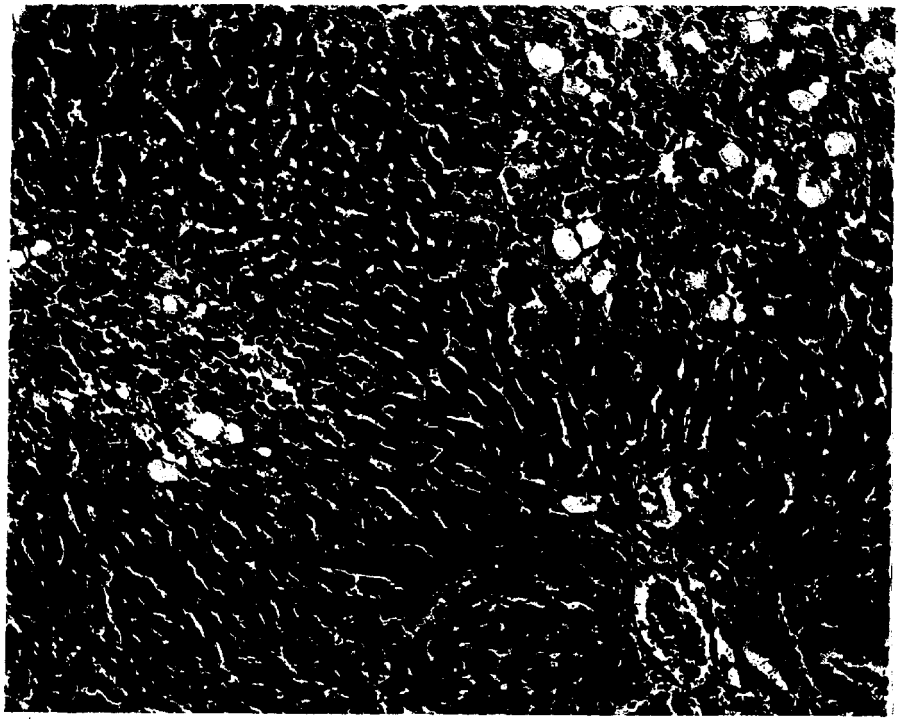


Figure 6

Figure 7

Liver from a rat given 5 ml/kg ethanol eighteen hours prior to exposure to 2000 ppm of carbon tetrachloride for four hours. Sacrificed two hours after exposure. H & E, 145X.

Moderate central lobular degenerative fatty infiltration can be observed.

Figure 8

Liver from a rat given 5 ml/kg ethanol eighteen hours prior to exposure to 2000 ppm of carbon tetrachloride for four hours. Sacrificed twenty hours after exposure. H & E, 145X.

The characteristic pathological changes were more pronounced following treatment with both ethanol and carbon tetrachloride. Severe central lobular necrosis with acute inflammation and infiltration of polymorphonuclear leucocytes. Lipidic accumulation, represented by spherical droplets in the necrotic areas and in the peripheral tissue.

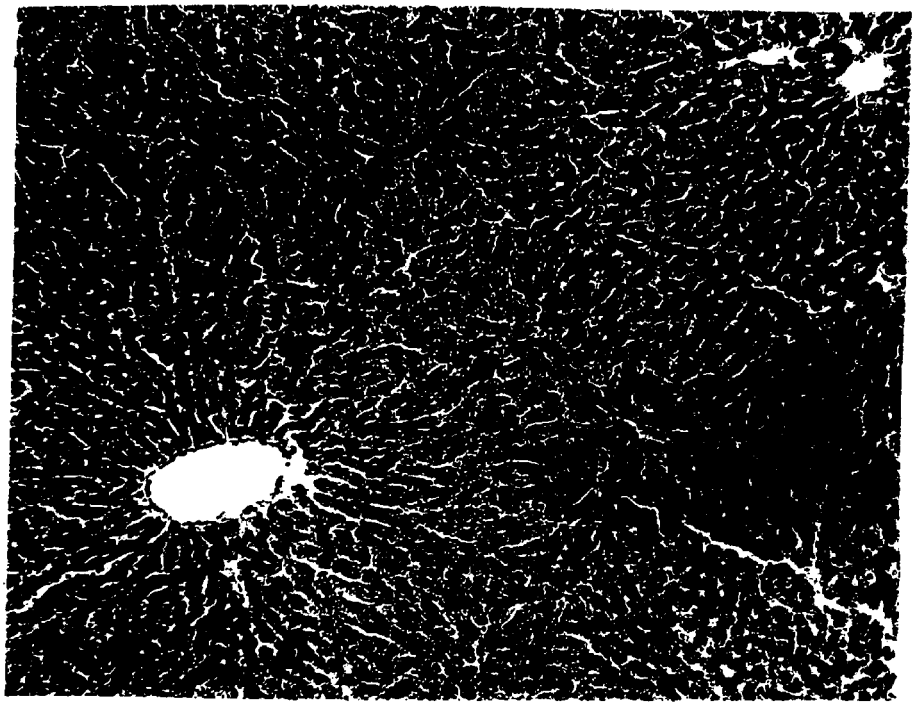


Figure 7

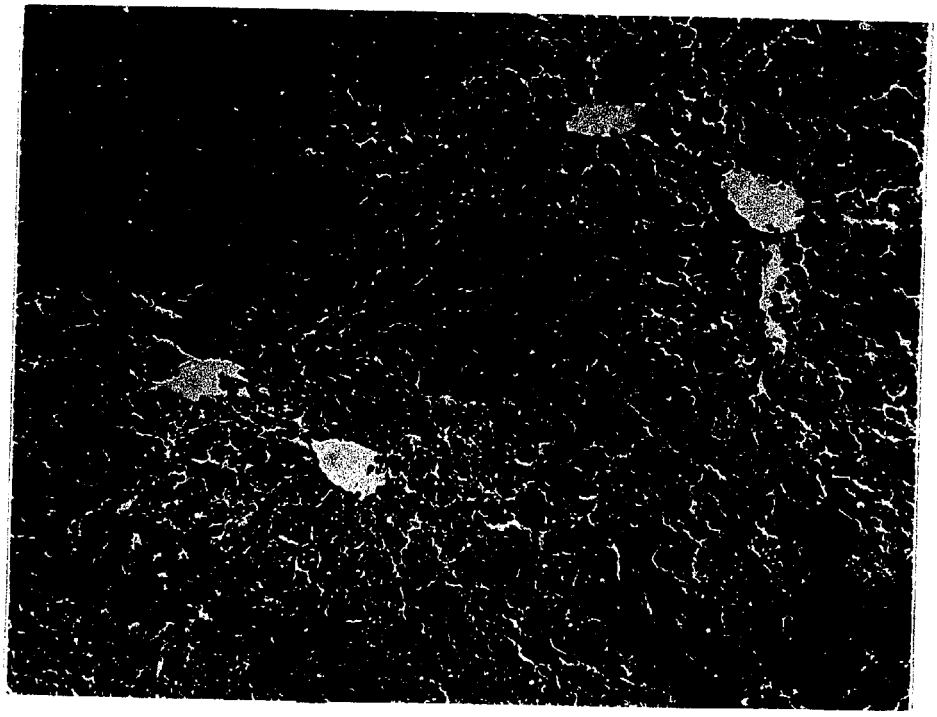


Figure 8

Figure 9

Liver from a rat twenty hours after exposure to 1000 ppm of carbon tetrachloride for two hours. H & E, 145X.

Minimal morphological changes were observed following exposure to this concentration of carbon tetrachloride. Minimal focal fatty accumulation may be observed. No necrosis of liver was present and examination of adrenal, spleen, kidney, pancreas, and lung revealed no abnormalities.

Figure 10

Liver from a rat given 5 ml/kg ethanol orally eighteen hours prior to exposure to 1000 ppm of carbon tetrachloride for two hours. Sacrificed twenty hours after exposure. H & E, 85X.

Diffuse degenerative fatty infiltration and focal early central lobular necrosis are present. Neutrophilic infiltrates are present, and hydropic degeneration has occurred. The morphological changes observed following treatment with this dose of carbon tetrachloride and ethanol are more severe than those produced by carbon tetrachloride alone. (See Figure 9).

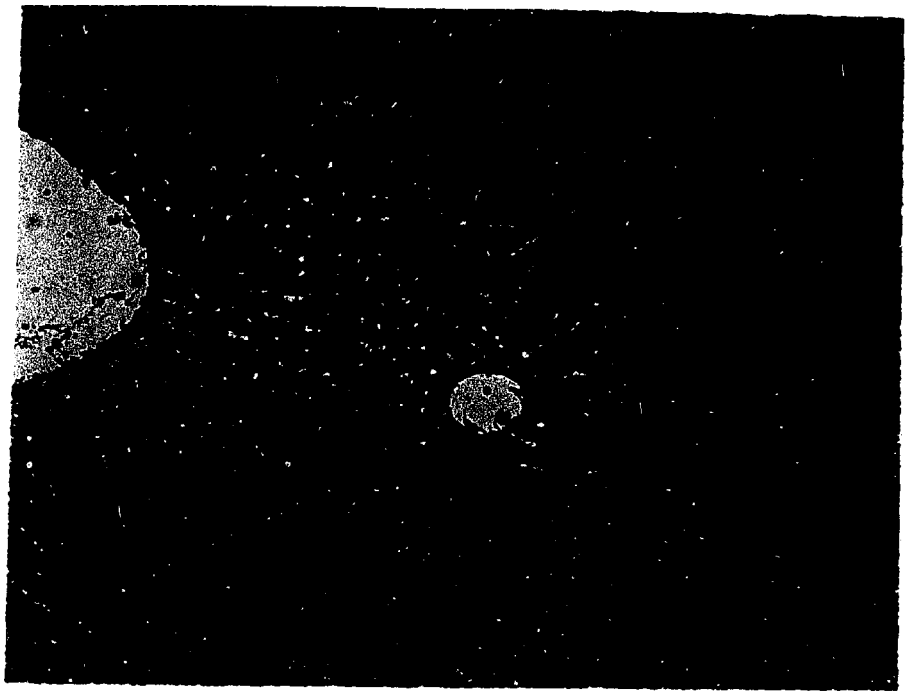


Figure 9

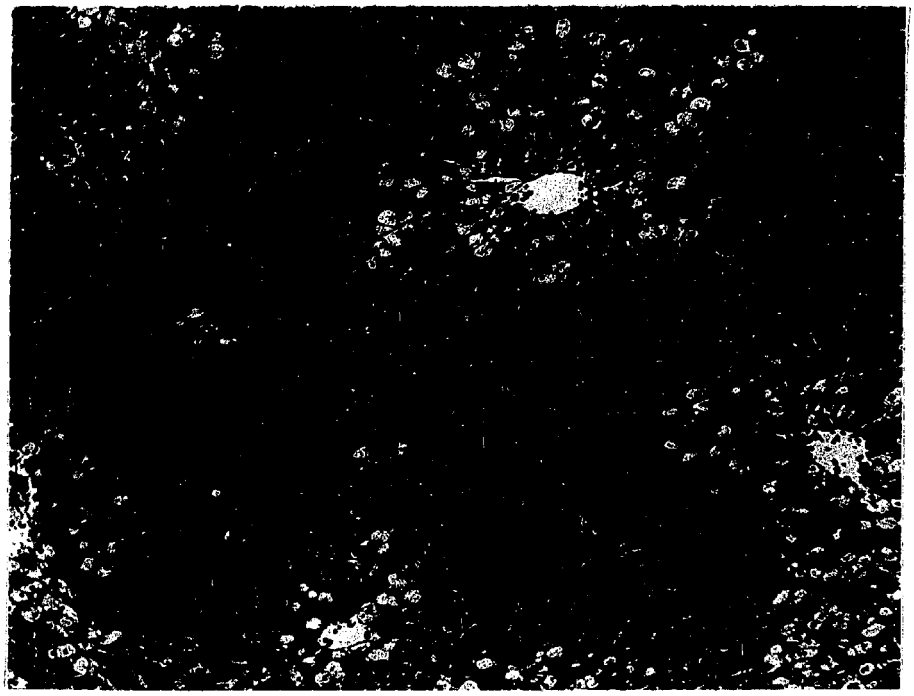


Figure 10

DISCUSSION

Examination of serum enzyme activity following carbon tetrachloride administration showed marked differences in response between normal and ethanol treated animals. Following exposure to 250 ppm, differences in enzyme activity could be detected dependent upon ethanol pre-treatment of the animal. These enzyme differences were still more apparent following exposure to 1000 ppm for two hours. Histopathological examination has confirmed morphological changes in ethanol treated animals following exposure to this concentration of the chlorinated hydrocarbon. Similarly, the ratio of liver pyridine nucleotides was altered in animals which received the combination of agents. However, a causal relationship between this ratio and the direct action of these agents cannot be assumed because of the arguments mentioned in the Methods Section II-D. It is of interest to note that these differences in response to carbon tetrachloride occurred eighteen hours following the single dose of ethanol. At this time, the ethanol has been metabolized and is no longer present in the tissues. The hypothesis that the toxicity of carbon tetrachloride is potentiated by direct interaction with alcohol is not compatible with these observations. Analysis of carbon tetrachloride in blood and liver did not reveal a correlation between the amount of hepatic lipid and the organic halide found.

The concentration of serum iron found was consistent with the results observed histopathologically. Greater morphological changes were observed following administration of carbon tetrachloride to alcohol pre-treated animals than in control animals. Similarly, serum enzyme activity was greater in alcohol treated rats than in corresponding control groups treated with carbon tetrachloride.

IV -- HEPATIC STEATOSIS INDUCED BY AGENTS OTHER THAN ETHANOL AND THE BIOLOGICAL RESPONSE OF THESE ANIMALS TO CARBON TETRACHLORIDE

INTRODUCTION

To investigate the role of liver lipid in the potentiation of carbon tetrachloride toxicity it would be pertinent to measure the potentiating effect of hepatic steatosis induced by means other than ethanol. If a causal relationship exists between the accumulation of liver lipid and the potentiated carbon tetrachloride toxicity, this increased toxic response would be observed when hepatic steatosis was induced by other agents. However, if the potentiation of toxicity is due to factors other than lipidic accumulation in the liver, the greatly enhanced toxic response would not be observed when lipid levels were elevated by other means. The possibility exists that agents which induce hepatic steatosis may potentiate by a biochemical chain of events similar to that of ethanol. For this reason it was decided to study the toxic response to carbon tetrachloride when hepatic steatosis was induced by several agents which act by different mechanisms to produce a fatty liver.

Fatty accumulation of the liver has been reported following various treatments. Among the methods commonly used for the production of experimental hepatic steatosis are: administration of ethionine, arsenic, rare earth salts, phosphorus, orotic acid, flectol H, lead,

chloroform, and high fat, or choline deficient diets. Several of these agents produce severe toxic symptoms, in addition to hepatic steatosis, and were considered unsuited to the study of enhanced toxic response to carbon tetrachloride. The contribution of such toxic materials as phosphorus or arsenic to the toxic response following carbon tetrachloride administration would be considerable and difficult to assess accurately. As reported by Snyder, Cress, and Kyker (165), the variability in production of fatty liver in male Sprague-Dawley rats by rare earth salts and its toxicity in the doses required did not permit its use in this study. Similarly, ethionine has often been employed to produce experimental hepatic steatosis in animals. Olivecrona (125) has documented the increase in hepatic lipid following ethionine administration. The biological sequence of events leading to fatty accumulation of the liver induced by ethionine has been investigated by several workers. A marked decrease in hepatic ATP levels has been reported by Farber and others (42, 43), following administration of ethionine. These workers suggested that the rapid formation of S-adenosylethionine and slow metabolism of this derivative effectively sequestered the available adenine supply. The resulting cellular deficit in adenine is reflected by decreased hepatic ATP levels and concomitant alteration in nucleic acid and protein synthesis, and accumulation of hepatic triglycerides. Hutterer and Leonard (75) have elucidated the role of increased fat uptake by hepatocytes. Using labelled oleic C^{14} acid a study of hepatic uptake and turnover showed that increased uptake, probably by pinocytosis, was a more important factor

in ethionine steatosis than increased synthesis or decreased secretion of triglycerides. This metabolic antagonist of methionine, however, exerts a profound toxic effect upon the experimental animal. Koch-Weser et al. (87), have reported decreased hepatic esterase levels, elevated bromosulphotalein retention and bilibubin levels following ethionine intoxication. Because of its high toxicity and possible interference with measurements of toxic response, ethionine was used to produce hepatic steatosis in only one study to be reported here.

Fatty accumulation of the liver was reported by Rikans, Arrata, and Cederquist (141) in animals maintained on a high fat diet supplemented with niacin. In animals placed on a high fat diet these authors noted a decrease in endogenous oxidation and an increase in fatty acid oxidase activity. Concomitantly, an increase in hepatic and blood pyridine nucleotides, measured by the method described by Robinson et al. (145) was observed. Although a depressed growth rate and food intake was observed in animals fed a high fat diet, efficiency of food utilization (gms. weight gain/100 K cal consumed) was not impaired. The increased intake of fat would serve to increase the choline requirement of animals maintained on this diet. The fatty accumulation of the liver observed could reflect a mild choline deficiency induced by this high fat diet.

The production of fatty accumulation of the liver by choline deficiency has been widely studied, and the use of choline deficient diets

is recognized as a classical method of inducing experimental liver changes. The lipotropic factor, choline, is involved in the formation of lipoprotein and plays an important part in fatty acid oxidation. Artom (4) has demonstrated that fatty acid oxidation is decreased in choline deficient animals. Recently, Tesluk and Stewart (173) have demonstrated, by gas chromatographic analysis, that the lipid accumulating in the liver in choline deficient animals resembles that found in adipose tissue, and dietary sources. The increase in oleic and linoleic acid found in liver was proportional to the amount of fatty change in the liver. Concurrently, the decrease in plasma triglyceride levels was proportionate to the amount of fatty accumulation of the liver. This pattern of change suggests that the accumulation of hepatic lipid is due to interference with transport rather than interference with fat oxidation. Lombardi et al. (105) measured plasma and liver triglycerides and phospholipid levels at short intervals after rats were placed on choline deficient diets. These workers found an increase in liver triglyceride within three to six hours. Within twenty-four hours rats on a choline deficient diet exhibited a five-fold increase in liver triglyceride compared to normal animals. By measuring the rate of uptake and distribution of 9-10 H^3 palmitic acid, these workers concluded that the triglyceride which accumulates rapidly in the liver of choline deficient animals is dietary in origin. The increase in hepatic

triglyceride was associated with a decrease in plasma phospholipid. This evidence gives support to the mechanism of decreased triglyceride transport as a causal factor in choline deficiency induced steatosis. In studies with perfused livers from choline deficient animals, Mookerjee and Haines (115) reported a marked decrease in triglyceride transport into the perfusate. These authors concluded that impairment of triglyceride output was a major factor in the development of fatty liver. Chronic choline deficiency, also, can lead to fibrosis and cirrhosis in addition to the early fatty changes observed. Winawer and co-workers (184) have described the biochemical changes associated with progressing pathology due to choline deficiency. Liver hydroxy-proline increased significantly with manifestations of early fibrosis, while significant increases in nucleic acid levels appeared with advanced cirrhosis. Since animals were maintained on choline deficient diets for relatively short periods in the present study to develop fatty changes, these more serious chronic effects did not occur and would not interfere with the measurements of toxicity.

Recently a series of papers by Handschumacher (65) and others (154) have described the fatty accumulation of the liver induced by incorporation of orotic acid into the diet of test animals. Several factors influence the fatty liver produced by orotic acid. Sidransky et al. (155) have observed that female rats developed a greater degree of hepatic lipid accumulation than males. That orchidectomized males

exhibited greater lipidic infiltration than castrate males given testosterone suggests that the androgen level influences the degree of hepatic steatosis. These workers also observed that the degree of fatty change was influenced by the quantity and quality of food consumption. Starvation of the animals, or replacement of dietary casein with purified amino acids effectively prevented the fatty liver. Handschumacher (65) noted an inhibition of fatty accumulation by the addition of 0.25% adenine to the diet. Hayano and co-workers (68) noted an inhibition of hepatic steatosis by the addition of 1% 5-aminoimidazolecarboxamide to the orotic acid diet. The addition of 0.5% of the nucleosides uridine, cytidine, and adenosine to the test diet showed a considerable preventive effect in the production of the characteristic fatty liver. This observation suggested that an imbalance of purines or pyrimidines in the diet was responsible for the production of fatty accumulation. Using electron microscopy, Novikoff and co-workers (121) showed that orotic acid caused the accumulation of small fat droplets within a vesicle of endoplasmic reticulum. The nuclear envelope showed accumulated lipid between its layers. A decrease in nucleoside diphosphatase activity accompanied the morphological changes in the endoplasmic reticulum. Upon administration of adenine, these lipidic changes disappeared and the nucleoside diphosphatase activity of the endoplasmic reticulum was restored. Similarly, Rubin and Pendleton (147) observed biochemical imbalances in the animals fed orotic acid. A marked increase of uridine nucleotides and

depletion of adenine nucleotides and hepatic ATP content was measured. After five days of orotic acid administration, when gross fatty accumulation was observed, hepatic ATP levels were reduced to 50% of normal values. Synthesis of the protein moiety of β lipoprotein, the form in which triglycerides are released from hepatocytes, was significantly decreased. These observations suggest that hepatic steatosis induced by orotic acid is caused by a specific defect in the synthesis of the protein required for transport of neutral fat from the liver. Despite the biochemical lesions which lead to the development of fatty liver, the administration of 1% dietary orotic acid produces little indication of generalized toxic response. Animals fed this diet made normal weight gains, were smooth-coated and active. Sidransky has remarked that cirrhosis did not appear in animals maintained on this diet for two years, although the liver was heavily infiltrated with fat.

By inducing fatty accumulation in the liver by some of the agents described above, the relationship of liver lipid to the toxicity of carbon tetrachloride was studied. Using the parameters of toxicity described in Section II, the toxic response of carbon tetrachloride was measured when various degrees of hepatic steatosis were induced by agents other than alcohol. By comparison of the response to carbon tetrachloride following ethanol ingestion with that produced in carbon tetrachloride treated animals with non-ethanol induced fatty livers, the relative contribution of hepatic lipid to the toxicity may be assessed. If

the ethanol induced fatty liver is causally related to the potentiated toxicity of carbon tetrachloride, an increased toxic response would be expected when hepatic levels are elevated by other means. However, if ethanol exerts its potentiating effect through some other mechanism, which may only be associated with steatosis, the increased toxicity is not likely to be observed when lipid accumulation was produced by other agents. To clarify the role of liver lipid in the potentiation of toxicity of carbon tetrachloride, a comparison of the toxic response was made in the various groups listed below.

I Normal animals, treated with carbon tetrachloride.

II Hepatic steatosis induced by ingestion of ethanol eighteen hours prior to carbon tetrachloride administration.

III Hepatic steatosis induced by agents other than ethanol, same dose of carbon tetrachloride given as in I and II.

IV Ethanol given to animals with pre-existing fatty liver induced as in III, carbon tetrachloride administered as preceding groups.

V Suitable control groups not receiving carbon tetrachloride were employed to determine whether ethanol, the hepatosteatogenic agent, or their combination would alter the toxic response.

IV-A -- BIOLOGICAL RESPONSE TO CARBON TETRACHLORIDE AND ETHANOL IN ANIMALS WITH HEPATIC STEATOSIS INDUCED BY CHOLINE DEFICIENCY

PROCEDURE

A group of male rats, 125-150 gms of the Sprague-Dawley strain were placed on a choline deficient diet for eleven days to produce a fatty liver. Animals were allowed access to both water and diet ad libitum. In those animals treated with ethanol, the dose was administered by intubation at a dosage of 5.0 ml/kg. Eighteen hours after the administration of ethanol, animals were exposed to 1000 ppm carbon tetrachloride for four hours. Twenty hours after exposure to carbon tetrachloride, animals were sacrificed by decapitation and blood collected for assay of serum enzyme activity. Sections of liver were reserved for analysis of total liver lipids and histopathological examination. Groups of normal animals were similarly treated with carbon tetrachloride and ethanol and served as controls for comparison of enzyme activity following treatment with carbon tetrachloride, ethanol, or their combination.

A group of rats was sacrificed eighteen hours after intubation of ethanol to determine the liver lipid levels at the time of exposure to carbon tetrachloride. Lipid analysis and measurement of serum enzyme activity was performed as described in Methods, Section II.

Concentration of carbon tetrachloride in blood of normal and

choline deficient animals was determined one hour after exposure to 6000 ppm for two hours. The concentration of the organic halide found in these animals was compared to that found in similarly treated groups which were intubated with 5.0 ml/kg ethanol eighteen hours prior to exposure. Analysis of carbon tetrachloride was performed as described in Section II.

RESULTS

ANALYSIS OF LIVER LIPIDS

After eleven days, rats maintained on the choline deficient diet attained a weight range of 200-225 gms. Rats maintained on normal diet were selected in this weight range to serve as controls. Rats fed the choline deficient diet exhibited mean total liver lipids of 12.19 $\pm$ 1.81% as shown in Table 8. Treatment of these animals with ethanol or carbon tetrachloride did not alter this grossly elevated liver lipid level. Rats maintained on a normal diet had a mean liver lipid value of 4.60 $\pm$ 0.14% in this same body weight range. Eighteen hours following intubation of 5 ml/kg ethanol, mean liver lipid levels were 5.20 $\pm$ 0.25%, and 4.69 $\pm$ 0.18% twenty hours later. Animals exposed to carbon tetrachloride exhibited a mean liver lipid value of 5.86 $\pm$ 0.17% twenty hours later which was significantly elevated above normal. (Table 8). Similarly exposed animals which were pretreated with ethanol had a liver lipid content of 5.18 $\pm$ 0.24%. Liver lipid content of choline deficient animals was greatly elevated above that of normal animals in all cases.

TABLE 8

TOTAL HEPATIC LIPID IN NORMAL AND CHOLINE DEFICIENT RATS FOLLOWING ADMINISTRATION OF ETHANOL AND/OR CARBON TETRACHLORIDE

Treatment	Percent Liver Lipid (± S. E.)
CDD	12.19 ± 1.81[a]
CDD + C	12.68 ± 1.61[a]
CDD + C + E	12.34 ± 1.25[a]
C + E	5.18 ± 0.24
C	5.86 ± 0.17[a]
E_{38}	4.69 ± 0.18
E_{18}	5.20 ± 0.25
NORMAL	4.60 ± 0.14

CDD -- Animals maintained on choline deficient diet to induce fatty liver.

E -- 5 ml/kg ethanol, oral.

E_{18} -- Lipid level at time of exposure to CCl_4, 18 hours after ethanol.

E_{38} -- 38 hours after ethanol, comparable to time of sacrifice of ethanol and carbon tetrachloride treated rats.

C -- 1000 ppm carbon tetrachloride for four hours.

At least six animals per group.

[a] $P < 0.05$ versus normal

SERUM ENZYME ACTIVITY

In a preliminary study, the serum GOT activity following exposure to a high concentration of carbon tetrachloride was determined in animals with hepatic steatosis induced by choline deficiency, or ethionine. Twenty hours after exposure to 6000 ppm of carbon tetrachloride for two hours, normal animals exhibited S-GOT activities of 24437 $\pm$ 3201 (Table 9). Similar animals which were given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to exposure had a mean S-GOT activity of 22462 $\pm$ 2666 at this time. One of the four animals died within twelve hours after exposure to carbon tetrachloride.

When 750 mg/kg ethionine was administered intraperitoneally to animals to induce fatty accumulation of the liver, S-GOT values rose to 844 $\pm$ 271 thirty six hours later (Table 9). Twenty hours after exposure to 6000 ppm of carbon tetrachloride, these animals had a mean S-GOT activity of 17545 $\pm$ 2474 . This value was comparable to that observed in normal animals exposed to this concentration of carbon tetrachloride.

Following exposure of animals with hepatic steatosis induced by choline deficiency, serum GOT values rose to 4788 $\pm$ 365 within twenty hours. This value was markedly less than that observed in normal animals exposed to this concentration of carbon tetrachloride. Two choline deficient animals, which were treated with ethanol, eighteen

TABLE 9

SERUM ENZYME ACTIVITY FOLLOWING CARBON TETRACHLORIDE EXPOSURE OF RATS WITH HEPATIC STEATOSIS INDUCED BY ETHANOL, ETHIONINE OR CHOLINE DEFICIENCY

Treatment	S-GOT Activity	% Liver Lipid (± SE)
Normal	248 ± 44	4.60 ± 0.14
C	24437 ± 3201[c]	6.06 ± 0.22[c]
C + E	22462[a] ± 2666[c]	6.43 ± 0.306[c]
Ethionine	844 ± 271	4.36 ± 0.37
Ethionine + C	17545 ± 2474[c]	5.39 ± 0.38
CDD	382 ± 134	6.26 ± 1.05
CDD + C	4788 ± 365[d]	8.16 ± 1.13[c]
CDD + C + E	13000;23000[b, c]	- - -

CDD -- Choline deficient diet.

C -- 6000 ppm for two hours.

E -- 5.0 ml/kg administered orally eighteen hours prior to carbon tetrachloride exposure.

Ethionine -- 75 mg/100 gm B.W., IP, 36 hours prior to carbon tetrachloride exposure.

All animals sacrificed twenty hours after carbon tetrachloride administration. Four animals per group.

[a] Mean of three animals.

[b] Two animals.

[c] $P < 0.05$ versus normal.

[d] $P < 0.05$ versus C + E or C.

hours prior to exposure, exhibited S-GOT activities of 13,000 and 23,000. Since a greatly elevated response in enzyme activity was observed following this treatment, it did not appear that choline deficiency inhibited enzyme release or activity.

Because of the generalized toxic effects, elevation of S-GOT activity, and variable extent of hepatic steatosis produced, ethionine was discarded as a useful agent in this study. It is of parenthetical interest to note that choline deficiency produced a greater degree of hepatic steatosis in young rats than in mature animals. For this reason, immature rats (B.W. < 150 gms) were maintained on choline deficient diets until they attained a body weight of 200-225 gms.

Comparison of serum enzyme activity in choline deficient and normal animals was made following exposure to a lower concentration of carbon tetrachloride. As noted in Table 2, differences in serum enzyme activity observed between normal and ethanol treated animals were more pronounced after exposure to lower levels of carbon tetrachloride. Comparison of serum enzyme activity was made in choline deficient rats pre-treated with ethanol and those which received carbon tetrachloride alone, to differentiate the effects of ethanol and the presence of hepatic lipid upon the response observed.

Twenty hours following exposure to 1000 ppm carbon tetrachloride for four hours, serum GOT or ICD activity in normal animals

TABLE 10

SERUM ENZYME ACTIVITY FOLLOWING ADMINISTRATION OF ETHANOL AND/OR CARBON TETRACHLORIDE TO NORMAL AND CHOLINE DEFICIENT RATS

	Serum Enzyme Activity		Potentiation Index	
Treatment	S-GOT (± S. E.)	S-ICD (± S. E.)	GOT	ICD
CDD	382 ± 134	900 ± 46	1.0	1.0
CDD + E	433 ± 80	910 ± 76	1.1	1.0
CDD + C	677 ± 89[a]	930 ± 107[a]	1.8	1.0
CDD + C + E	7150 ± 1802	10125 ± 915	18.7	11.3
C + E	2481 ± 105	2700 ± 381	10.0	3.8
C	296 ± 44	750 ± 31	1.2	1.1
E	234 ± 30	738 ± 93	0.9	1.0
NORMAL	248 ± 44	706 ± 57	1.0	1.0

CDD -- Animals maintained on choline deficient diet to induce fatty liver.

E -- 5 ml/kg ethanol administered orally.

C -- 1000 ppm carbon tetrachloride for four hours.

At least six animals per group. All animals sacrificed twenty hours after exposure.

[a] $P < 0.05$ versus C + E.

was not significantly elevated in comparison to untreated animals (Table 10). Similarly, intubation of ethanol caused no significant change in serum enzyme activity, thirty eight hours after dosage. However, when animals were pre-treated with ethanol eighteen hours prior to exposure to carbon tetrachloride, serum GOT and ICD activity rose to 2481 $\pm$ 105 and 2700 $\pm$ 381 respectively. This represents an increase of 10.0 and 3.8 times the normal value.

When hepatic lipid content was increased by choline deficiency, the animals exhibited a mean serum GOT and ICD activity of 382 $\pm$ 134 and 900 $\pm$ 46 respectively. Although the serum enzyme activity was not statistically different from normal animals, the generalized toxic effects of the diet, characterized by rough coat, slow weight gains, must be considered. Thirty eight hours after ingestion of 5.0 ml/kg of ethanol, serum enzyme activity of choline deficient animals was comparable to that of the control group on a similar diet and not statistically different from that of normal animals. If the alcohol potentiated response to carbon tetrachloride can be attributed to increased hepatic lipid, a greatly elevated serum enzyme activity would be expected following exposure of the animals with the choline deficiency induced steatosis. However, measurement of serum enzyme activity in such animals twenty hours after exposure showed levels of serum GOT and ICD of 677 $\pm$ 89 and 930 $\pm$ 107, respectively. Neither of these values were statistically different

from corresponding values measured in animals with similar choline deficiency. (Compare CDD with CDD + C). However, when a group of similar choline deficient animals was pre-treated with ethanol prior to exposure, grossly elevated serum enzyme activity was observed. Twenty hours after exposure, these choline deficient ethanol treated animals exhibited levels of 7150 $\pm$ 1802, and 10125 $\pm$ 915 for serum GOT and ICD, respectively. (Table 10). These values represent an increase of 18.7 and 11.3 times the normal value.

CONCENTRATION OF CARBON TETRACHLORIDE IN BLOOD

Following inhalation of 6000 ppm for two hours concentration of carbon tetrachloride was determined in normal animals and those pre-treated with 5 ml/kg ethanol eighteen hours prior to exposure. Similar groups of choline deficient animals (untreated or alcohol treated) were exposed at the same time to this concentration of carbon tetrachloride. Because of the rapid excretion of carbon tetrachloride through the lungs following exposure, animals were serially sacrificed at timed intervals one hour after exposure. Mean blood concentrations of carbon tetrachloride in choline deficient animals were similar to those of normal, or ethanol treated animals. Results of this analyses are summarized in Table 11. Individual values of blood concentration of carbon tetrachloride found in variously treated groups were compared with time of sacrifice. This relationship can be expressed by the function: $X = 0.026 - \frac{Y}{3100}$

TABLE 11

CARBON TETRACHLORIDE CONCENTRATION IN BLOOD OF NORMAL AND CHOLINE DEFICIENT RATS

Treatment	Blood Concentration mg CCl_4/gm (± SE)
CDD + C	0.027 ± 0.003
CDD + E + C	0.030 ± 0.003
C	0.025 ± 0.009
E + C	0.021 ± 0.003

CDD -- Animals maintained on choline deficient diet to induce hepatic steatosis.

E -- 5 ml/kg ethanol, orally administered eighteen hours prior to carbon tetrachloride.

C -- 6000 ppm carbon tetrachloride for two hours.

At least five animals per group.

where X is concentration of CCl_4 in mg/gm blood, Y is time after exposure in minutes.

Liver concentrations of carbon tetrachloride were too low to be consistently determined following this level of exposure.

HISTOLOGICAL STUDIES

Examination of liver sections from choline deficient rats showed the characteristic hepatic accumulation of lipid. As shown in Figure 13, prominent lipidic droplets could be detected within the liver parenchyma of the choline deficient animal. No disruption of the hepatic architecture was observed.

Twenty hours after exposure to 1000 ppm carbon tetrachloride for four hours, examination of liver sections revealed fatty accumulation but no architectural changes in hepatic structure. Comparison of Figure 11 with Figure 13 shows that the degree of lipidic infiltration due to carbon tetrachloride exposure is similar to that of the unexposed choline deficient animal. No necrosis was observed in either case.

The liver from a choline deficient animal given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to carbon tetrachloride exposure exhibited central lobular necrosis and infiltration of polymorphonuclear leucocytes, as shown in Figure 12. The morphological changes observed in ethanol pre-treated choline

Figure 11

Liver from a choline deficient rat twenty hours after exposure to 1000 ppm carbon tetrachloride for four hours. H & E, 145X.

The liver shows degenerative changes which are produced by the choline deficient diet. Fatty accumulation, but no necrosis, is present. Hepatic architecture is intact.

Figure 12

Liver from a choline deficient rat given 5 ml/kg ethanol orally eighteen hours prior to exposure to 1000 ppm carbon tetrachloride for four hours. The rat was sacrificed twenty hours after carbon tetrachloride exposure. H & E, 145X.

Central lobular necrosis and infiltration of polymorphonuclear leucocytes can be observed. Moderate lipidic accumulation is present in necrotic areas and in midzonal areas. The morphological changes are more severe than those produced by carbon tetrachloride alone. (See Figure 11).



Figure 11

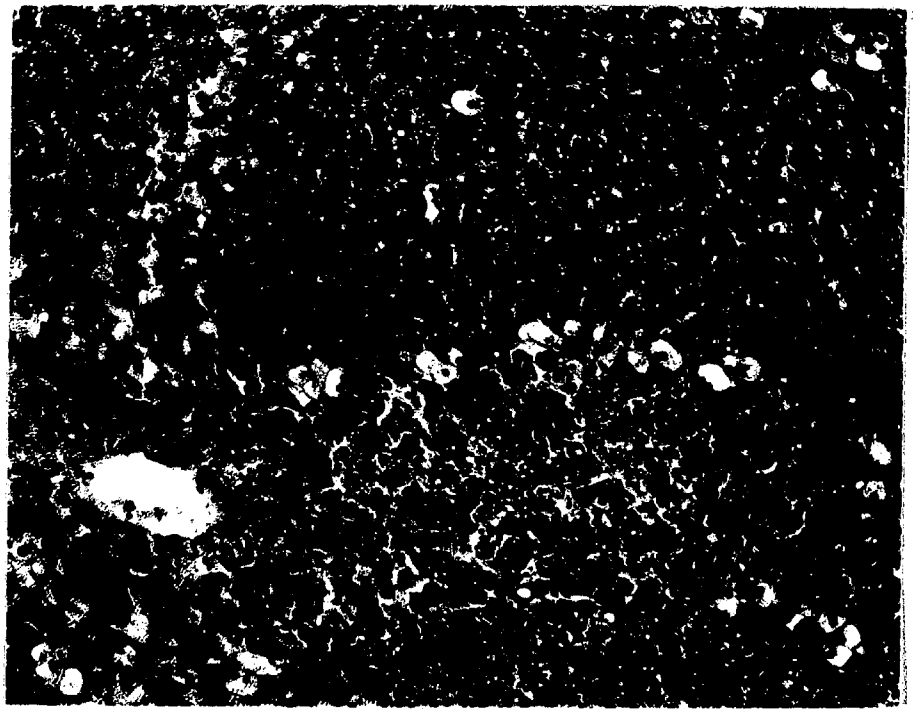


Figure 12

deficient rats were more prominent than in similar animals which received only carbon tetrachloride. At this low level of carbon tetrachloride exposure, necrosis was observed only in ethanol treated animals, irrespective of the degree of hepatic steatosis.

Examination of sections of heart, lung, kidney, adrenal, and spleen revealed no abnormalities.

DISCUSSION

Eighteen hours after the ingestion of ethanol,at the time of CCl_4 exposure, essentially all of the administered ethanol has been metabolized or excreted. Therefore, little direct interaction with carbon tetrachloride could be attributed to the presence of alcohol in the tissues. Histopathological examination revealed a slight lipidic accumulation eighteen hours after ethanol ingestion. Lipid analysis also indicated a slight increase in the mean value of total hepatic lipid at this time. If this residual effect of increased liver lipid was directly related to the potentiation of carbon tetrachloride toxicity by ethanol, an increased toxic response would be expected in animals with steatosis induced by choline deficiency. Examination of serum enzyme activity following treatment with carbon tetrachloride in animals with normal or elevated hepatic lipid levels did not reveal a great difference in response to the hydrocarbon. Only after treatment with both carbon tetrachloride and ethanol was a gross elevation of serum

enzymes observed. The serum enzyme activity induced by carbon tetrachloride alone was independent of the amount of hepatic lipid. The argument that enzyme activity was depressed in choline deficient animals did not appear to be tenable since a large serum enzyme response was observed following treatment of these animals with alcohol and carbon tetrachloride. The grossly elevated serum enzyme activity which has been related to toxic tissue damage, was observed only when animals were treated with the combination of ethanol and carbon tetrachloride and was independent of hepatic lipid levels. Similarly, liver necrosis was observed only in animals treated with ethanol prior to exposure and was also independent of hepatic lipid accumulation.

IV-B -- BIOLOGICAL CHANGES IN ANIMALS WITH DIETARY HEPATIC STEATOSIS GIVEN CARBON TETRACHLORIDE AND/OR ETHANOL

INTRODUCTION

Increased hepatic lipid has been reported frequently in animals fed a high fat diet. To determine whether an elevated hepatic lipid level would cause increased concentration of carbon tetrachloride in blood or liver, analysis of the hydrocarbon was performed in animals given various treatments. Comparison of hepatic and blood levels of carbon tetrachloride was made between animals with normal or fatty livers.

PROCEDURE

Fatty accumulation of the liver was induced in male Sprague-Dawley rats, 175-200 gms, fed a fortified corn meal diet into which 30% corn oil was incorporated. Eighteen hours prior to administration of carbon tetrachloride, a group of the animals was given 5 ml/kg ethanol orally as a 50% solution. Carbon tetrachloride was administered to animals by intraperitoneal injection of 0.6 ml/kg CCl_4. Two hours after the administration of the carbon tetrachloride, animals were sacrificed and analysis of the hydrocarbon was performed as described earlier. Analysis of the organic halide was performed in animals with normal livers after similar treatment with ethanol and/or carbon tetrachloride.

Sections of liver were reserved for histopathological examination and determination of total lipid.

RESULTS

Animals fed the enriched corn meal diet all exhibited rough coat, diarrhea, and moderate generalized lipidic degeneration of the liver. Two hours after the injection of carbon tetrachloride, a group of six of these animals developed a hepatic level of 0.0285 $\pm$ 0.155 mg CCl_4/gm and 0.071 $\pm$ 0.047 mg CCl_4/gm blood. (Table 12). In comparison, similar animals which were pre-treated with ethanol eighteen hours prior to carbon tetrachloride exhibited 0.374 $\pm$ 0.210 mg CCl_4/gm liver, and 0.064 $\pm$ 0.040 mg CCl_4/gm blood. Histopathological examination of liver sections of these animals showed moderate lipidic degeneration of the liver in a central and mid-lobular distribution. (Figures 15 and 16). No significant difference in total liver lipid was observed between these groups. Those animals which received only carbon tetrachloride exhibited a mean lipid value of 6.79 $\pm$ 0.63%. Similarly, animals which were treated with ethanol prior to carbon tetrachloride injection showed 6.56 $\pm$ 0.39% total hepatic lipid at the time of sacrifice.

Animals which were maintained on a normal diet showed slight lipidic degeneration of the liver upon histopathological examination. (Figures 17 and 18). Analysis revealed 0.264 $\pm$ 0.110 mg CCl_4/gm liver and 0.048 $\pm$ 0.009 mg CCl_4/gm blood at this time. A similar

TABLE 12

CONCENTRATION OF CARBON TETRACHLORIDE IN BLOOD AND LIVER OF ANIMALS WITH DIETARY INDUCED STEATOSIS

Treatment	Concentration of Carbon Tetrachloride Liver mg CCl_4/gm	Blood mg CCl_4/gm	% Total Liver Lipid
FC	0.285 ± 0.155	0.071 ± 0.047	6.79 ± 0.63[a]
FEC	0.374 ± 0.210	0.064 ± 0.040	6.56 ± 0.39[a]
C	0.264 ± 0.110	0.048 ± 0.009	4.75 ± 0.35
EC	0.390 ± 0.170	0.043 ± 0.016	4.87 ± 0.55

F -- Fatty accumulation of the liver induced by high fat-cornmeal diet.

E -- 5 ml/kg ethanol administered orally eighteen hours prior to carbon tetrachloride injection.

C -- 0.6 ml/kg carbon tetrachloride intraperitoneal.

All animals sacrificed two hours after administration of carbon tetrachloride. At least six animals per group, except FEC = 4.

[a] $P < 0.05$ versus normal.

Figure 13

Hepatic steatosis induced by choline deficiency. Oil Red 0, 145X.

Moderate lipidic accumulation can be observed in the liver of a rat maintained on a choline deficient diet for eleven days. Hepatic architecture is intact. Prominent lipidic droplets can be detected within the parenchyma.

Figure 14

Hepatic steatosis produced by high fat diet. Oil Red 0, 145X.

Prominent fatty change can be observed in the liver of a rat maintained on a high fat diet. Hepatic architecture is intact. No abnormalities in heart, kidney, or spleen were observed.



Figure 13

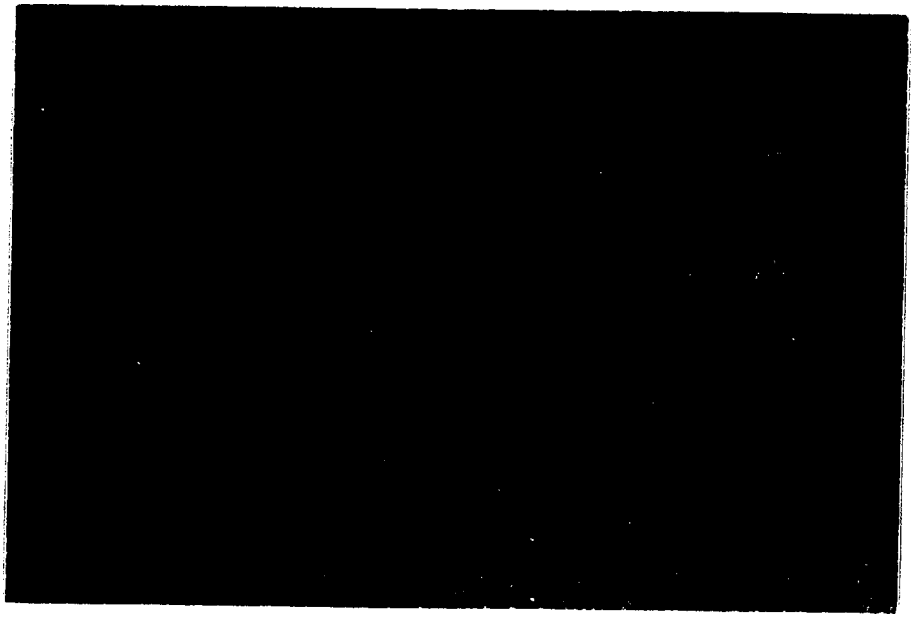


Figure 14

Figure 15

Liver of a rat maintained on a high fat diet and sacrificed two hours after intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Oil Red 0, 145X.

Moderate generalized lipidic degeneration of the liver can be observed.

Figure 16

Liver of a rat maintained on a high fat diet and given an oral dose of 5 ml/kg ethanol eighteen hours prior to injection of 0.6 ml/kg carbon tetrachloride. The animal was sacrificed two hours after administration of the carbon tetrachloride. Oil Red 0, 145X.

A moderate generalized lipidic degeneration of the liver can be observed.



Figure 15

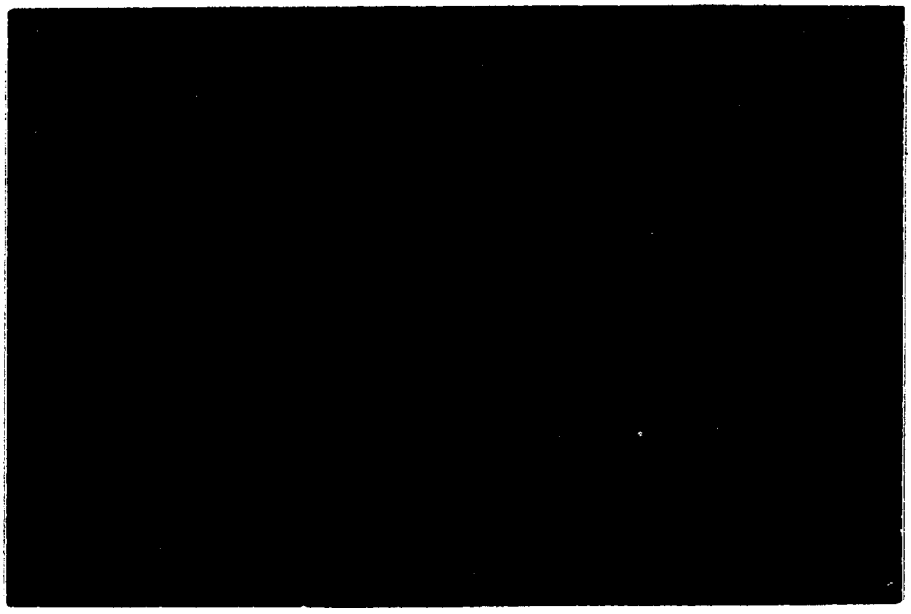


Figure 16

Figure 17

Liver of a rat two hours after injection of 0.6 ml/kg carbon tetrachloride. Oil Red 0, 145X.

Very slight lipidic degeneration of the liver can be observed.

Figure 18

Liver of a rat given an oral dose of 5 ml/kg ethanol eighteen hours prior to intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. The rat was sacrificed two hours after administration of carbon tetrachloride. Oil Red 0, 145X.

Slight lipid degeneration of the liver extending to mid-lobular and peripheral zones can be observed.

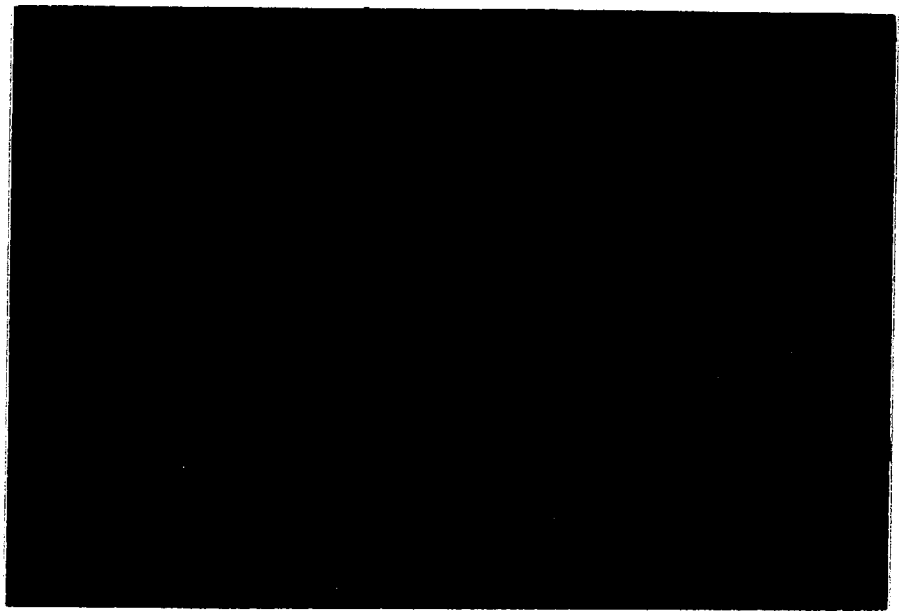


Figure 17

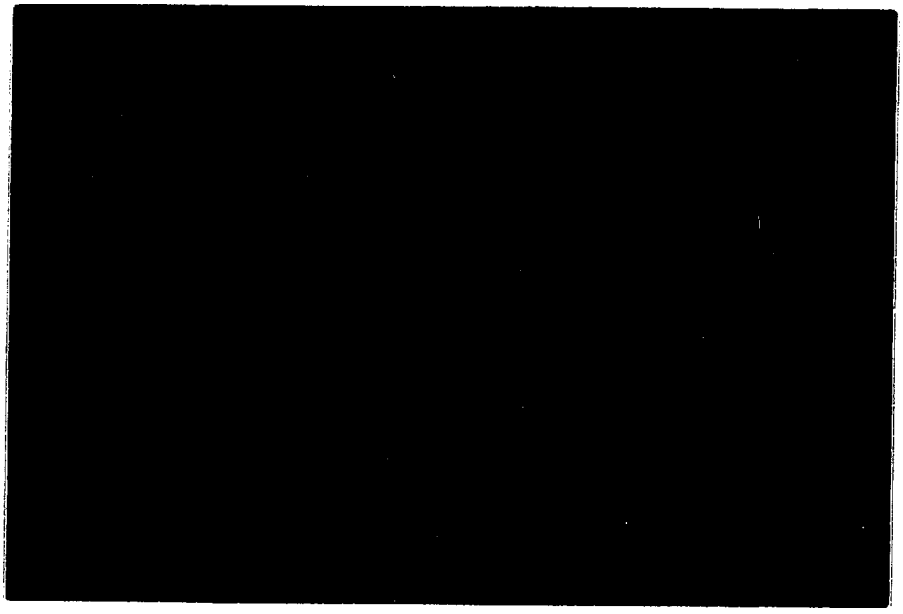


Figure 18

group of six animals which were treated with ethanol prior to administration of carbon tetrachloride exhibited 0.390 ± 0.170 mg CCl_4 gm liver and 0.043 ± 0.016 mg CCl_4/gm blood. At the time of sacrifice, animals which received only carbon tetrachloride exhibited a total hepatic lipid value of 4.75 ± 0.35%. Analysis of total liver lipid in animals which received both ethanol and carbon tetrachloride revealed a mean value of 4.87 ± 0.55 at this time.

DISCUSSION

No significant differences were found in the carbon tetrachloride concentrations in blood or liver of exposed rats, with or without prior ethanol treatment. In addition, no correlation appeared to exist between the hepatic lipid level and the concentration of carbon tetrachloride in the liver. Animals were allowed food ad libitum until the time of administration of carbon tetrachloride. The presence of food, and especially the large quantity of corn oil in the diet, may have affected the distribution of carbon tetrachloride in the rat. In addition, ethanol treated animals suffered a loss of appetite following intubation. The possibility exists that the lack of food in the gastro intestinal tract of these animals may have affected the results observed.

Animals fed the high fat diet also suffered from chronic diarrhea. The stress of the diet and physiological changes may play an important part in the interpretation of these results. Because of the toxic effects of the high fat diet, this method of inducing hepatic steatosis was not continued.

In subsequent determinations of carbon tetrachloride concentrations, food was removed from animals eighteen hours prior to its administration. Animals were given isocaloric doses of ethanol or sucrose.

A significant difference in hepatic lipid levels was observed between animals placed on the two diets. In spite of the elevated liver lipid level caused by the corn meal diet, the concentration of carbon tetrachloride found in the livers of these animals (FC) was comparable to that found in the normal (C) group. The presence of a pre-existent fatty liver did not appear to increase the concentration of carbon tetrachloride within that organ. Subsequent analyses of the hydrocarbon was made to determine whether alcohol caused an increased concentration in blood and liver. No differences of statistical significance could be assigned to the concentrations of carbon tetrachloride found in the blood of the variously treated groups.

IV-C -- BIOLOGICAL RESPONSE TO CARBON TETRACHLORIDE AND ETHANOL IN ANIMALS WITH OROTIC ACID INDUCED HEPATIC STEATOSIS

PROCEDURE

A group of male Sprague Dawley rats, 125-150 gms was maintained (for ten days) on a Nutritional Biochemical Co. normal protein diet in which 1% orotic acid was incorporated. Gross examination of the liver at this time revealed an extensive accumulation of hepatic lipid. The liver was friable, light tan in color and bulged outward when the capsule was cut. In other respects, all animals maintained on this diet appeared normal with smooth coats and regular body weight increases. A similar control group was maintained on the normal protein diet, but without the addition of the orotic acid. All animals were allowed drinking water ad libitum. Ethanol was administered by stomach tube as a 50% aqueous solution, each animal receiving 5 ml/kg ethanol. Carbon tetrachloride was given by two routes of administration. Intraperitoneally, carbon tetrachloride was administered in a dose of 0.6 ml/kg body weight, and orally it was administered by stomach tube at the rate of 2.5 ml/kg body weight in an equal volume of corn oil. Carbon tetrachloride was given, in all cases, eighteen hours after administration of ethanol.

Twenty hours after administration of carbon tetrachloride, animals were sacrificed by decapitation and blood collected for the determination of serum levels of glutamic oxaloacetic transaminase, isocitric dehydrogenase, and glutamic pyruvic transaminase. Carbon tetrachloride in blood and liver was measured as described in Section II. Determinations of serum iron, and liver nucleotide ratios were performed as described in Methods II-D & E. Sections of heart, liver, lung, kidney, spleen and adrenal in 10% formalin were reserved for histopathological examination following staining with hemotoxylin and eosin, or Oil Red O. Liver lipid levels were determined as described in Section II-A.

RESULTS

TOTAL LIVER LIPID CONTENT AFTER ADMINISTRATION OF CARBON TETRACHLORIDE AND/OR ETHANOL

Animals fed a normal protein diet containing 1% orotic acid developed a striking fatty liver within ten days. The liver at this time was light tan in color and analysis showed 13.32 $\pm$ 0.75% total lipid (Table 13). Except for the marked accumulation of liver fat, animals appeared normal. Control animals maintained on the same diet without orotic acid had 4.54 $\pm$ 0.19% liver lipid.

The administration of ethanol to animals fed the orotic acid

diet caused a striking increase in liver lipid accumulation. Eighteen hours after the administration of alcohol, liver lipids rose to 21.26 ± 1.33%. The mean value, thirty eight hours after ingestion of ethanol was 18.03 ± 1.01 still significantly elevated above levels of rats fed orotic acid but not given alcohol.

Two hours after the intraperitoneal injection of 0.6 ml/kg CCl_4, animals on the orotic acid containing diet exhibited a liver lipid level of 13.50 ± 0.64% comparable to that of untreated orotic acid fed animals. Twenty hours after the injection of carbon tetrachloride, the lipid level rose to 17.53 ± 1.67%. Similar results were obtained with the alcohol pretreated animals on the orotic acid diet. Two hours after administration of carbon tetrachloride (twenty hours after ethanol ingestion), the liver lipid level was 13.34 ± 1.15%. Twenty hours later the mean value of lipid determinations rose slightly to 15.46 ± 0.70%.

Control animals not given orotic acid had a mean liver lipid value of 5.29 ± 0.57% eighteen hours after the ingestion of ethanol. Twenty hours later, lipid values were 5.73 ± 0.87%. After the intraperitoneal injection of carbon tetrachloride, liver lipid levels were 5.11 ± 0.47% two hours after dosage and rose to 7.99 ± 0.75 at twenty hours. In comparison, animals which were pretreated with ethanol showed a rapid increase to 6.93 ± 0.59% liver lipid two hours

TABLE 13

TOTAL LIVER LIPID IN NORMAL OR OROTIC ACID FED RATS FOLLOWING ADMINISTRATION OF ETHANOL AND/OR CARBON TETRACHLORIDE

Treatment	Percent Total Liver Lipid (± S.E.) 2 Hours after CCl_4 Administration	20 Hours after CCl_4 Administration
O	13.32 ± 0.75[a]	
OE	21.26 ± 1.33[a]	18.03 ± 1.01[a]
OC	13.50 ± 0.64a	17.53 ± 1.67[a]
OEC	13.34 ± 1.15[a]	15.46 ± 0.70[a]
Normal	4.53 ± 0.19	
E	5.29 ± 0.57	5.73 ± 0.87
C	5.11 ± 0.47	7.99 ± 0.75[b]
EC	6.93 ± 0.59[b]	7.94 ± 0.39[b]

O -- 1% dietary orotic acid.

C -- 0.6 ml/kg CCl_4 IP.

E -- 5 ml/kg ethanol, oral.

At least 5 animals per group.

[a] $P < 0.001$ versus normal.

[b] $P < 0.005$ versus normal.

after injection with carbon tetrachloride. Twenty hours after treatment with carbon tetrachloride, these alcohol pretreated animals exhibited elevated liver lipid levels of 7.94 ± 0.39. This amount of liver lipid was comparable to that found in alcohol. untreated animals which were given the same dose of carbon tetrachloride.

DETERMINATION OF SERUM ENZYME LEVELS

Twenty hours after administration of carbon tetrachloride, serum enzyme activity was measured in groups of rats treated with ethanol, carbon tetrachloride, orotic acid, or their combination. Comparison of serum enzyme activity following administration of carbon tetrachloride was made to determine whether a pre-existent fatty liver augmented the toxic response to carbon tetrachloride.

Following the intubation of 5 ml/kg ethanol, the activity of serum glutamic oxaloacetic transaminase (S-GOT) was not significantly altered in normal animals or those with orotic acid induced fatty livers as shown in Table 14. Twenty hours after the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride into animals with fatty livers induced by orotic acid, the activity of S-GOT increased to 865 ± 165. A similar group of animals which had been treated with ethanol eighteen hours prior to carbon tetrachloride injection exhibited a greatly elevated S-GOT level with a mean value

of 17,562 ± 434. In contrast, normal animals exhibited an increase in S-GOT activity to 5333 ± 1389 after the injection of carbon tetrachloride. A similar group of animals pretreated with ethanol prior to administration of carbon tetrachloride showed a mean S-GOT value of 9475 ± 1426.

Examination of serum isocitric dehydrogenase (S-ICD) activity revealed a pattern of response to carbon tetrachloride in treated groups of animals similar to that above. Those animals with a fatty liver induced by orotic acid showed a S-ICD value of 1263 ± 166 following administration of carbon tetrachloride alone. When a similar group of animals was given ethanol prior to carbon tetrachloride, a greatly elevated response of 48,594 ± 10,489 was observed. In control animals this same dose of the chlorinated hydrocarbon resulted in an increase of S-ICD activity to 2275 ± 657. When ethanol was given to a similar group eighteen hours prior to carbon tetrachloride, S-ICD values rose markedly to 23,250 ± 5141.

A similar response in serum glutamic pyruvic transaminase (S-GPT) was observed following treatment with ethanol, carbon tetrachloride, or their combination. In animals with fatty liver induced by orotic acid administration, the mean value of S-GPT activity was 439 ± 58 when this dosage of carbon tetrachloride alone was given. In similar animals, when the carbon tetrachloride dosage

was preceded by ethanol intubation, the value observed was 9412 $\pm$ 2758.

In control animals, the mean value of S-GPT activity was 1368 $\pm$ 156 when carbon tetrachloride was given alone. When treatment with ethanol preceded dosage with the halogenated hydrocarbon the mean value rose to 2665 $\pm$ 566.

If the ethanol induced elevation of liver lipid levels were causally related to the potentiation of carbon tetrachloride toxicity, an increased toxic response to the halogenated hydrocarbon would be observed in animals given orotic acid. Serum enzyme activity, used as a measure of toxic tissue response to the hydrocarbon, did not reveal increased tissue damage in animals with the orotic acid induced fatty accumulation of the liver. Following dosage with carbon tetrachloride, the potentiation index of S-GOT, S-GPT, and S-ICD was 2.9, 22.0, and 1.6, respectively, in animals with fatty liver caused by orotic acid. Similarly treated animals with normal lipid levels exhibited potentiation indexes of 17.8, 68.4, and 2.8 for S-GOT, S-GPT, and S-ICD, respectively. In all cases, the enzyme response following carbon tetrachloride administration was lower in animals with the elevated liver lipid levels than those with normal levels. In the case of S-GOT and S-GPT levels, this difference was statistically

significant ($P < 0.05$). Measurement of serum enzyme activity in control animals fed the orotic acid diet, however, showed no deviation from normal values. In animals with markedly elevated lipid values induced by orotic acid, potentiation indexes of 58.5, 470.6, and 60.7 for S-GOT, S-GPT and S-ICD were observed in alcohol pretreated animals after injection with carbon tetrachloride. A group of normal animals, similarly treated with alcohol and carbon tetrachloride had potentiation indexes of 31.6, 133.3, and 29.1 for the respective enzymes. In this instance, the enzyme response in animals with orotic acid induced fatty liver was greater than in the corresponding normal group treated with the same combination of alcohol and hydrocarbon. The greatly increased enzyme response was elicited only when prior ingestion of alcohol accompanied the administration of carbon tetrachloride. The elevated liver lipid levels appeared to be independent of potentiated serum enzyme activity following carbon tetrachloride dosage alone. The greatly elevated serum enzyme response, indicative of greater tissue damage, was produced only when ethanol was given prior to carbon tetrachloride injection in both normal animals and those with fatty liver. No direct correlation between amount of liver lipid and elevated enzyme activity was observed. Since a marked increase in serum enzyme levels was

TABLE 14

SERUM ENZYME LEVELS FOLLOWING ADMINISTRATION OF CARBON TETRACHLORIDE AND/OR ETHANOL TO NORMAL AND OROTIC ACID FED RATS

	Serum Enzyme Activity (± S.E.)			Potentiation Index		
Treatment	S-GOT	S-GPT	S-ICD	S-GOT	S-GPT	S-ICD
OE	230 ± 27	21 ± 0.6	546 ± 37	0.8	1.1	0.7
OC	865 ± 165[a]	439 ± 58[a]	1263 ± 166[a]	2.9[a]	22.0[a]	1.6[a]
OEC	17562 ± 434	9412 ± 2758	48594 ± 10489	58.5	470.6	60.7
Normal	300 ± 14	20 ± 0.2	800 ± 100	1.0	1.0	1.0
E	280 ± 13	22 ± 1.6	837 ± 165	0.9	1.1	1.0
C	5333 ± 1389[b]	1368 ± 156[b]	2275 ± 657	17.8[b]	68.4[b]	2.8
EC	9475 ± 1426[a]	2665 ± 566[a]	23250 ± 5141[a]	31.6[a]	133.3[a]	29.1[a]

O -- Fatty liver induced by 1% dietary orotic acid.

E -- 5 ml/kg ethanol.

C -- 0.6 ml/kg carbon tetrachloride injected intraperitoneally.

All animals sacrificed 20 hours after carbon tetrachloride administration.

At least five animals per group.

[a] $P < 0.05$, OC versus EC

[b] $P < 0.05$, OC versus C

observed in the carbon tetrachloride and ethanol treated orotic acid fed animals, it did not appear that orotic acid inhibited enzyme activity or the release of enzymes from damaged cells.

DETERMINATION OF CARBON TETRACHLORIDE CONCENTRATIONS IN BLOOD AND LIVER OF NORMAL AND OROTIC ACID FED RATS

Two hours after the intraperitoneal injection of carbon tetrachloride, 0.273 $\pm$ 0.023 mg CCl_4 per gm was found in the liver of control animals. Animals which had been pretreated with ethanol eighteen hours prior to injection with carbon tetrachloride exhibited a level of 0.277 $\pm$ 0.047 mg/gm liver (Table 15). Similarly, animals with the orotic acid induced fatty liver exhibited a concentration of 0.320 $\pm$ 0.074 mg CCl_4/gm liver two hours after injection of the hydrocarbon. Ingestion of ethanol, eighteen hours prior to carbon tetrachloride injection, in similar animals did not alter the carbon tetrachloride concentration found. The concentration in liver of alcohol pretreated animals with fatty liver was 0.290 $\pm$ 0.048 mg CCl_4/gm liver.

Determination of carbon tetrachloride concentrations in blood of these animals revealed a similar pattern. Animals with normal liver lipid levels had 0.095 $\pm$ 0.017 mg CCl_4/gm blood, those animals pretreated with ethanol had 0.103 $\pm$ 0.024 mg CCl_4/gm blood

at this time. Determination of blood concentration of carbon tetrachloride in animals with orotic acid induced fatty liver showed 0.100 ± 0.026 mg CCl_4/gm, and similar animals pretreated with ethanol showed 0.092 ± 0.028 mg CCl_4/gm. Apparently, the prior treatment with ethanol or the elevated liver lipid levels did not affect the concentration of carbon tetrachloride found in blood or liver after intraperitoneal injection of the hydrocarbon.

When carbon tetrachloride was given by the oral route at a rate of 2.5 ml/kg to animals with the orotic acid induced fatty liver 0.727 ± 0.252 mg/gm was recovered. From similar animals, which were pretreated with ethanol eighteen hours previously, 0.700 ± 0.193 mg/gm carbon tetrachloride was recovered in liver after the same dosage. When animals with normal liver lipid levels were given this dose of the hydrocarbon, 0.342 ± 0.084 mg CCl_4/gm was found in the liver. Similar animals which had been treated with ethanol eighteen hours previously exhibited a concentration of 0.367 ± 0.126 mg CCl_4/gm liver of carbon tetrachloride at this time. Since the intubation of ethanol caused a loss of appetite, all food was removed at the time of ethanol administration. Animals not receiving ethanol, were given an isocaloric dose of sucrose at this time. This procedure would minimize differences in absorption

due to the presence of food in the gastro intestinal tract, and to irritation caused by intubation. The mean values of carbon tetrachloride found in the liver of animals with orotic acid induced fatty liver were higher than those of the corresponding normal group, possibly due to large individual variations in absorption, this difference was not statistically significant at the 95% confidence level.

A similar pattern of carbon tetrachloride concentration in blood was observed in the various groups treated. In animals with the orotic acid induced fatty liver, 0.019 ± 0.008 mg CCl_4/gm blood was measured. This value was comparable to the 0.016 ± 0.003 mg/gm found in similar animals pretreated with alcohol. In the normal group, 0.030 ± 0.011 mg CCl_4/gm blood was recovered. The corresponding group, pretreated with ethanol exhibited a corresponding value of 0.035 ± 0.019 mg CCl_4/gm blood at this time. Differences between any of the groups were not statistically significant at the 95% confidence level. In comparing individual values of carbon tetrachloride concentration found in blood and liver with the corresponding value of total hepatic lipid for each animal, no correlation was found.

TABLE 15

CARBON TETRACHLORIDE CONCENTRATIONS IN BLOOD AND LIVER OF NORMAL AND OROTIC ACID FED ANIMALS

Concentration of Carbon Tetrachloride in Blood and Liver

Treatment	Liver (mg/gm ± S. E.)	Blood (mg/gm ± S. E.)
O + E + C_i	0.290 ± 0.048	0.092 ± 0.028
O + C_i	0.320 ± 0.074	0.100 ± 0.026
C_i	0.273 ± 0.023	0.095 ± 0.017
E + C_i	0.277 ± 0.047	0.103 ± 0.024
O + E + C_o	0.700 ± 0.193	0.016 ± 0.003
O + C_o	0.727 ± 0.252	0.019 ± 0.008
E + C_o	0.367 ± 0.126	0.035 ± 0.019
C_o	0.342 ± 0.084	0.030 ± 0.011

O -- 1% dietary orotic acid.

E -- 5 ml/kg ethanol administered orally eighteen hours prior to carbon tetrachloride.

C_i -- 0.6 ml carbon tetrachloride injected intraperitoneally. (Six animals/group).

C_o -- 2.5 ml/kg carbon tetrachloride administered orally in an equal volume of corn oil. (10 animals/group).

All animals sacrificed 20 hours after carbon tetrachloride administration.

DETERMINATION OF APPARENT LIVER PYRIDINE NUCLEOTIDE RATIOS IN NORMAL AND OROTIC ACID FED ANIMALS FOLLOWING ETHANOL AND/OR CARBON TETRACHLORIDE ADMINISTRATION

Determination of pyridine nucleotide levels in animals treated with combinations of orotic acid, ethanol or carbon tetrachloride was performed according to the method of Gallagher as described in Section II. Groups of animals were sacrificed two, or twenty hours following the intraperitoneal injection of 0.6 ml carbon tetrachloride. Comparison of nucleotide levels was made in animals pretreated with orotic acid or ethanol to determine whether gross differences occurred with respect to normal control values.

Two hours after the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride animals with the orotic acid induced fatty liver which were pretreated with ethanol exhibited a nucleotide ratio of 1.52 ± 0.08 (Table 16). Compared to normal animals, this value represents a decrease of 27% in the nucleotide ratio. Values determined in the various groups treated with combinations of orotic acid, carbon tetrachloride, or ethanol were not significantly altered as compared to normal at this time.

Twenty hours after treatment with carbon tetrachloride,

TABLE 16

LIVER NUCLEOTIDE RATIOS IN NORMAL AND OROTIC ACID FED RATS FOLLOWING THE ADMINISTRATION OF CARBON TETRACHLORIDE AND/OR ETHANOL

APPARENT LIVER PYRIDINE NUCLEOTIDE LEVELS

Treatment	2 Hours After CCl_4 Administration	% of Normal 2 Hours After CCl_4	20 Hours After CCl_4 Administration	% of Normal 20 Hours After CCl_4
O	2.08 ± 0.44	106		
OE	1.97 ± 0.43	100	1.43 ± 0.33	73
OC	1.95 ± 0.34	99	1.23 ± 0.08[a]	63
OEC	1.52 ± 0.09[a]	78	1.25 ± 0.11[a]	63
E	1.90 ± 0.19	97	2.42 ± 0.24	123
C	2.09 ± 0.07	107	1.93 ± 0.07	98
EC	1.99 ± 0.10	102	1.08 ± 0.19[a]	55
Normal	1.96 ± 0.04			

O -- Fatty accumulation of the liver induced by 1% dietary orotic acid.

E -- 5 ml/kg ethanol, oral.

C -- 0.6 ml/kg carbon tetrachloride by intraperitoneal injection.

At least five animals/group.

[a] $P < 0.05$ versus normal.

the liver nucleotide ratio was depressed in animals with orotic acid induced fatty liver. The mean value of the nucleotide ratio measured in animals which received ethanol thirty eight hours previously and were maintained on the orotic acid diet, was depressed, but individual variation did not permit the assignment of statistical significance to this difference. In animals with normal liver lipid levels, the nucleotide ratio was depressed only in animals which were pretreated with ethanol prior to injection with carbon tetrachloride. Thirty eight hours after the oral dosage of ethanol animals exhibited a slight increase in the mean nucleotide ratio, but this elevation was not statistically significant.

DETERMINATION OF SERUM IRON CONCENTRATION IN NORMAL AND OROTIC ACID FED ANIMALS FOLLOWING ADMINISTRATION OF ETHANOL AND/OR CARBON TETRACHLORIDE

Twenty hours after an intraperitoneal injection of 0.6 ml/kg carbon tetrachloride, animals with gross fatty accumulation of the liver induced by orotic acid exhibited a mean serum iron concentration of 13.1 $\pm$ 1.0 ppm (Table 18). Similar animals which had been given 5.0 ml/kg ethanol eighteen hours prior to dosage with the hydrocarbon showed a serum iron level of 25.4 $\pm$ 1.2 ppm at this time. Following the same dosage of carbon tetrachloride, animals with normal liver lipid levels had a mean serum iron concentration of 16.7 $\pm$ 1.3 ppm. Similar animals which were pretreated with 5.0 ml/kg ethanol exhibited a mean serum iron level of 21.0 $\pm$ 1.0 ppm twenty hours after injection of this dose of carbon tetrachloride. Serum iron concentration in animals treated with ethanol or orotic acid was not significantly different from that of normal controls. These results agreed with the qualitative estimation of pathological changes observed in liver sections observed in liver sections of variously treated animals.

It was of interest to note that molybdenum was detected in the sera of animals treated with carbon tetrachloride, but not in the controls. This suggests that an increase in xanthine oxidase, which

contains molybdenum may have occurred. An increase in serum xanthine oxidase following carbon tetrachloride administration has been demonstrated enzymatically by several workers (10, 32, 177, 178). The method of analysis employed, however, did not permit the quantitative estimation of the amount of molybdenum present.

TABLE 17

SERUM IRON CONCENTRATION IN NORMAL AND OROTIC ACID FED ANIMALS FOLLOWING CARBON TETRACHLORIDE AND/OR ETHANOL ADMINISTRATION

Treatment	Serum Iron Concentration (ppm)	Hepatic Damage
OC	13.1 ± 1.0	+
OEC	25.4 ± 1.2 [b]	++++
C	16.7 ± 1.3 [a]	++
EC	21.0 ± 1.0 [a]	+++
Normal	10.0 ± 1.0	

O -- Fatty liver induced by 1% dietary orotic acid.

E -- 5.0 ml/kg ethanol administered orally eighteen hours prior to carbon tetrachloride dosage.

C -- 0.6 ml/kg carbon tetrachloride administered by intraperitoneal injection.

All animals sacrificed twenty hours after carbon tetrachloride administration.

[a] $P < 0.05$ versus normal.

[b] $P < 0.001$ versus normal.

HISTOLOGICAL STUDIES OF NORMAL AND OROTIC ACID FED RATS TREATED WITH ETHANOL AND/OR CARBON TETRACHLORIDE

Animals fed 1% dietary orotic acid for ten days developed a striking fatty accumulation of the liver as shown in Figures 19 and 20. Hepatic architecture was intact and examination of heart, lung, liver, kidney, spleen, pancreas, and adrenals revealed no abnormalities. Because of the extensive lipidic accumulation produced by this treatment, it was necessary to compare histological changes observed using the general H & E stain with those found in tissues stained for fat with Oil Red O. Similarly, comparison of tissue sections at two and twenty hours after the administration of carbon tetrachloride revealed progressive morphological hepatic changes.

Eighteen hours after the intubation of 5.0 ml/kg ethanol, the orotic acid fed animals showed a very severe accumulation of hepatic lipid (Figures 21 and 22). This tissue section represented the condition of the liver at the time of injection of carbon tetrachloride. Thirty-eight hours following ethanol ingestion, the orotic acid fed animals exhibited severe accumulation of lipid distributed throughout the hepatic parenchyma. The hepatic architecture was intact, although large lipidic vacuoles were observed within the

parenchymal cells. (Figures 23 and 24). Examination of liver sections from normal rats showed slight lipid accumulation of hepatic lipid eighteen hours after ingestion of 5.0 ml/kg ethanol. (Figure 33). This degree of lipidic accumulation was similar to that illustrated in Figure 4. Thirty-eight hours after ethanol ingestion the liver of these animals appeared unremarkable and similar to normal animals. (Figure 34).

Two hours after the injection of carbon tetrachloride to orotic acid fed rats very severe lipidic infiltration could be observed in the liver parenchyma, but hepatic architecture remained intact. (Figures 25 and 26). Twenty hours later, coarse vacuolization, due to lipidic infiltrates, was noted. An occasional cell in the central area exhibited hydropic degeneration. However, no necrosis was observed and hepatic architecture was intact.

In comparison, normal rats treated with carbon tetrachloride showed a marked vauolor change of the hepatic parenchyma which was not associated with lipid within two hours after injection. (Figures 35 and 36). A moderate fatty infiltration in predominantly central lobular areas was observed at this time. Twenty hours after injection of carbon tetrachloride, normal rats showed extensive cellular disruption with severe lipidic infiltration in these areas. (Figures 37 and 38). In the periportal areas, intact cells were ob-

served. The pathological changes were more severe in normal rats than those fed orotic acid (See Figures 27 and 28).

Animals which were fed orotic acid and given an oral dose of ethanol prior to carbon tetrachloride injection showed large vacuole formation in the liver two hours later. A somewhat greater distribution of lipid vacuoles was observed in the mid-zonal and periportal areas (Figures 29 and 30). Twenty hours after carbon tetrachloride administration, these animals exhibited severe central lobular necrosis and prominent lipidic accumulation of the liver. Similarly, normal animals given an oral dose of ethanol showed moderate lipidic infiltration two hours after intraperitoneal injection of carbon tetrachloride (Figures 39 and 40). No disruption of hepatic architecture was noted at this time. Twenty hours after carbon tetrachloride administration, these animals exhibited severe central lobular necrosis. Severe fatty accumulation could be observed in the necrotic areas (Figures 41 and 42), with infiltration of polymorphonuclear leucocytes.

The most extensive histopathological changes following carbon tetrachloride administration were observed in normal and orotic acid fed animals which were treated with ethanol. It appeared that normal animals developed more extensive hepatic injury than orotic acid fed animals given the same dose of carbon

tetrachloride. This observation was not compatible with the hypothesis that lipidic accumulation of the liver is causally related to the increased hepatotoxicity of carbon tetrachloride. If the presence of increased amounts of hepatic lipid is the cause of increased hepatotoxicity of carbon tetrachloride, extensive damage, as observed in Figures 41 and 42, would be expected.

Figure 19

Liver of a rat maintained on normal protein diet into which 1% orotic acid was incorporated. H & E, 145X.

Prominent lipidic infiltration can be observed throughout the hepatic parenchyma. Hepatic architecture is intact.

Figure 20

Liver of rat maintained on normal protein diet into which 1% orotic acid was incorporated. Oil Red 0, 145X.

Prominent accumulation of lipid is present, distributed as fine vacuoles throughout the parenchyma.

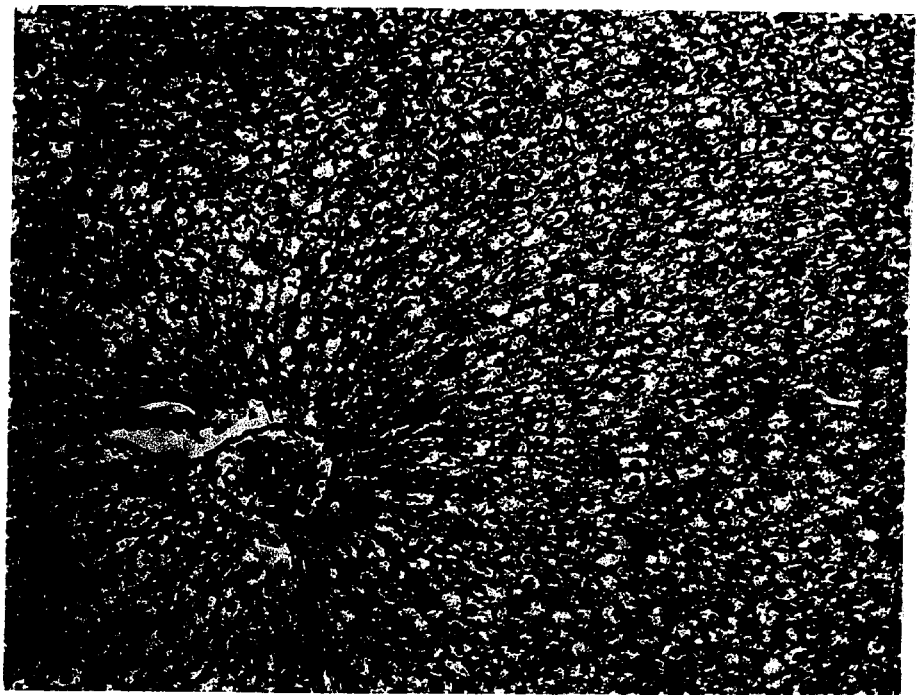


Figure 19

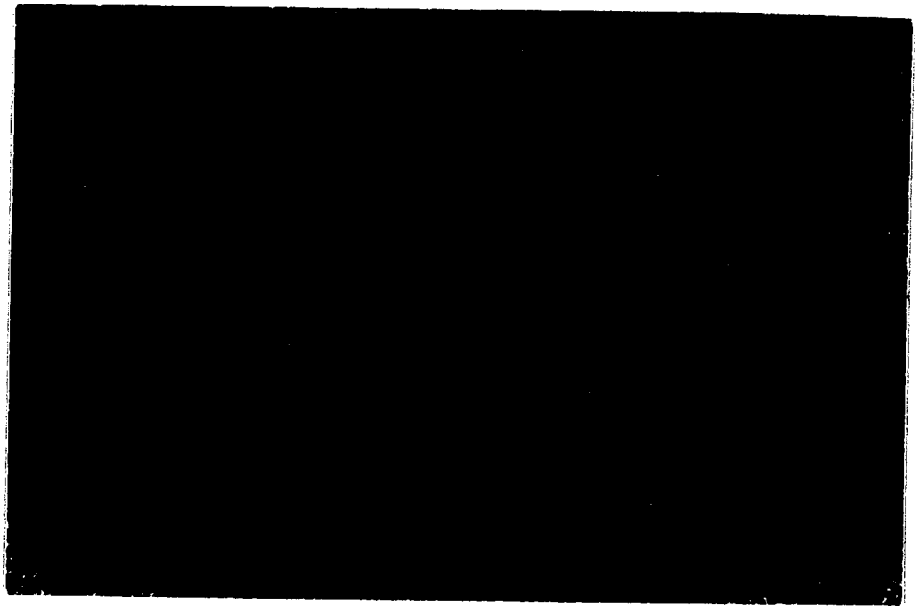


Figure 20

Figure 21

Liver from a rat given 1% dietary orotic acid and 5 ml/kg ethanol orally, eighteen hours prior to sacrifice. H & E, 145X.

Very severe lipidic accumulation can be observed in the hepatic parenchyma. The vacuoles are somewhat larger than those in Figure 20.

Figure 22

Liver from a rat given 1% dietary orotic acid and 5 ml/kg ethanol orally, eighteen hours prior to sacrifice. Oil Red 0, 145X.

Very severe accumulation of lipid distributed throughout the hepatic parenchyma can be observed. Hepatic architecture is intact, although cell components appear to be displaced by lipid vacuoles.

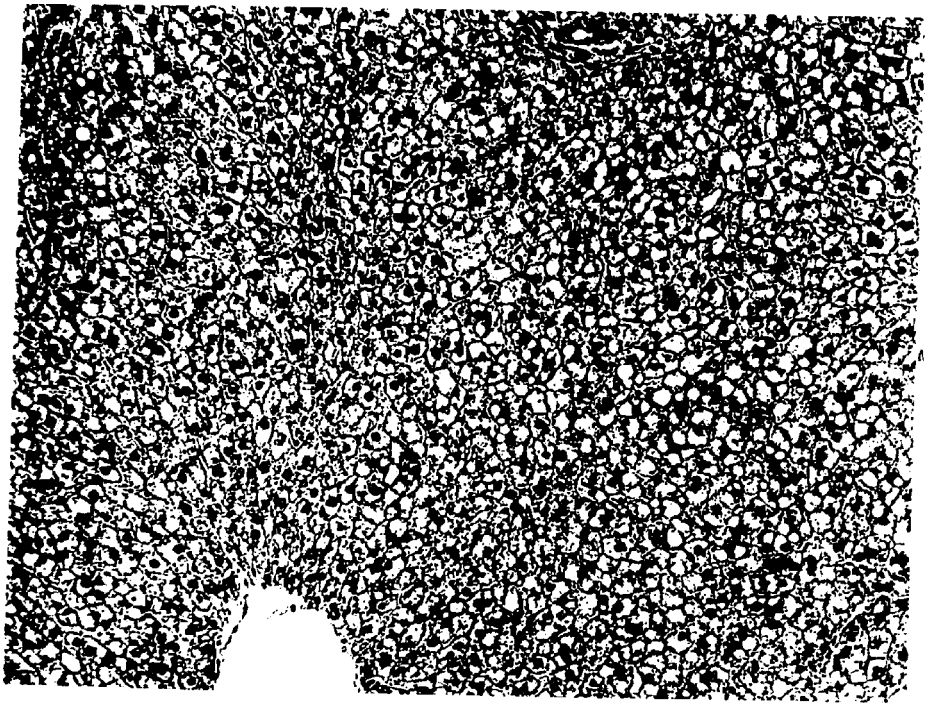


Figure 21

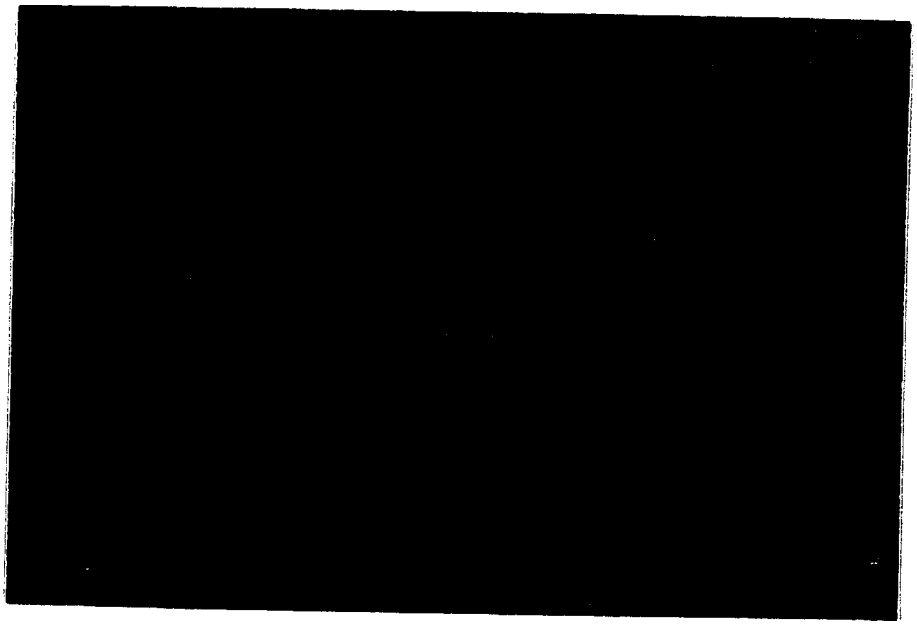


Figure 22

Figure 23

Liver from rat given 1% dietary orotic acid and 5 ml/kg ethanol thirty-eight hours prior to sacrifice. H & E, 145X.

The liver exhibits a moderately severe large vacuole formation within the parenchymal cells.

Figure 24

Liver from rat given 1% dietary orotic acid, and 5 ml/kg ethanol thirty-eight hours prior to sacrifice. Oil Red O, 145X.

A heavy infiltration of lipid vacuoles, indicated as red droplets, is noted.

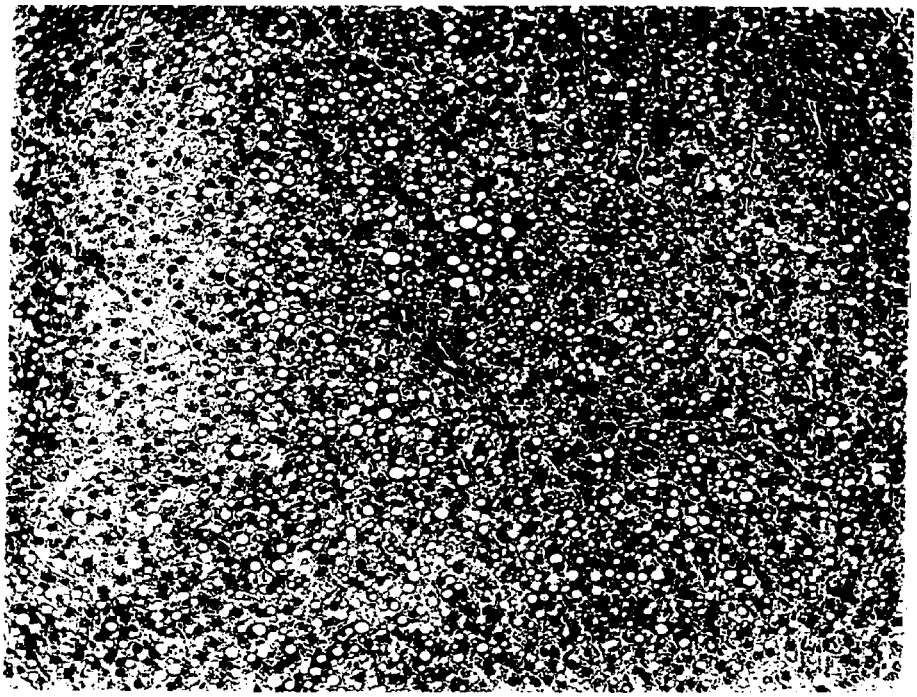


Figure 23

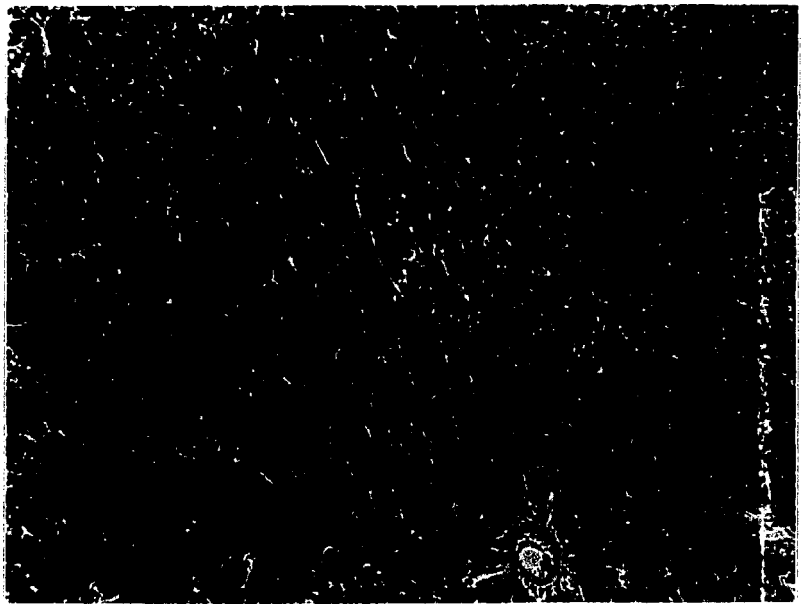


Figure 24

Figure 25

Liver from rat given 1% dietary orotic acid and 0.6 ml/kg carbon tetrachloride, intraperitoneally. Animal was sacrificed two hours after injection. H & E, 145X.

Very severe lipidic infiltration can be noted in the liver parenchyma. Hepatic architecture is intact.

Figure 26

Liver from rat given 1% dietary orotic acid and 0.6 ml/kg carbon tetrachloride intraperitoneally. Animal was sacrificed two hours after administration of carbon tetrachloride. Oil Red O, 145X.

Severe lipidic infiltration can be observed within hepatic cord cells.



Figure 25

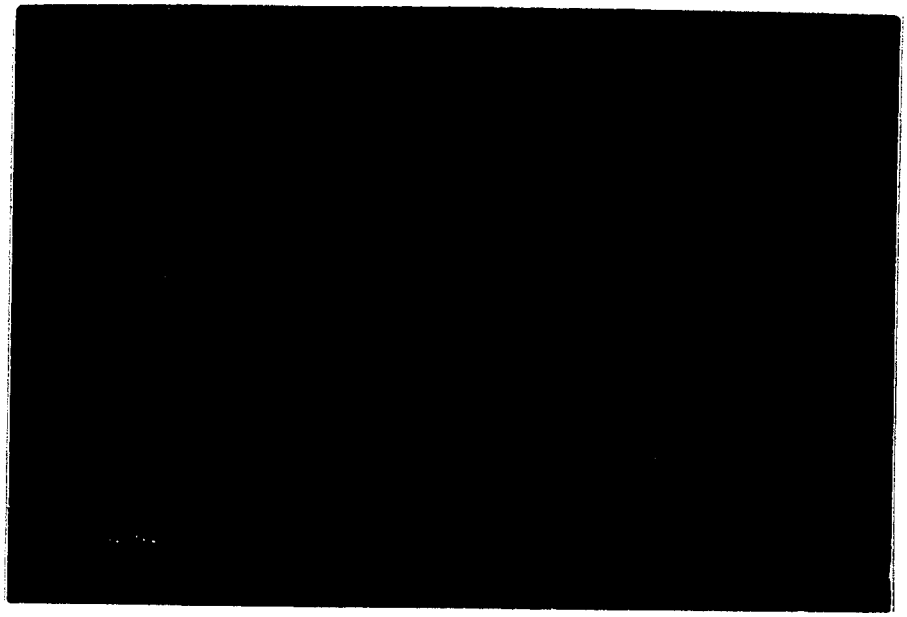


Figure 26

Figure 27

Liver from a rat given 1% dietary orotic acid for ten days, and intraperitoneal injection of 0.6 ml carbon tetrachloride twenty hours prior to sacrifice. H & E, 145X.

The liver exhibits coarse vacuolization of the parenchyma. An occasional cell exhibits hydropic degeneration in the central area. This specimen is representative of the most severe morphological changes observed.

Figure 28

Liver from a rat given 1% dietary orotic acid for ten days, and intraperitoneal injection of 0.6 ml/kg carbon tetrachloride twenty hours prior to sacrifice. Oil Red O, 145X.

Severe lipidic accumulation can be observed.

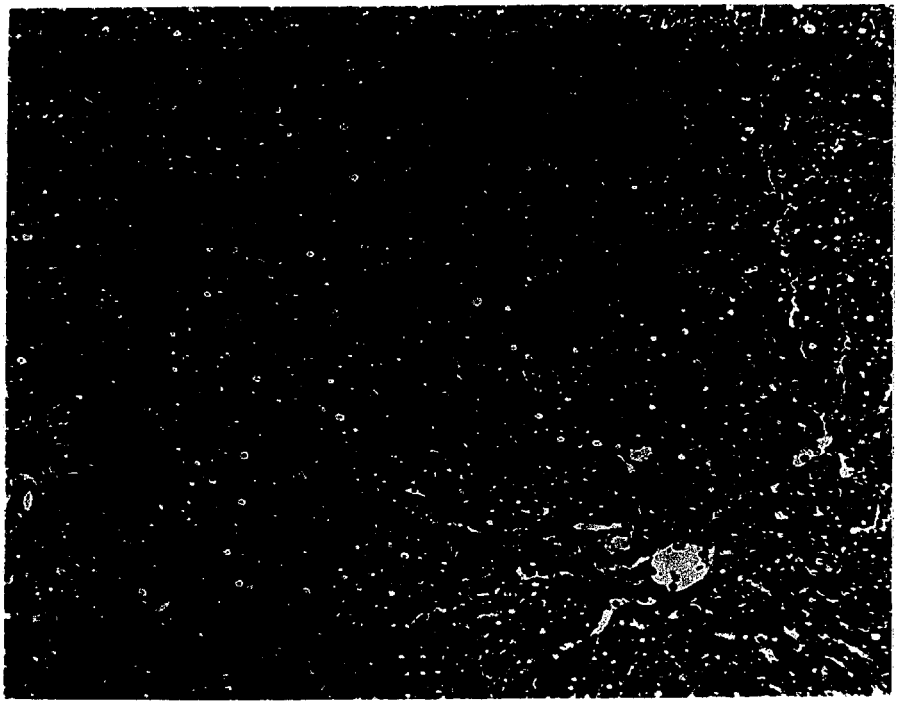


Figure 27

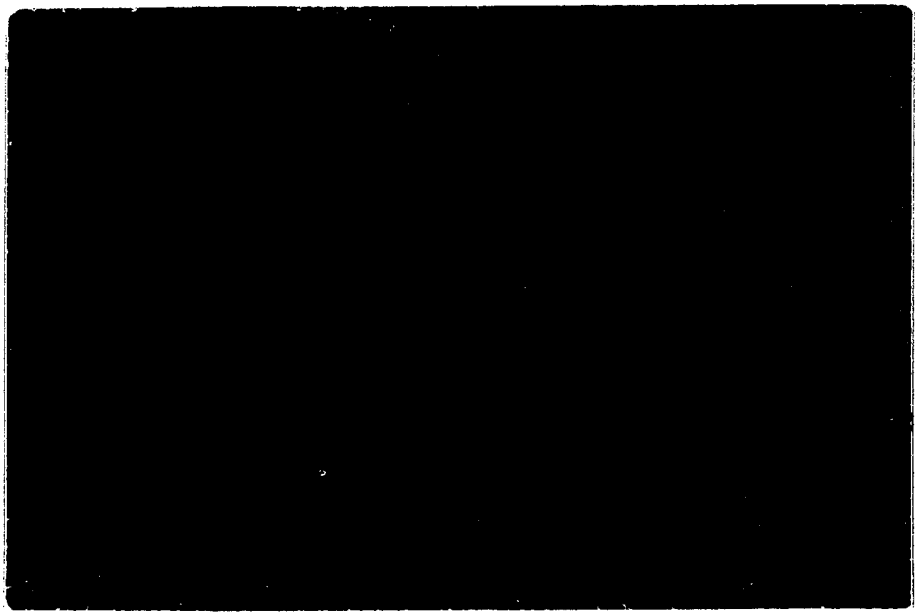


Figure 28

Figure 29

Liver from a rat given 1% dietary orotic acid for ten days and 5 ml/kg ethanol eighteen hours prior to the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Animal was sacrificed two hours after carbon tetrachloride administration. H & E, 145X.

Prominent lipidic infiltration and large vacuole formation can be observed in the liver. Some dilatation of central veins in sinusoids is noted.

Figure 30

Liver from a rat given 1% dietary orotic acid for ten days and 5 ml/kg ethanol eighteen hours prior to the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Animal was sacrificed two hours after carbon tetrachloride administration. Oil Red 0, 145X.

Prominent fatty accumulation of the liver can be observed. A somewhat greater distribution of lipid may be observed in the mid-zonal and periportal areas.

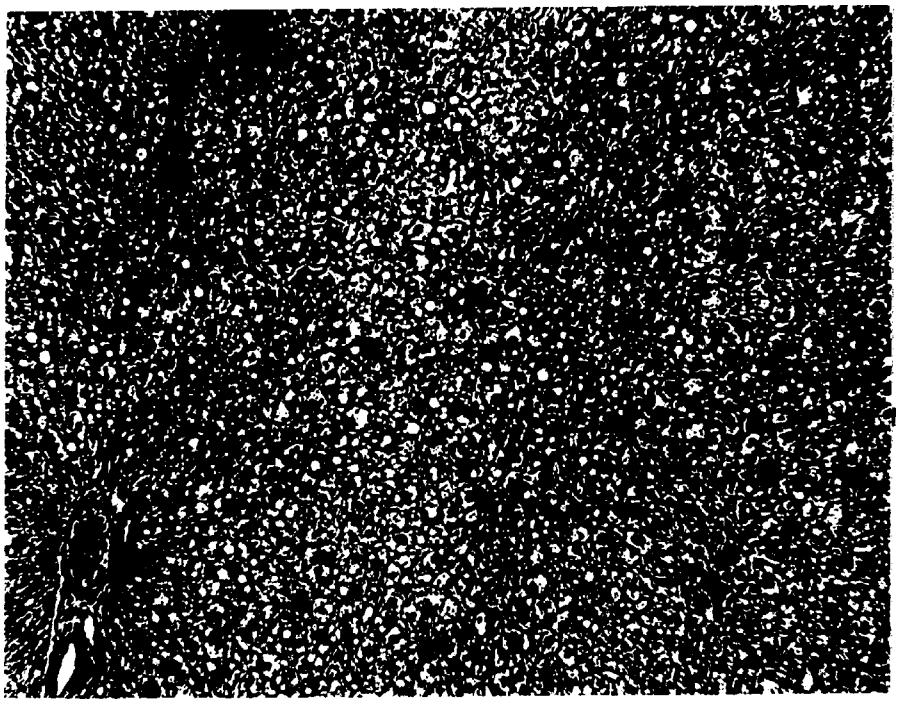


Figure 29



Figure 30

Figure 31

Liver from a rat given 1% dietary orotic acid for ten days and 5.0 ml/kg ethanol eighteen hours prior to the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Animal was sacrificed twenty hours after carbon tetrachloride administration. H & E, 145X.

Severe central lobular necrosis is present. Prominent lipidic accumulation can be observed as large vacuoles. Focal hydropic degeneration is also evident.

Figure 32

Liver from a rat given 1% dietary orotic acid for ten days and 5 ml/kg ethanol eighteen hours prior to the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Animal was sacrificed twenty hours after carbon tetrachloride administration. Oil Red O, 145X.

Extensive accumulation of lipid may be observed in the zones of necrosis.

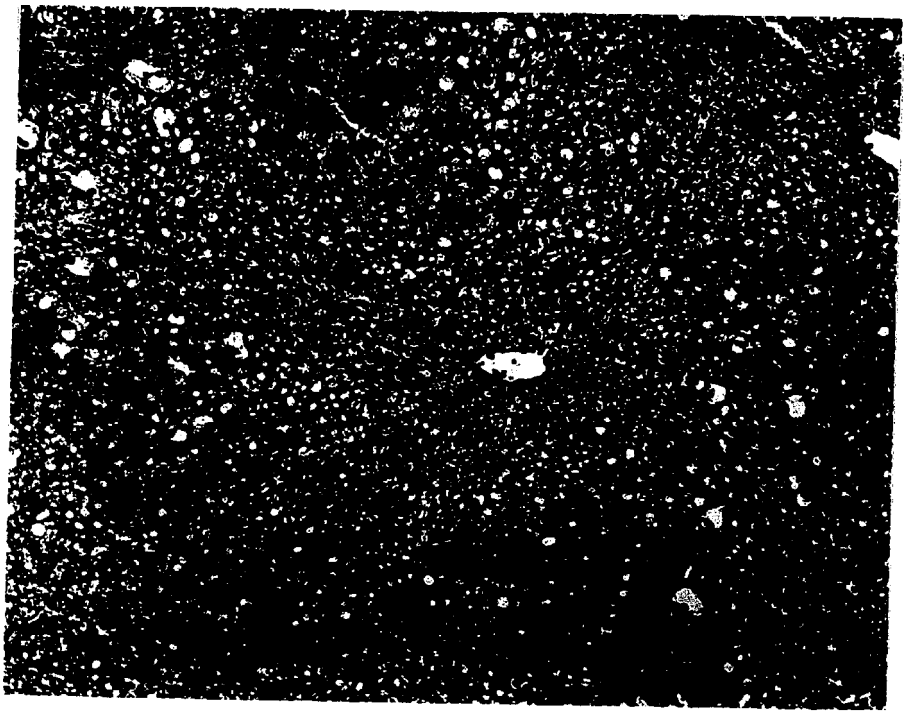


Figure 31

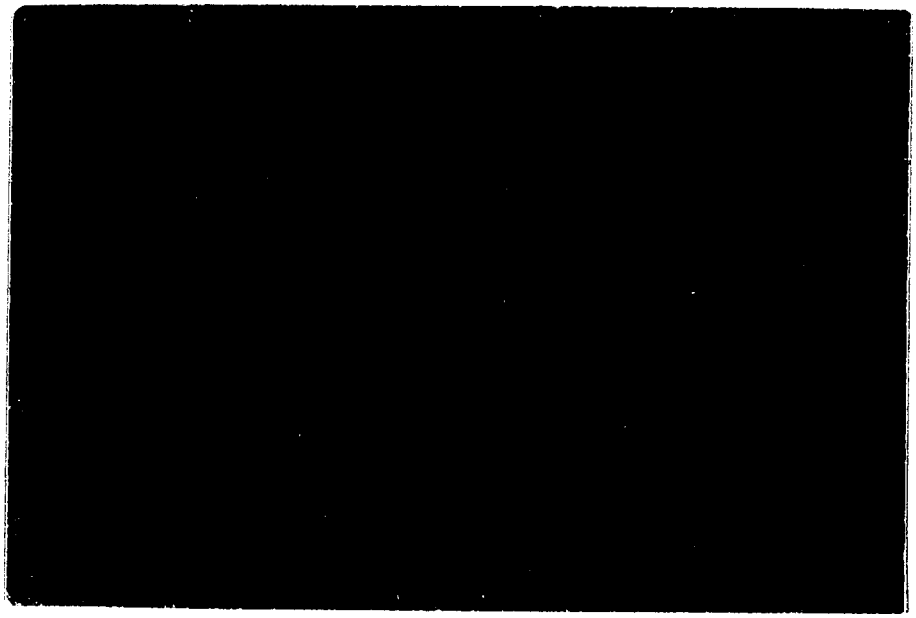


Figure 32

Figure 33

Liver of a rat maintained on a normal protein diet, and given 5.0 ml/kg ethanol eighteen hours prior to sacrifice. H & E, 145X.

Slight lipidic accumulation can be observed within the hepatic parenchyma. Hepatic architecture is intact. Lipidic accumulation is comparable to Fig. 4.

Figure 34

Liver of a rat maintained on a normal protein diet and given 5.0 ml/kg ethanol thirty-eight hours prior to sacrifice. H & E, 145X.

Liver is unremarkable. Hepatic structure is intact and normal. Lipidic accumulation is comparable to Fig. 3.



Figure 33



Figure 34

Figure 35

Liver of a rat maintained on a normal protein diet and given 0.6 ml/kg carbon tetrachloride intraperitoneally two hours prior to sacrifice. H & E, 145X.

Marked vacuolar change in liver parenchyma. Moderate lipidic accumulation which is not associated with vacuolar changes.

Figure 36

Liver of a rat maintained on normal protein diet and given 0.6 ml/kg carbon tetrachloride intraperitoneally two hours prior to sacrifice. Oil Red O, 145X.

Moderate fatty infiltration predominantly in central lobular areas.

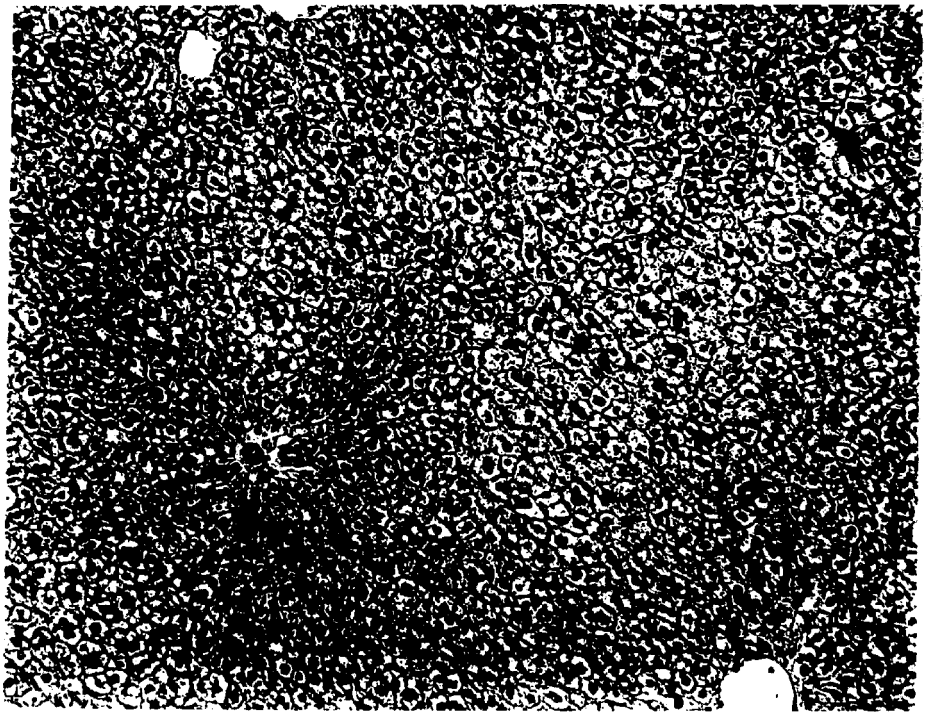


Figure 35



Figure 36

Figure 37

Liver from a rat maintained on a normal protein diet and injected intraperitoneally with 0.6 ml/kg carbon tetrachloride twenty hours prior to sacrifice. H & E, 145X.

Extensive cellular disruption with severe lipidic infiltration. Intact cells remain in the portal area.

Figure 38

Liver from a rat maintained on normal protein diet and injected intraperitoneally with 0.6 ml/kg carbon tetrachloride twenty hours prior to sacrifice. Oil Red O, 145X.

Prominent lipidic infiltration in the central lobular zone.

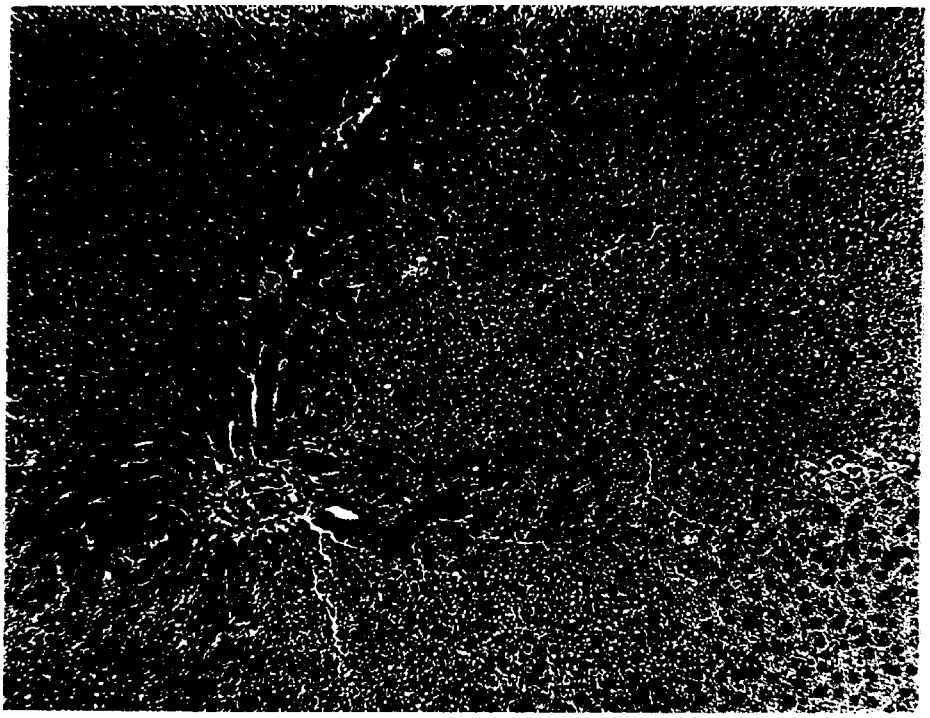


Figure 37

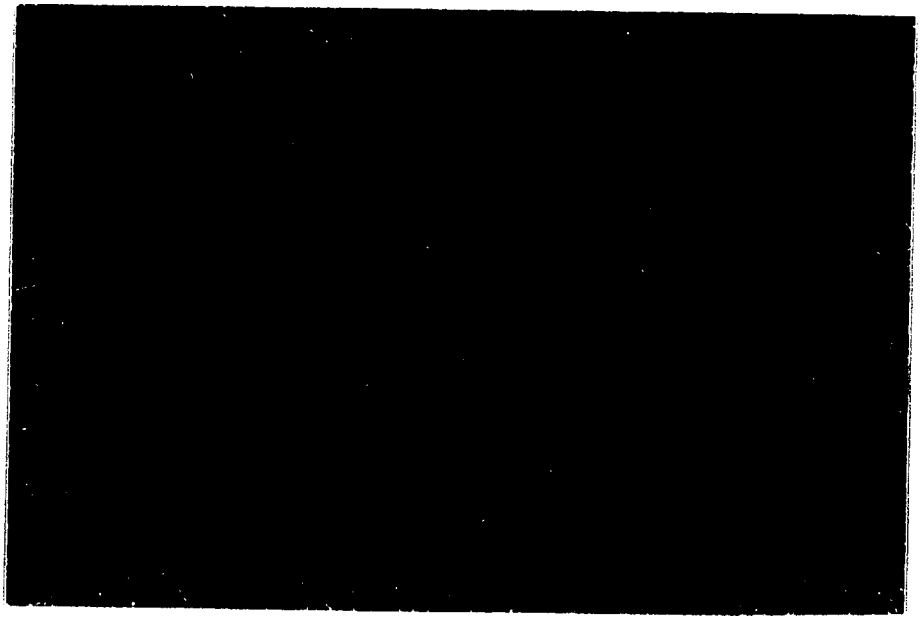


Figure 38

Figure 39

Liver from a rat maintained on normal protein diet and given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Animal was sacrificed two hours after administration of the carbon tetrachloride. H & E, 145X.

Moderate lipidic infiltration can be observed throughout the hepatic parenchyma. No disruption of hepatic architecture is noted.

Figure 40

Liver from a rat maintained on normal protein diet and given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Animal was sacrificed two hours after administration of carbon tetrachloride. Oil Red O, 145X.

Moderate generalized lipidic infiltration of the liver can be observed.

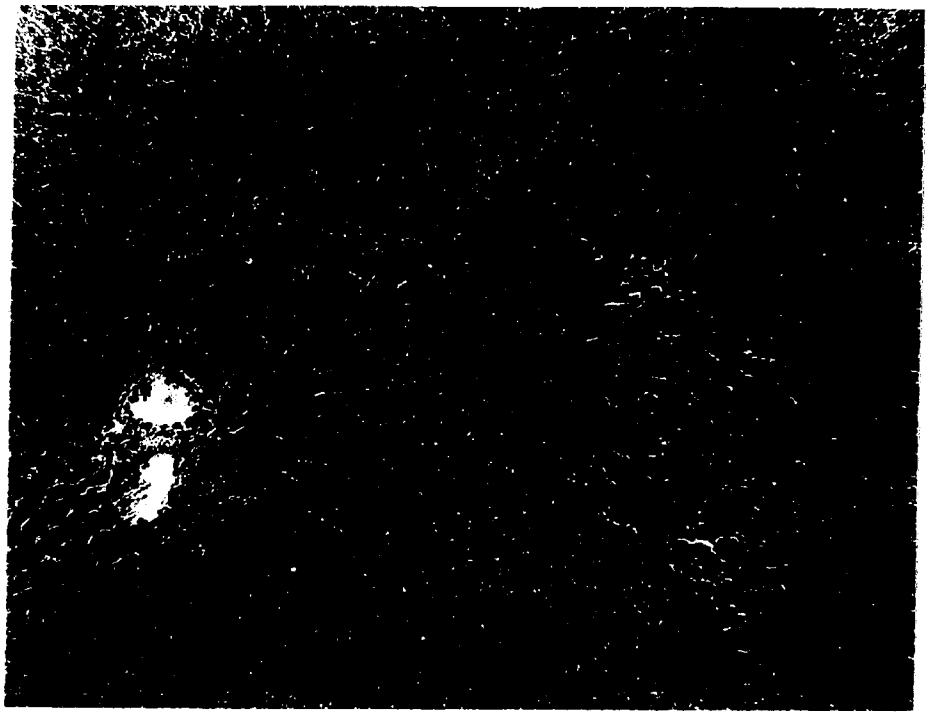


Figure 39

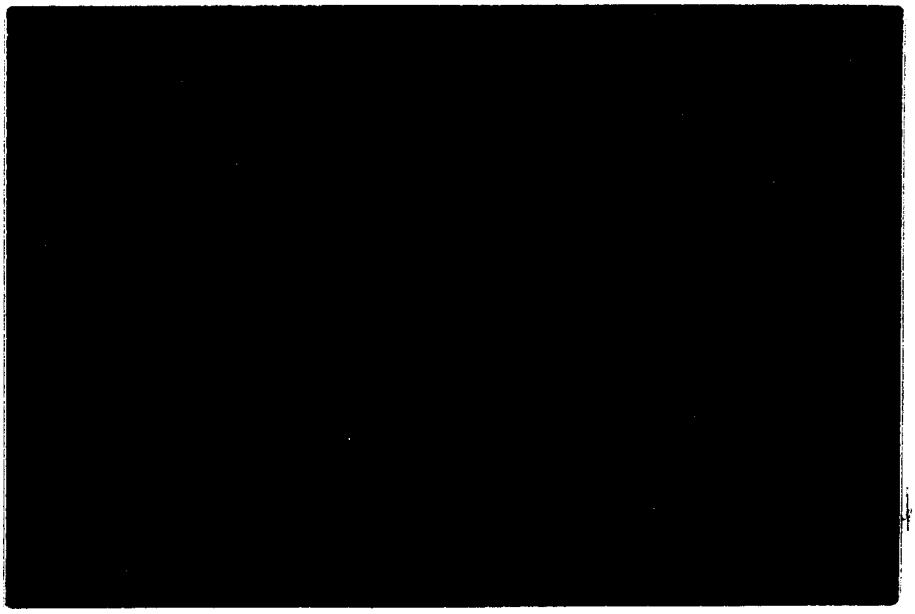


Figure 40

Figure 41

Liver from a rat maintained on a normal protein diet and given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Animal was sacrificed twenty hours after administration of carbon tetrachloride. H & E, 145X.

Severe central lobular necrosis of the liver is noted. Marked central lobular fatty infiltration can be observed in the necrotic area. Infiltration of polymorphonuclear leucocytes is present.

Figure 42

Liver from a rat maintained on a normal protein diet and given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to the intraperitoneal injection of 0.6 ml/kg carbon tetrachloride. Animal was sacrificed twenty hours after administration of carbon tetrachloride. Oil Red O, 145X.

Severe central lobular fatty infiltration can be observed.

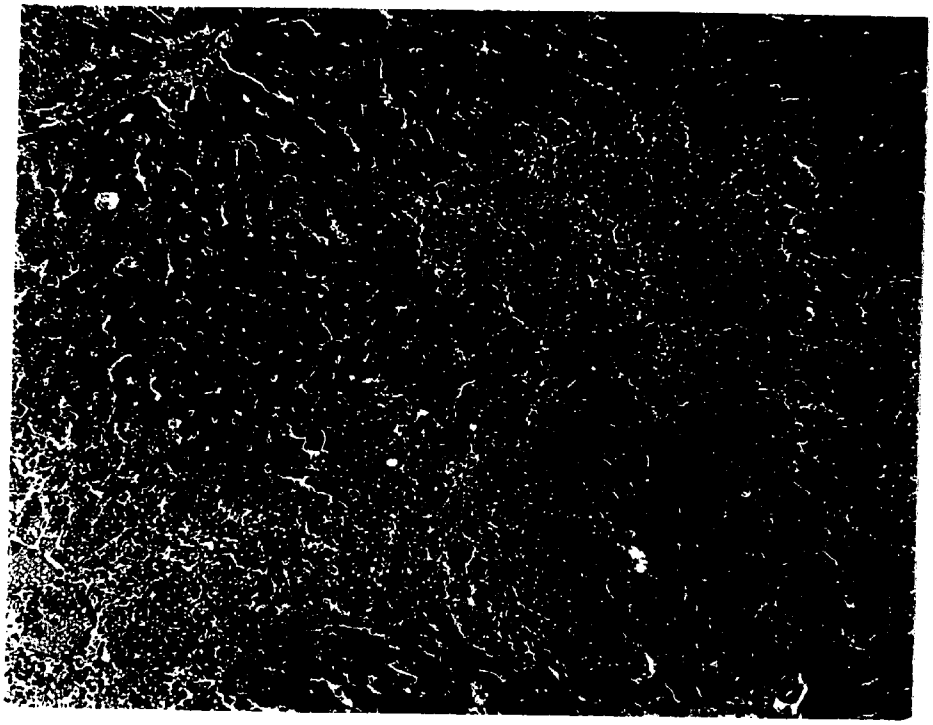


Figure 41



Figure 42

DISCUSSION

In comparing the biological response to carbon tetrachloride and/or ethanol in normal and orotic acid fed rats, several observations relative to the hepatotoxicity of the chlorinated hydrocarbon can be made. Examination of serum enzyme levels showed that marked elevation in activity occurred in both normal and orotic acid fed animals given carbon tetrachloride. A greatly increased serum enzyme response was elicited by the combination of ethanol-carbon tetrachloride treatment and was independent of the amount of total hepatic lipid present. Serum enzyme measurements were compatible with observed morphological changes in hepatic structure. The greatest elevation in serum enzyme activity was measured in normal and orotic acid fed animals which received both ethanol and carbon tetrachloride. These groups of animals also exhibited the most severe hepatic damage upon histopathological examination. Normal animals exhibited a somewhat greater degree of morphological hepatic damage than orotic acid fed animals given the same dose of carbon tetrachloride. Upon examination of serum enzyme activity, normal animals also had somewhat higher mean values of S-GOT and S-GPT than orotic acid fed animals given this dose of the halogenated hydrocarbon. Similarly, when hepatic steatosis was induced by choline deficiency, the large increases in serum enzyme activity were associated with ethanol ingestion, independent of the amount of hepatic lipid

present. Although the liver of choline deficient animals showed fatty accumulation, great elevation in serum enzyme levels following carbon tetrachloride inhalation was observed only when animals were given ethanol.

A similar correlation can be observed between values of serum iron concentration and histopathological alterations. Again, the greatest increases in serum iron concentration were observed in animals given both ethanol and carbon tetrachloride.

The most severe histopathological changes were observed in animals which had the orotic acid induced fatty liver and received both ethanol and carbon tetrachloride. Such animals exhibited submassive necrosis of the liver and extensive lipidic infiltration in the zones of necrosis. In contrast, orotic acid fed animals which were given carbon tetrachloride showed little disruption of hepatic architecture, with only an occasional cell undergoing hydropic degeneration in the central area. Normal animals treated with carbon tetrachloride alone, showed less liver damage than those animals which received both ethanol and the chlorinated hydrocarbon. Severe central lobular necrosis and moderately severe fatty accumulation was observed in animals treated with both ethanol and carbon tetrachloride. In animals treated with carbon tetrachloride alone, fatty degeneration, and moderate necrosis was observed. These histopathological changes were compatible with increases noted in serum iron concentration. The highest concentration of serum iron

was observed in animals treated with both carbon tetrachloride and ethanol. The agreement between the several methods utilized to measure biological effects lends support to their usefulness in the evaluation of a toxic response.

If the potentiating action of ethanol upon carbon tetrachloride toxicity is causally related to the increased amounts of hepatic lipid following alcohol ingestion, an increased toxicity to carbon tetrachloride would be expected when hepatic lipids were grossly increased by orotic acid ingestion. Examination of serum enzyme activity, serum iron concentration, and histopathological changes failed to demonstrate a marked increase in toxic response following carbon tetrachloride administration when hepatic lipid levels were increased by orotic acid ingestion. This would imply that, although increased hepatic lipid content may be associated with enchanced toxicity of carbon tetrachloride, the mechanism of potentiation of toxicity is not causally related to elevated lipid content. The potentiated toxicity of carbon tetrachloride was observed in all animals previously treated with ethanol. In contrast, the potentiated response was not observed in animals with marked hepatic steatosis if these animals did not receive ethanol. Since this biological response to carbon tetrachloride was elicited in both groups of animals, only after ethanol ingestion, this would suggest that biochemical and physiological factors, other than hepatic lipid content, are more directly related to the mechanism of potentiation.

To illustrate this point, measurement of pyridine nucleotide ratios in ethanol treated normal and orotic acid fed animals showed a decrease following carbon tetrachloride administration. Although this gross determination of nucleotide ratio suffers from the disadvantages described in the methods section, the alterations in the values observed suggest cellular imbalances that may be related to the mechanisms of potentiation. Recently, Slater, Sawyer and Strauli (158, 159) measured liver pyridine nucleotide levels in rats following treatment with carbon tetrachloride or ethanol. Following the intubation of carbon tetrachloride, a rapid decrease in $NADPH_2$ and a concomitant increase in NADP was observed within one hour. These changes persisted for eighteen hours after dosage with the hydrocarbon. When the animals were given the antihistamine drug, Phenergan, which prevents, or substantially delays necrosis caused by carbon tetrachloride, these changes in NADP and $NADPH_2$ concentrations were not observed. Although the changes in nucleotide concentrations may be an indication of general cellular disturbance, the prevention of both necrosis and nucleotide imbalance by Phenergan suggests a relationship between these events. The changes in pyridine nucleotide concentrations which are prevented by Phenergan, may be part of the necrogenic chain of reactions observed following carbon tetrachloride intoxication, as suggested by Slater et al. (Abbreviations, p. xix).

Similarly, these workers measured pyridine nucleotide levels in rats following acute ethanol intoxication. The ratio of concentration of NAD to $NADH_2$ decreased rapidly within thirty minutes of administration of the ethanol and remained depressed for thirteen hours afterwards. In contrast, the NADP and $NADPH_2$ concentrations remained constant during this period of study. Since variations in the NAD:$NADH_2$ or NADP:$NADPH_2$ ratios occurred independently, these alterations were not sufficient to initiate transhydrogenase-type reactions. Although these cellular imbalances may occur following administration of ethanol or carbon tetrachloride, no causal relationship to the mechanism of potentiation can be assumed.

It has been suggested that the accumulation of hepatic lipid following ethanol administration would increase the concentration of carbon tetrachloride (because of its favorable water-oil partition coefficient) in this organ. Determination of carbon tetrachloride concentrations in blood and liver failed to reveal an increase in concentration of the organic halide in animals treated with ethanol in comparison with those which did not receive ethanol. The mean values of carbon tetrachloride concentrations found in alcohol treated rats when the compound was administered by intraperitoneal injection or inhalation, were indistinguishable from those found in alcohol untreated animals. Animals with gross hepatic steatosis induced by

agents other than ethanol showed similar concentrations of the organic halide. When carbon tetrachloride was administered orally to rats fed orotic acid, the mean value of carbon tetrachloride concentration in the liver was greater than that found in normal animals. Although statistical significance could not be assigned to this difference, perhaps a greater number of animals (ten used in this study) would indicate a significant increase in concentration of carbon tetrachloride in the livers of orotic acid fed rats. The mean value of carbon tetrachloride concentration in the liver of orotic acid fed animals was indistinguishable from that found in similarly fed animals pretreated with ethanol. The possibility exists that the carbon tetrachloride may be present in the lipid but not contribute to the potentiated response observed. Employing the method of analysis used in this study, it (7) has been shown that body fat had the highest concentration of carbon tetrachloride following administration of large doses. Because of the marked fatty accumulation induced by orotic acid (20% total lipid in some cases), it is possible that the liver possesses some of the characteristics of adipose tissue in these animals. Since the carbon tetrachloride was administered orally in an equal volume of corn oil to the orotic acid fed animal, the vehicle may have influenced the concentration

of the hydrocarbon found in the liver. As suggested by Hutterer and Leonard (75), an increased uptake of lipid by pinocytosis, may have occurred in this case. Nevertheless, carbon tetrachloride concentrations found in normal animals or those with severe hepatic steatosis were independent of administration of ethanol. It appeared, therefore, that ethanol did not influence the amount of carbon tetrachloride found in the blood or liver.

V -- BIOLOGICAL RESPONSE TO CARBON TETRACHLORIDE AND ETHANOL WHEN HEPATIC STEATOSIS WAS INHIBITED

INTRODUCTION

Adrenalectomy, cordotomy, hypophysectomy, and ganglionic blocking agents which interfere with mobilization of fatty acids have been shown to be effective in preventing ethanol induced fatty liver. The following experiments were performed to clarify the relationship of hepatic lipid levels to the observed toxicity of carbon tetrachloride. The accumulation of hepatic lipid following ethanol administration was prevented by adrenalectomy, cordotomy, and hypothermia and the toxic response to carbon tetrachloride was evaluated by the techniques described earlier. In this way, it should be possible to differentiate the affect of fatty liver from other physiological or metabolic changes induced by ethanol. (Suitable control groups were employed to determine the effects of the surgical manipulation, alcohol, and carbon tetrachloride upon the parameters of toxicity evaluation.)

If the presence of hepatic lipid is causally related to the potentiation of carbon tetrachloride toxicity, some degree of protection would be expected when normal lipid levels were maintained following ethanol ingestion. However, if the fatty liver is not directly related to potentiation of toxicity, the enhanced response would be expected following the administration of ethanol.

V-A -- BIOLOGICAL RESPONSE TO CARBON TETRACHLORIDE AND ETHANOL WHEN HEPATIC STEATOSIS WAS INHIBITED BY ADRENALECTOMY

PROCEDURE

Rats used were 175-200 gm males (Sprague Dawley strain) maintained on Purina Rat Chow and water ad libitum. Bilateral adrenalectomy was performed under ether anesthesia ten days prior to use. Adrenalectomized animals were supplied with 0.9% NaCl instead of tap water. Ethanol was administered by stomach tube as a 50% v/v aqueous solution. Each animal received 5 ml ethanol per kg of body weight. Carbon tetrachloride was given by several routes of administration. Intraperitoneally, carbon tetrachloride was given in a dose of 0.6 ml/kg B.W. Orally, it was administered by stomach tube in a dose of 2.5 ml/kg B.W. in an equal volume of corn oil. By the inhalation route, animals were exposed to concentrations of 1000, 2000, or 4000 ppm of carbon tetrachloride for 2 or 4 hours.

Animals were stunned and sacrificed by decapitation. Blood was collected for analysis of serum enzymes. (See Methods, Section II-B). Analysis of carbon tetrachloride in blood and liver was performed as described in Methods, Section II-E. Sections of heart, liver, lung, kidney, spleen, and adrenal (in control animals) were preserved in 10% formalin and reserved for histopathological exam-

ination. Liver lipid levels were determined as described in the Methods Section.

RESULTS

LIVER LIPID ANALYSIS OF INTACT AND ADRENALECTOMIZED RATS

Determination of total liver lipid was performed in intact and bilaterally adrenalectomized animals following treatment with carbon tetrachloride, ethanol, or the combination of both.

Eighteen hours after an oral dose of 5 ml/kg of ethanol, the total liver lipid level increased from a normal value of 4.60 to 5.20% (Table 18) in intact rats. This increase in total liver lipid was transient, returning to normal levels within 38 hours after the ingestion of the alcohol. Eighteen hours after administration of this dose of ethanol, a group of intact rats was exposed to 2000 ppm carbon tetrachloride for 2 hours. Immediately after exposure, the animals were sacrificed and the liver lipids were determined. These animals had liver lipid levels comparable to those given only ethanol, 5.22 and 5.20% respectively. However, twenty hours after the exposure to carbon tetrachloride, the rats previously intoxicated with ethanol exhibited a marked rise in liver lipid content, reaching a value of 6.59% at time of sacrifice. Comparable groups of rats, not given ethanol,

but exposed to the same concentration of carbon tetrachloride showed liver lipid levels similar to controls when sacrificed immediately following the exposure. Twenty hours after exposure to 2000 ppm carbon tetrachloride for 2 hours, these animals exhibited a mean lipid content of 5.00% compared to 4.60% for the untreated control group. Only animals which received both carbon tetrachloride and ethanol exhibited grossly elevated liver lipid values.

In contrast to the results observed with intact animals following ethanol administration, no increase in liver lipid was induced in adrenalectomized animals given the same dose of alcohol. Thirty eight hours after ingestion of this dose of ethanol, mean lipid values were 4.06 ± 0.29 in comparison with 4.79 ± 0.35 for untreated adrenalectomized animals. Mallov suggested that the inhibition of ethanol induced fatty accumulation may be due to an increased rate of fat catabolism and decreased dietary intake.

When a group of adrenalectomized animals was exposed to 2000 ppm of carbon tetrachloride, no significant effect on liver lipid content was noted at the termination of the exposure or twenty hours later (Table 18).

Adrenalectomized rats pretreated with ethanol eighteen hours before exposure to 2000 ppm carbon tetrachloride showed no

TABLE 18

HEPATIC LIPID LEVELS FOLLOWING INHALATION OF 2000 PPM CARBON TETRACHLORIDE IN INTACT AND ADRENALECTOMIZED RATS

	PERCENT TOTAL LIVER LIPID ± SE		
Treatment	At Time of CCl_4 Exposure	At Termination Of CCl_4 Exposure	20 Hours After CCl_4
AE	4.22 ± 0.26		4.06 ± 0.29
A	4.79 ± 0.35		
E	5.20 ± 0.25		4.71 ± 0.17
Normal	4.60 ± 0.14		
AEC		4.76 ± 0.08	4.90 ± 0.26
AC		4.92 ± 0.08	4.21 ± 0.29
EC		5.22 ± 0.12[a]	6.59 ± 0.51[a]
C		4.91 ± 0.12	5.00 ± 0.28

A -- Bilateral adrenalectomy.

C -- 2000 ppm CCl_4 for two hours.

E -- 5 ml/kg ethanol, oral, 18 hours prior to CCl_4 exposure.

At least 6 animals per group.

[a] $P < 0.05$ versus normal.

significant increase in liver lipid within twenty hours after exposure to the hydrocarbon. Treatment of the adrenalectomized animal with carbon tetrachloride, ethanol, or the combination failed to alter liver lipid values.

The effect of a higher concentration of carbon tetrachloride on lipid levels in similarly treated animals was studied. When a group of twelve intact rats was exposed to 4000 ppm of carbon tetrachloride for four hours, liver lipid levels at the termination of the exposure were already elevated to 6.07 $\pm$ 0.16% compared to 4.60 $\pm$ 0.14% for normal animals (Table 19). Twenty hours after the exposure lipid values had decreased to 5.47 $\pm$ 0.12%. Intact rats which had been treated with 5 ml/kg ethanol eighteen hours prior to exposure to the same concentration of carbon tetrachloride showed a similar increase in liver lipid values at the termination of exposure. Twenty hours after carbon tetrachloride exposure, the liver lipid values were still markedly elevated (6.22 $\pm$ 0.19%).

In contrast, bilaterally adrenalectomized animals exposed to this concentration of carbon tetrachloride did not exhibit elevated liver lipid levels following the inhalation of the hydrocarbon (Table 19). Twenty hours after exposure to carbon tetrachloride, the mean liver lipid values of these exposed animals was increased to 5.14

TABLE 19

HEPATIC LIPID LEVELS FOLLOWING INHALATION OF 4000 PPM CARBON TETRACHLORIDE IN INTACT AND ADRENALECTOMIZED RATS

PERCENT TOTAL LIVER LIPID ± SE

Treatment	At Time of CCl_4 Exposure	At Termination Of CCl_4 Exposure	20 Hours After CCl_4
AE	4.22 ± 0.26		4.48 ± 0.05
A	4.84 ± 0.35		
E	5.20 ± 0.25		4.65 ± 0.17
Normal	4.60 ± 0.14		
AEC		4.75 ± 0.14	5.31 ± 0.11
AC		4.83 ± 0.16	5.14 ± 0.05
EC		6.12 ± 0.14 [a]	6.22 ± 0.19 [a]
C		6.07 ± 0.16 [a]	5.47 ± 0.12 [a]

A -- Bilateral adrenalectomy

C -- 4000 ppm CCl_4 for four hours

E -- 5 ml/kg ethanol, oral, 18 hours prior to CCl_4 exposure

At least 6 animals per group

[a] $P < 0.05$ versus normal.

± 0.05% lipid. This small increase in liver lipid was not statistically different from the values of the unexposed adrenalectomized animals. Adrenalectomized animals which had been pretreated with 5 ml/kg ethanol eighteen hours prior to exposure did not exhibit a significant increase in hepatic lipid twenty hours after carbon tetrachloride administration. It was observed in all cases that bilateral adrenalectomy prevented the fatty accumulation observed following treatment with ethanol, carbon tetrachloride, or their combination. Similarly, hepatic lipid content was determined in intact animals two hours after the oral administration of 2.5 ml/kg carbon tetrachloride in an equal volume of corn oil (Table 20). Comparison of carbon tetrachloride concentrations in blood and liver of intact or adrenalectomized rats was made with the amounts of hepatic lipid found in each group (Table 24).

Adrenalectomized animals treated with ethanol and/or carbon tetrachloride had hepatic lipid levels comparable to untreated adrenalectomized animals. Adrenalectomized rats treated with ethanol prior to carbon tetrachloride administration had mean lipid levels which were somewhat lower than similarly treated intact animals. The variations in hepatic lipid values may be attributed to the inhibition of fatty accumulation following ethanol adminis-

TABLE 20

HEPATIC LIPID LEVELS OF INTACT AND ADRENALECTOMIZED RATS GIVEN ORAL DOSES OF CARBON TETRACHLORIDE AND/OR ETHANOL

Treatment	Percent Total Lipid (± S. E.)
AEC	4.76 ± 0.07
AC	4.92 ± 0.08
EC	5.22 ± 0.12
C	4.91 ± 0.12

A -- Bilateral adrenalectomy

E -- 5.0 ml/kg ethanol, given orally, eighteen hours prior to carbon tetrachloride administration

C -- 2.5 ml/kg carbon tetrachloride administered orally in an equal volume of corn oil

All animals sacrificed two hours after carbon tetrachloride administration

tration to the adrenalectomized rat.

SERUM ENZYME ACTIVITY IN INTACT AND ADRENALECTOMIZED ANIMALS FOLLOWING ADMINISTRATION OF ETHANOL AND/OR CARBON TETRACHLORIDE

Serum enzyme levels of GOT, ICD, or GPT following ethanol administration alone were comparable to those determined in adrenalectomized or intact rats (Table 21).

Adrenalectomized rats exposed to 4000 ppm of carbon tetrachloride for four hours exhibited serum enzyme levels of GOT, GPT, and ICD, which were increased 22.4, 15.3, and 7.4 fold above control values. Adrenalectomized animals which were pre-treated eighteen hours prior to carbon tetrachloride exposure with ethanol showed corresponding elevations of GOT, GPT, and ICD of 100., 23.4, and 26.0 times that of control group values. Serum GOT response to carbon tetrachloride in adrenalectomized animals pre-treated with ethanol was significantly greater than the response in adrenalectomized animals without alcohol pre-treatment. Intact rats showed serum GOT, GPT, and ICD increases of 44.3, 92.6, and 52.7 times the normal value following exposure to 4000 ppm of carbon tetrachloride for four hours. Intact rats dosed with 5 ml/kg ethanol eighteen hours prior to this same exposure showed corresponding enzyme elevations of 91.3, 97.6, and 66.7.

Adrenalectomized rats exposed to 2000 ppm of carbon tetra-

TABLE 21

SERUM ENZYME LEVELS IN INTACT AND ADRENALECTOMIZED RATS EXPOSED TO 4000 PPM OF CARBON TETRACHLORIDE

Treatment	SERUM ENZYME ACTIVITY (± SE)			POTENTIATION INDEX		
	S-GOT	S-GPT	S-ICD	S-GOT	S-GPT	S-ICD
AEC	15,100 ± 1803[b]	422 ± 106	16,583 ± 5378[a]	100.0[b]	23.4	26.0[a]
AC	3,388 ± 995[a]	275 ± 63[a]	4,734 ± 2144	22.4[a]	15.3[a]	7.4
AE	194 ± 25	22 ± 3	654 ± 153	1.3	1.2	1.0
A	151 ± 17	18 ± 1	638 ± 10	1.0	1.0	1.0
EC	22,660 ± 1962[c]	1952 ± 198	40,710 ± 6652	91.4[c]	97.6	66.7
C	11,000 ± 1598[a]	1852 ± 243[a]	32,190 ± 7581[a]	44.4[a]	92.6[a]	52.8[a]
E	240 ± 20	20 ± 1	638 ± 4	1.0	1.0	1.0
Normal	248 ± 44	20 ± 1	610 ± 10	1.0	1.0	1.0

A -- Bilateral adrenalectomy

E -- 5 ml/kg ethanol administered orally eighteen hours prior to carbon tetrachloride

C -- 4000 ppm carbon tetrachloride for four hours

All animals sacrificed twenty hours after exposure to carbon tetrachloride

At least six animals per group

[a] $P < 0.05$ versus normal [b] $P < 0.05$ versus AC [c] $P < 0.05$ versus C

chloride for two hours (Table 22) exhibited serum enzyme values of GOT, GPT, and ICD 13.4, 8.0, and 15.0 times that of unexposed adrenalectomized animals. Bilaterally adrenalectomized animals treated with 5 ml/kg ethanol eighteen hours prior to exposure to the same carbon tetrachloride concentration showed serum GOT, GPT, and ICD indexes of 31.6, 10.8, and 32.2 compared to similarly exposed intact rats whose corresponding serum enzyme levels were 12.8, 12.9, and 6.2 times those of normal rats. In comparison, intact rats treated with ethanol eighteen hours prior to carbon tetrachloride exposure exhibited corresponding serum enzyme values which were elevated 21.5, 16.0, and 29.5 times the normal value. At this level of exposure to carbon tetrachloride, both intact and adrenalectomized animals pretreated with ethanol exhibited a greatly elevated serum enzyme response. Although some overlap of values occurred, mean serum enzyme levels of alcohol pretreated rats were elevated above those animals which received only carbon tetrachloride. These elevated serum levels were observed in both intact and adrenalectomized animals which were given the combination of ethanol and carbon tetrachloride. This blurring of the serum enzyme picture could be explained as a result of extensive tissue damage, and consequent efflux of large amounts of enzyme into the serum. The great elevation of serum enzymes following these exposures to carbon tetrachloride tended to mask differences

TABLE 22

SERUM ENZYME ACTIVITY IN INTACT AND ADRENALECTOMIZED RATS EXPOSED TO 2000 PPM OF CARBON TETRACHLORIDE

Treatment	SERUM ENZYME ACTIVITY (+ SE) S-GOT	S-GPT	S-ICD	POTENTIATION INDEX S-GOT	S-GPT	S-ICD
AEC	4766 ± 398[b]	198 ± 28[a]	20561 ± 4076[a]	31.6[b]	10.8	32.2[a]
AC	2025 ± 400[a]	146 ± 3[a]	9615 ± 3067[a]	13.4[a]	8.0[a]	15.0[a]
AE	194 ± 25	22 ± 3	654 ± 153	1.3	1.2	1.0
A	151 ± 17	18 ± 1	638 ± 10	1.0	1.0	1.0
EC	5320 ± 432[a]	320 ± 124[a]	18000 ± 3055[a]	21.5[a]	16.0[a]	29.5[a]
C	3172 ± 428[a]	257 ± 22[a]	3788 ± 1278	12.8[a]	12.9[a]	6.2
E	240 ± 20	20 ± 1	638 ± 37	1.0	1.0	1.0
Normal	248 ± 44	20 ± 1	610 ± 10	1.0	1.0	1.0

A -- Bilateral adrenalectomy

E -- 5 ml/kg ethanol, administered orally

C -- 2000 ppm carbon tetrachloride for two hours

All animals sacrificed 20 hours after exposure to carbon tetrachloride

At least six animals per group

[a] $P<0.05$ versus normal.

[b] $P<0.05$ versus AC.

TABLE 23

SERUM ENZYME LEVELS IN INTACT AND ADRENALECTOMIZED RATS EXPOSED TO 1000 PPM CARBON TETRACHLORIDE

Treatment	SERUM ENZYME ACTIVITY (+ SE) S-GOT	S-GPT	S-ICD	POTENTIATION INDEX S-GOT	S-GPT	S-ICD
AEC	1982 ± 204[a]	178 ± 44[a]	2446 ± 212[a]	13.1[a]	9.7[a]	3.8[a]
AC	264 ± 42	37 ± 10	740 ± 155	1.7	2.0	1.2
AE	194 ± 25	22 ± 3	654 ± 153	1.2	1.2	1.0
A	151 ± 17	18 ± 10	638 ± 10	1.0	1.0	1.0
EC	2200 ± 142[b]	195 ± 55[b]	1850 ± 151[b]	8.9[b]	5.4[b]	4.3[b]
C	289 ± 17	41 ± 13	525 ± 123	1.2	1.1	1.2
E	241 ± 20	29 ± 11	450 ± 105	1.0	0.8	1.0
Normal	248 ± 44	36 ± 7	427 ± 141	1.0	1.0	1.0

A -- Bilateral adrenalectomy

E -- 5 ml/kg ethanol administered orally eighteen hours prior to carbon tetrachloride

C -- 1000 ppm carbon tetrachloride for two hours

All animals sacrificed twenty hours after exposure to carbon tetrachloride

At least six animals per group

[a] $P < 0.005$ versus C or AC.

[b] $P < 0.005$ versus normal or C.

in enzyme release between alcohol pretreated and untreated rats. This blurring can be resolved by decreasing the dose of carbon tetrachloride to a level that causes slight or minimal increases in serum enzyme levels in the normal animal.

When intact or adrenalectomized rats were exposed to 1000 ppm for two hours, serum enzyme levels were not greatly elevated above values of normal unexposed animals. However, in adrenalectomized animals pretreated with ethanol eighteen hours prior to exposure to carbon tetrachloride, the serum enzyme levels of GOT, GPT, and ICD were elevated 13.1, 9.7, and 3.8 times the normal value (Table 23). Similarly, intact rats pretreated with 5.0 ml/kg ethanol eighteen hours prior to carbon tetrachloride exposure, exhibited serum GOT, GPT, and ICD levels which were 8.8, 5.3, and 4.3 times the normal value. Only animals which were pretreated with ethanol exhibited greatly increased serum enzyme levels. Although adrenalectomized animals did not develop ethanol induced fatty liver, the enzyme response characteristic of increased tissue damage, was comparable to that of ethanol treated intact rats exposed to the same concentration of carbon tetrachloride.

CARBON TETRACHLORIDE CONCENTRATION IN BLOOD AND LIVER IN INTACT AND ADRENALECTOMIZED RATS FOLLOWING ADMINISTRATION OF CARBON TETRACHLORIDE AND/OR ETHANOL

It has been suggested that the fatty accumulation in the liver caused

by pretreatment with ethanol may serve to increase the concentration of the lipid soluble hydrocarbon in this organ. To determine whether alcohol might exert its synergistic effect on the toxicity of carbon tetrachloride by this mechanism of increased solubility, analyses were made to determine the effect of pretreatment with ethanol on the concentration of the organic halide in blood and liver.

Carbon tetrachloride concentrations were measured in normal rats and in animals pretreated with 5 ml/kg BW ethanol eighteen hours prior to administration of the organic halide. Blood and liver samples from bilaterally adrenalectomized rats, which do not develop fatty liver following ethanol pretreatment, were also analyzed after administration of carbon tetrachloride. By comparison of tissue and blood concentrations of carbon tetrachloride after various treatments, the role of pre-existent fatty liver in the potentiation of carbon tetrachloride toxicity may be evaluated.

A group of intact rats was exposed to 4000 ppm carbon tetrachloride for four hours. Half of these animals had been treated with 5.0 ml ethanol/kg prior to the time of exposure. Similarly treated groups of bilaterally adrenalectomized animals were exposed to the same concentration of carbon tetrachloride. Since an air lock was not available to remove animals individually after exactly four hours

of exposure, all animals were exposed as a group and sacrificed at time intervals after removal from the chamber. The rate of excretion through the lungs following exposure can be described as an exponential function. However, during the short time interval in question, this relationship can be expressed as the linear function, $Y = 0.022 - \frac{t}{5000}$. Where Y is the blood concentration of carbon tetrachloride in mg/gm, t is the time after exposure in minutes.

Blood concentrations found in adrenalectomized and intact animals were similar and independent of prior treatment with ethanol (Table 24). Due to the low concentration of carbon tetrachloride and the smaller samples used in tissue analysis, the organic halide was not detected in the liver in any of the groups mentioned.

The blood and liver concentration of carbon tetrachloride was also determined following administration of the organic halide by intraperitoneal injection.

A group of six intact rats were injected intraperitoneally with 0.60 ml/kg BW carbon tetrachloride. A similar group which had been dosed with 5 ml/kg BW eighteen hours previously were also injected with the same dose of the hydrocarbon. Corresponding groups of bilaterally adrenalectomized animals (no ethanol, or ethanol

eighteen hours prior to injection) were given the same dose of carbon tetrachloride. Exactly two hours after injection of carbon tetrachloride, animals were sacrificed and analysis of carbon tetrachloride in blood and liver was performed (Table 24). At this time, intact rats exhibited a level of 0.308 $\pm$ 0.100 mg carbon tetrachloride per gram liver. This concentration was comparable to that observed in rats which had received ethanol eighteen hours prior to carbon tetrachloride injection.

Analyses of liver from bilaterally adrenalectomized rats intraperitoneally injected with carbon tetrachloride showed a level of 0.379 $\pm$ 0.122 mg per gram liver. Adrenalectomized rats which had been given 5 ml/kg ethanol prior to carbon tetrachloride injection had a concentration of 0.350 $\pm$ 0.050 mg carbon tetrachloride per gram liver. It was observed in all cases that the concentration of carbon tetrachloride found in the liver was independent of pretreatment with ethanol. Although adrenalectomized animals did not develop the ethanol induced fatty accumulation of the liver, the concentration of carbon tetrachloride found was comparable to that observed in intact rats. Analysis of blood for carbon tetrachloride showed similar results. Blood of intact rats contained 0.136 $\pm$ 0.039 mg carbon tetrachloride per gm following intraperitoneal injection of the hydrocarbon. All other groups showed comparable

blood levels regardless of previous treatment (Table 24).

Similarly, analyses of carbon tetrachloride in blood and liver were performed when the hydrocarbon was given by the oral route. Carbon tetrachloride was administered as a 50% solution in corn oil at the rate of 2.5 ml/kg BW of carbon tetrachloride. Exactly two hours after administration of carbon tetrachloride, animals were sacrificed and analysis of the organic halide was made in blood and liver. Intact rats intubated with this dose of carbon tetrachloride developed a concentration of 0.144 ± 0.085 mg CCl_4 per gram liver at this time. Intact rats which were pretreated with an oral dose of 5 ml/kg BW ethanol eighteen hours prior to administration of carbon tetrachloride had 0.223 ± 0.105 mg of the hydrocarbon per gram liver. Bilaterally adrenalectomized rats, administered the same oral dose of carbon tetrachloride, developed a level of 0.147 ± 0.058 mg CCl_4 per gram liver. A similar group of adrenalectomized animals, which had been pretreated with ethanol eighteen hours prior to dosing with the hydrocarbon, exhibited a level of 0.277 ± 0.068 mg CCl_4 per gram liver. Due to individual variations, no differences of statistical significance could be assigned to blood and liver concentrations in any of the various groups. The differences observed in the mean values of animals given carbon tetrachloride by the oral route could be ascribed to variation in gastro-intestinal absorption. Following

TABLE 24

CONCENTRATION OF CARBON TETRACHLORIDE IN BLOOD AND LIVER OF INTACT AND ADRENALECTOMIZED RATS

Treatment	CONCENTRATION OF CARBON TETRACHLORIDE Liver (mg CCl_4/gm ± SE)	Blood (mg CCl_4/gm ± SE)
Inhalation		
AEC	n. d.	0.020 ± 0.002
AC		0.020 ± 0.003
EC		0.021 ± 0.003
C		0.020 ± 0.003
Intraperitoneal		
AEC	0.350 ± 0.050	0.125 ± 0.052
AC	0.379 ± 0.122	0.104 ± 0.041
EC	0.363 ± 0.043	0.137 ± 0.041
C	0.308 ± 0.100	0.136 ± 0.039
Oral		
AEC	0.277 ± 0.068	0.045 ± 0.010
AC	0.147 ± 0.058	0.025 ± 0.010
EC	0.223 ± 0.105	0.037 ± 0.020
C	0.144 ± 0.085	0.028 ± 0.012

A -- Bilateral adrenalectomy

E -- 5.0 ml/kg ethanol given orally eighteen hours prior to carbon tetrachloride administration

C -- Carbon tetrachloride inhalation - 4000 ppm CCl_4 for four hours

C -- Intraperitoneal injection of 0.6 ml CCl_4/kg

C -- Oral intubation of 2.5 ml CCl_4/kg in an equal volume of corn oil

intubation with ethanol, a loss of appetite was observed in treated animals. The lack of food or irritation in the gastro-intestinal tract of these animals could account for variations in absorption which were reflected in values of carbon tetrachloride found.

HISTOPATHOLOGICAL STUDIES IN INTACT AND ADRENALECTOMIZED RATS TREATED WITH CARBON TETRACHLORIDE AND/OR ETHANOL

Histopathological examination of tissue stained with H & E and Oil Red O was performed to determine the changes produced by treatment with ethanol, carbon tetrachloride or their combination in intact and bilaterally adrenalectomized rats.

At the time of exposure to carbon tetrachloride, intact rats which had been treated eighteen hours previously with ethanol showed small vacuoles of lipid within the parenchymal cells of the liver (Figure 4). In contrast, bilaterally adrenalectomized animals treated with the same dose of ethanol did not exhibit fatty infiltration of the liver at this time (Figures 43 and 44). Thirty eight hours after the ingestion of 5.0 ml/kg ethanol, liver sections of intact or adrenalectomized rats stained for lipid were essentially negative.

Immediately following exposure to 4000 ppm carbon tetrachloride for four hours, examination of liver sections from intact rats stained for fat showed a few vacuoles of lipid within the liver

parenchymal cells. Rats which had been pretreated with ethanol prior to exposure to this concentration of carbon tetrachloride showed numerous vacuoles of lipid within the parenchymal cells at the termination of exposure.

Bilaterally adrenalectomized rats, both alcohol pretreated and untreated, did not exhibit vacuoles of lipid in the liver parenchyma before or at the termination of the four hour exposure to 4000 ppm carbon tetrachloride.

Twenty hours following exposure to 4000 ppm of carbon tetrachloride for four hours, liver of intact rats exhibited central lobular necrosis with moderately abundant acute inflammatory cellular infiltrate. Moderately severe degenerative fatty infiltrate was observed in cells surrounding areas of necrosis (Figure 46). Twenty hours after exposure to carbon tetrachloride, alcohol pretreated intact rats exhibited central lobular necrosis with scant mononuclear and polymorphonuclear infiltration in the liver. Severe peripheral lobular degenerative fatty infiltrate was confirmed upon examination of fat stained sections (Figure 48). A basophilic hyaline deposition was observed in renal tubules in one animal.

Liver sections of bilaterally adrenalectomized rats examined twenty hours after exposure to this concentration of carbon tetrachloride showed marked central lobular degenerative changes with

infiltrate of acute inflammatory cells. Fat stained sections showed vacuoles of lipid within these degenerating parenchymal cells (Figure 45). Similarly, ethanol pretreated adrenalectomized rats showed marked central lobular degenerative parenchymal changes with infiltrate of acute inflammatory cells (Figure 47). Fat stained sections confirmed the presence of lipid vacuoles within the degenerating parenchymal cells twenty hours after exposure to carbon tetrachloride.

Intact animals exposed to 1000 ppm of carbon tetrachloride for two hours showed no evidence of necrosis or retrogressive change within the liver (Figure 9). In contrast, intact animals which had been pretreated with ethanol prior to exposure to this low concentration of carbon tetrachloride showed pronounced central lobular necrosis and pronounced degenerative fatty infiltration in the parenchyma of the liver (Figure 10). Bilaterally adrenalectomized animals exposed to 1000 ppm of carbon tetrachloride for two hours showed no evidence of necrosis in the liver twenty hours after the exposure (Figure 50). Adrenalectomized animals pretreated with ethanol prior to this carbon tetrachloride exposure showed pronounced central lobular necrosis (Figure 49). Although fatty accumulation induced by pretreatment with ethanol was inhibited, necrosis developed in these animals. On examination of fat stained sections, fine lipid droplets were observed in degenerating

Figure 43

Liver from an adrenalectomized rat. H & E, 145X.

Hepatic architecture is intact. Fat stained section negative.

Figure 44

Liver from an adrenalectomized rat given 5.0 ml/kg ethanol eighteen hours prior to sacrifice. H & E, 145X.

No disruption of hepatic architecture. No accumulation of lipid observed.

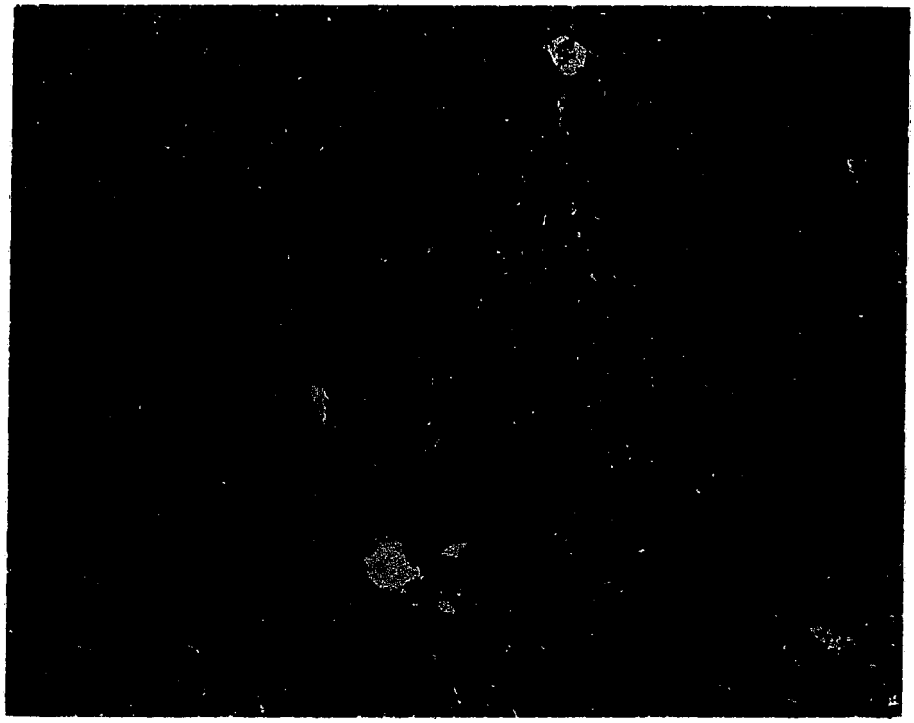


Figure 43

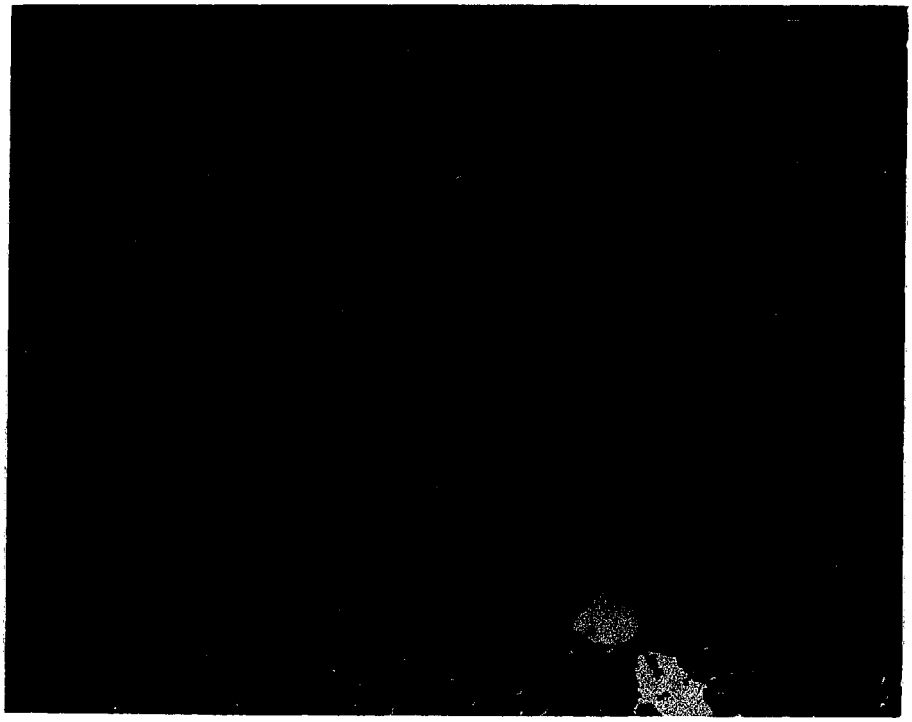


Figure 44

Figure 45

Liver from an adrenalectomized rat exposed to 4000 ppm carbon tetrachloride for four hours. Sacrificed twenty hours after exposure. H & E, 145X.

Marked central lobular degenerative changes with infiltrate of acute inflammatory cells can be observed. Vacuoles of lipid are present within the degenerating cells.

Figure 46

Liver from an intact rat exposed to 4000 ppm carbon tetrachloride for four hours. Sacrificed twenty hours after exposure. H & E, 145X.

Central lobular necrosis with moderately abundant acute inflammatory cellular infiltrate can be observed. Moderately severe fatty infiltration is present in cells surrounding areas of necrosis.

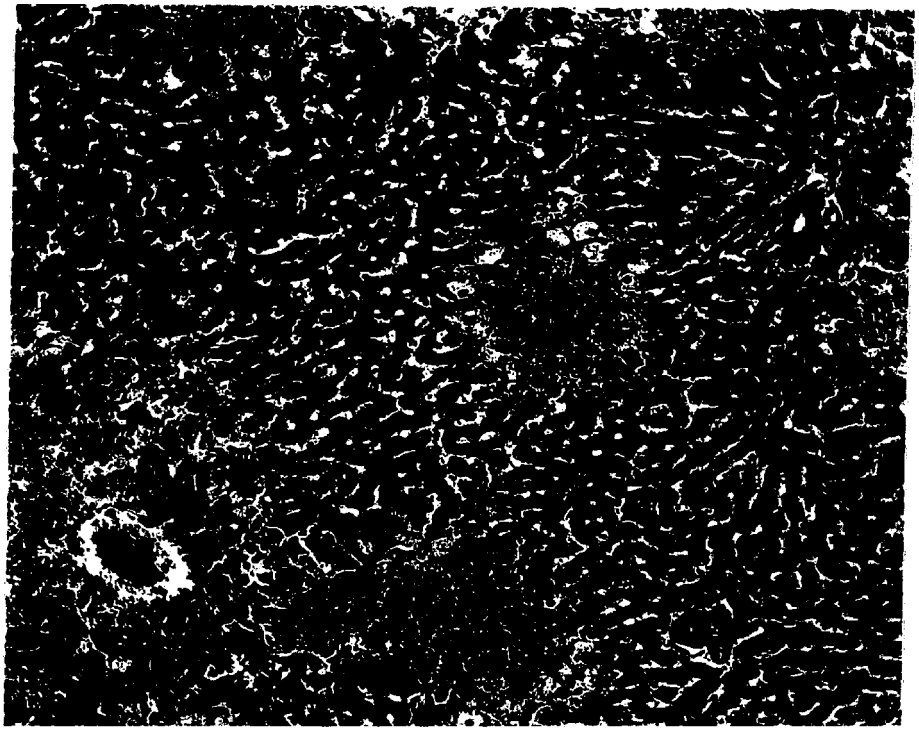


Figure 45

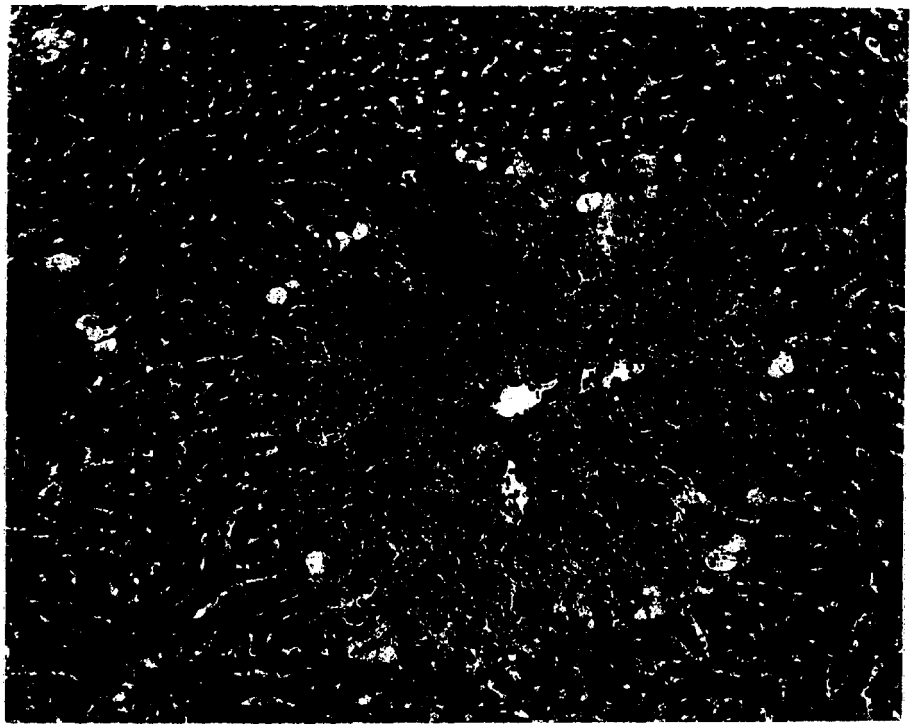


Figure 46

Figure 47

Liver from an adrenalectomized rat given 5.0 ml/kg ethanol, orally, eighteen hours prior to exposure to 4000 ppm carbon tetrachloride for four hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Marked central lobular degenerative changes with infiltration of acute inflammatory cells can be observed. Lipid vacuoles may be detected within these degenerating cells.

Figure 48

Liver from an intact rat given an oral dose of 5.0 ml/kg ethanol, eighteen hours prior to exposure to 4000 ppm carbon tetrachloride for four hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Central lobular necrosis with scant polymorphonuclear leucocyte infiltration can be observed. Severe peripheral lobular degenerative fatty infiltrate can be observed.

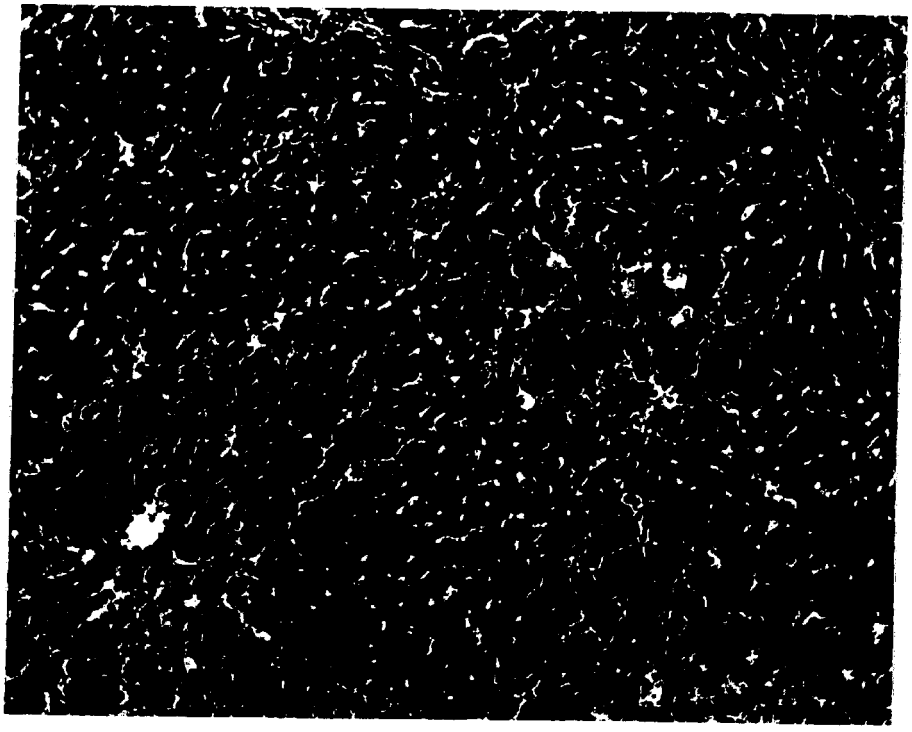


Figure 47

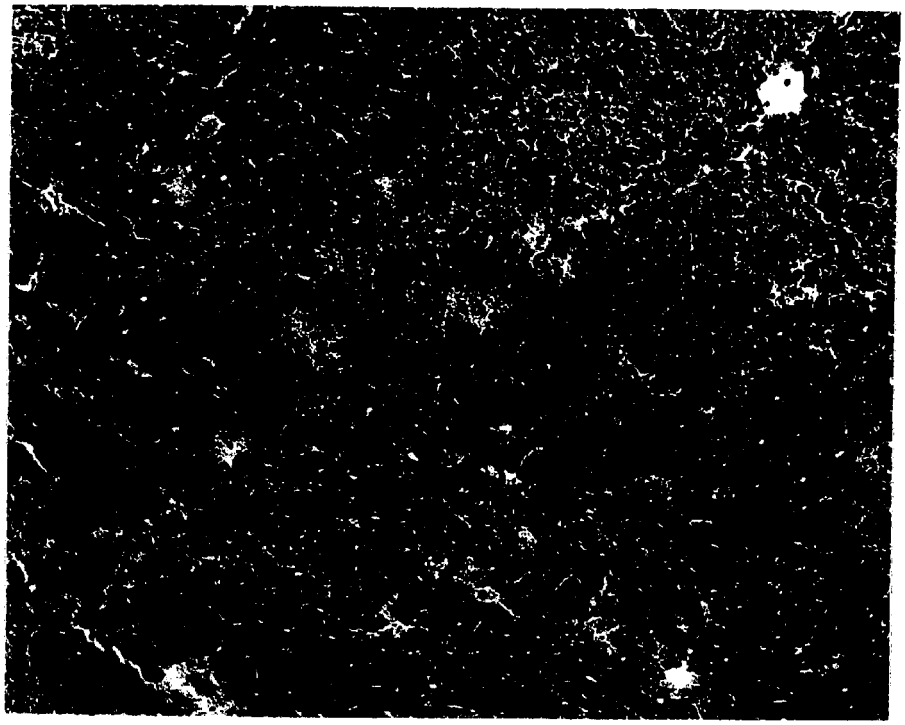


Figure 48

Figure 49

Liver from an adrenalectomized rat given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

The liver exhibits central lobular necrosis. Surviving cells show degenerative changes.

Figure 50

Liver from an adrenalectomized rat exposed to 1000 ppm carbon tetrachloride twenty hours prior to sacrifice. H & E, 145X.

Hepatic parenchyma shows minimal degenerative changes following exposure to carbon tetrachloride. No disruption of hepatic architecture is evident.

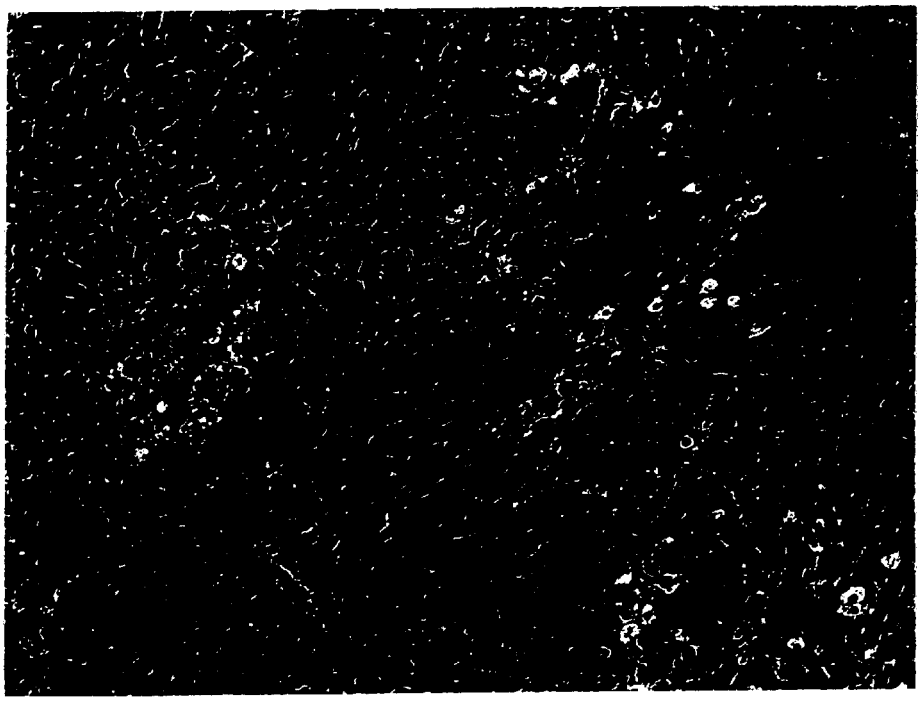


Figure 49



Figure 50

liver cells. Histopathological examination of this group of animals showed the development of necrosis only in animals which had been pretreated with ethanol. The development of necrosis was independent of the pre-existence of ethanol induced fatty accumulation of the liver. Although adrenalectomized animals did not exhibit the fatty change following ethanol administration, necrosis was produced in this group of animals.

DISCUSSION

The analyses of total liver lipid following ethanol or carbon tetrachloride administration confirm the results of Mallov (109) and Brody et al (20, 26, 170) bilateral adrenalectomy effectively prevented fatty accumulation of the liver observed following an intoxicating dose of ethanol. This prevention of liver lipid accumulation may be due to a more rapid rate of fat catabolism in the adrenalectomized animal. At the time of exposure to carbon tetrachloride, intact animals pretreated with ethanol exhibited slightly elevated liver lipid levels, whereas similarly treated adrenalectomized animals did not. Twenty hours after exposure to 4000 ppm carbon tetrachloride, intact animals pretreated with ethanol exhibited grossly elevated liver lipid values. Bilateral adrenalectomy effectively prevented lipid accumulation of the liver following administration of carbon tetrachloride and/or ethanol. Intact and adrenalectomized rats which had been treated with 5.0 ml/kg ethanol eighteen hours prior to exposure to carbon tetrachloride showed serum enzyme levels which were greater

than corresponding values of exposed rats not given ethanol. Although adrenalectomized rats did not develop ethanol induced fatty liver, the serum enzyme response of adrenalectomized ethanol pretreated rats was considerably elevated above the levels of corresponding control groups. If the increase in liver lipid caused by ethanol is the mechanism of potentiation of toxicity of carbon tetrachloride, increased tissue damage would not be expected in animals which did not develop the ethanol induced fatty liver. Since elevated serum enzyme levels can be related to the extent or degree of tissue damage, greater tissue changes occurred in all animals which had been given ethanol, irrespective of the development of the ethanol induced fatty liver.

Following inhalation of high concentrations of carbon tetrachloride, the serum enzyme response is greatly elevated and tends to mask the increase in serum enzyme activity observed in alcohol pretreated exposed animals. By exposing animals to a low concentration of carbon tetrachloride which causes minimal changes in serum enzyme activity, the effect of ethanol pretreatment on such exposed animals can be observed. Animals exposed to 1000 ppm carbon tetrachloride showed only slight changes in serum GOT, GTP, and ICD. However, rats given 5.0 ml/kg ethanol eighteen hours prior to this exposure showed greatly elevated serum enzyme levels even though fatty

liver accumulation was prevented by adrenalectomy in some of these animals. This observation suggests that pre-existent fatty liver is not the causative mechanism in the potentiation of carbon tetrachloride intoxication. Comparison of serum enzyme response with liver lipid levels found in individual animals also failed to reveal a correlation between these variables. Histopathological examination also indicated that a greater degree of tissue damage occurred in all ethanol pretreated exposed rats, including adrenalectomized rats whose livers did not contain excess lipid.

No statistically significant differences of carbon tetrachloride concentration were observed in blood or liver of carbon tetrachloride treated rats, irrespective of pretreatment with ethanol. No correlation was observed between individual values of carbon tetrachloride found in the liver compared to the corresponding value of total hepatic lipid. Variation in values of carbon tetrachloride administered orally may be related to the presence of food and the variability in absorption from the gastro-intestinal tract.

The development of greatly elevated serum enzyme levels without concomitant induction of ethanol fatty liver, and the similarity in carbon tetrachloride concentrations developed in liver suggests that factors other than physical solubility of the hydrocarbon may influence the potentiation of toxicity of carbon tetrachloride. Alterations in cellular components or metabolism caused by ingestion

of ethanol may be the fundamental biochemical lesion that influences the toxicity of carbon tetrachloride. The potentiation of toxicity may be due to biochemical imbalances within the cell, associated with lipid accumulation, but not dependent upon the presence of the lipid.

V-B -- BIOLOGICAL RESPONSE TO CARBON TETRACHLORIDE IN INTACT AND ADRENALECTOMIZED ANIMALS AS A FUNCTION OF TIME AFTER ETHANOL INGESTION

The accumulation of liver lipid in the intact rat following an oral dose of ethanol has been well documented. To examine the relationship of liver lipid to the potentiation of carbon tetrachloride toxicity, it would be pertinent to measure the toxic response to the hydrocarbon relative to the time after administration of the alcohol. If potentiation of toxicity of carbon tetrachloride is associated with the accumulation of liver lipid induced by ethanol, the toxic response should be greatest when the liver lipid levels reach their maximum values following a dose of ethanol. Although increased toxicity and lipid accumulation may occur simultaneously this does not imply a causal relationship. To clarify the nature of this relationship, the toxic response was measured in alcohol treated animals in which lipid accumulation was inhibited by adrenalectomy. It has been reported earlier (109, 110, 170, 26) that the adrenalectomized rat does not develop the characteristic accumulation of liver lipid following ingestion of ethanol. If potentiation of toxicity of carbon tetrachloride is causally related to the presence of the alcohol-induced fatty liver, protection against the enhanced toxicity would be observed in adrenalectomized animals treated with ethanol. However, if potentiation of toxicity is observed in such animals, the mechanism of action would appear to be related to other

functions of ethanol and not to be causally related to the presence of the lipid.

PROCEDURE

Male Sprague-Dawley rats, 175-200 gms, were bilaterally adrenalectomized under ether anesthesia, and maintained on Rockland Rat Chow and 0.9% saline, ad libitum, for ten days prior to dosage with ethanol. Groups of six adrenalectomized animals were given 5 ml/kg ethanol by intubation at 0, 4, 8, 12, 16 or 24 hours prior to exposure to 1000 ppm carbon tetrachloride for two hours. All animals were sacrificed twenty hours after exposure to carbon tetrachloride and blood was collected for serum enzyme analysis. Sections of liver were reserved for histopathological examination and determination of liver lipid. Liver lipid levels were determined in similar groups of six surgically altered animals which received only ethanol at 0, 8, 18, or 24 hours prior to sacrifice.

Similar groups of intact animals which were pre-treated with an oral dose of 5 ml/kg ethanol were exposed to 1000 ppm carbon tetrachloride for two hours 0, 2, 4, 8, 12, 16, 18, or 24 hours following ingestion of the alcohol. Twenty hours after exposure to the chlorinated hydrocarbon, all animals were sacrificed and blood collected for serum enzyme analysis. To determine the extent of liver lipid accumulation following this dose of ethanol, groups of

six intact rats were sacrificed at 0, 1, 3, 6, 9, 12, 15, 18, or 24 hours following administration of the alcohol, and portions of the liver analyzed for total lipid.

RESULTS

DETERMINATION OF TOTAL HEPATIC LIPID

Following an oral dose of 5.0 ml/kg ethanol, intact rats exhibited an elevation in total liver lipid within nine hours after administration of the alcohol (Table 1). Twenty-four hours after alcohol ingestion, liver lipid levels returned to normal values. Histopathological examination of the liver of these animals confirmed the presence of small droplets of lipid within the parenchyma eighteen hours after ethanol ingestion (Figure 4). In contrast, bilaterally adrenalectomized animals did not exhibit an increase in total liver lipid following the same oral dosage of ethanol (Table 25). Histopathological examination of liver sections stained with H & E or Oil Red O failed to reveal an accumulation of lipid droplets within the liver parenchyma of adrenalectomized rats eighteen hours after ingestion of ethanol (Figures 43 and 44).

Determination of total liver lipid in adrenalectomized rats exposed to 1000 ppm of carbon tetrachloride at various times after ethanol ingestion was performed and the results are summarized in Table 26. Twenty hours after exposure, these animals exhibited no increase in hepatic lipid. It was noted that the lowest value of

total hepatic lipid occurred in animals eighteen hours following ingestion of alcohol. Similarly the lowest level of hepatic lipid was found in animals which were exposed to carbon tetrachloride eighteen hours after ethanol ingestion. Adrenalectomy was effective in inhibiting fatty changes observed following ethanol or carbon tetrachloride administration.

TABLE 25

TOTAL LIVER LIPID IN ADRENALECTOMIZED RATS FOLLOWING ETHANOL ADMINISTRATION

Time after Ethanol Administration (Hours)	Percent Total Liver Lipid (± S. E.)
0	4.84 ± 0.35
8	4.68 ± 0.13
18	4.22 ± 0.26
24	4.48 ± 0.05

5.0 ml/kg ethanol administered orally.

Six adrenalectomized animals per group.

TABLE 26

TOTAL LIVER LIPID FOLLOWING CARBON TETRACHLORIDE EXPOSURE OF ETHANOL TREATED ADRENALECTOMIZED RATS

Time Interval of Ethanol-CCl_4 Exposure (Hours)	Percent Total Liver Lipid (± S. E.)
0	4.83 ± 0.18
4	4.64 ± 0.10
8	4.98 ± 0.15
12	4.72 ± 0.10
18	4.08 ± 0.12
24	4.19 ± 0.18
No Ethanol	4.70 ± 0.13

All animals sacrificed 20 hours following exposure to carbon tetrachloride.

5 ml/kg ethanol administered orally.

1000 ppm carbon tetrachloride for 2 hours.

At least six adrenalectomized animals per group.

SERUM ENZYME ACTIVITY IN INTACT AND ADRENALECTOMIZED ANIMALS EXPOSED TO CARBON TETRACHLORIDE AT VARIOUS TIMES AFTER ETHANOL INGESTION

The activity of S-GOT was determined in intact animals exposed to this low concentration (1000 ppm for two hours) of carbon tetrachloride at various times after ingestion of ethanol (Table 27). It was observed that animals which received ethanol within two hours of exposure to carbon tetrachloride did not exhibit greatly elevated serum enzyme levels twenty hours after exposure. Animals which received ethanol four hours prior to exposure to the hydrocarbon exhibited significantly elevated serum enzyme levels in comparison to exposed animals which were not dosed with ethanol. When the time interval between ethanol ingestion and carbon tetrachloride exposure was increased to 8, 12, 16, or 18 hours, the mean serum enzyme levels, twenty hours after exposure, reached their maximum values. This increase in serum enzyme activity is reflected by the value of the potentiation index which increased sharply at the eight hour interval to 10.3, reached a maximum value at the sixteen hour interval, then rapidly decreased. When

the time interval between ethanol-carbon tetrachloride dosage was extended to twenty-four hours, the elevation in serum enzyme activity was not significantly different from control values. This increased efflux of glutamic oxaloacetic transaminase into the serum during this time interval of ethanol-carbon tetrachloride administration occurs during the period of maximum lipid accumulation induced by alcohol. Whether a causal or associated relationship exists between increased liver lipid levels and elevated serum enzyme activity is a question which can be partially resolved by examination of serum enzyme response in adrenalectomized animals.

From the data previously presented in Table 25, it was observed that bilateral adrenalectomy prevented the accumulation of liver lipid in rats given this oral dose of 5 ml/kg ethanol. Groups of adrenalectomized animals were exposed to 1000 ppm carbon tetrachloride for two hours at 0, 4, 8, 12, 18, or 24 hours following dosage with ethanol, and the S-GOT activity was determined (Table 28). When carbon tetrachloride was administered 8, 12, or 18 hours following dosage with ethanol greatly elevated serum enzyme activity was observed. The potentiation index at these time intervals of ethanol-carbon tetrachloride dosage was 6.2, 18.7, and 16.5 respectively. Although these adrenalectomized

animals did not develop the alcohol induced fatty accumulation of the liver, the greatly elevated serum enzyme response was still observed.

TABLE 27

ACTIVITY OF S-GOT IN INTACT RATS EXPOSED TO CARBON TETRACHLORIDE AT VARIOUS TIMES AFTER ETHANOL INGESTION

Time Interval Between Ethanol Administration and CCl_4 Exposure	Activity S-GOT (± S. E.)	Potentiation Index
0	222 ± 11	0.9
2	202 ± 47	0.8
4	605 ± 79	2.4
8	2552 ± 844	10.3
12	2700 ± 562[a]	10.9
16	4586 ± 2203	18.5
18	2200 ± 142[a]	8.9
24	369 ± 70	1.5
No Ethanol	289 ± 16	1.2
Normal	248 ± 44	1.0

1000 ppm carbon tetrachloride for two hours.

5 ml/kg ethanol administered orally.

At least six animals per group.

All animals sacrificed 20 hours after CCl_4 exposure.

[a] $P < 0.05$ versus normal.

TABLE 28

ACTIVITY OF S-GOT IN ADRENALECTOMIZED RATS EXPOSED TO CARBON TETRACHLORIDE AT VARIOUS TIMES AFTER ETHANOL INGESTION

Time Interval Between Ethanol Administration and CCl_4 Exposure	S-GOT Activity (± S. E.)	Potentiation Index
0	555 ± 38	2.2
4	226 ± 24	0.9
8	1527 ± 169 [a]	6.2
12	4631 ± 668 [a]	18.7 [a]
18	4238 ± 502 [a]	16.5 [a]
24	798 ± 193	3.2
No Ethanol	232 ± 36	0.9
Normal	248 ± 44	1.0

5 ml/kg ethanol administered orally.

1000 ppm carbon tetrachloride for two hours.

At least six animals per group.

All animals sacrificed 20 hours after CCl_4 exposure.

[a] $P < 0.05$ versus normal.

RESPIRATION RATE AND HEART RATE FOLLOWING ETHANOL INGESTION

Hitchcock (174) has reported stimulation of respiration following dosage of alcohol in humans. However, large doses of alcohol, causing severe intoxication, depress respiration. Increased respiration rate could lead to increased absorption of CCl_4 during exposure periods. To determine the effect upon respiratory rate induced by this dosage of ethanol to the experimental animals, the heart rate, respiratory rate, and EKG were measured employing the E & M Instrument Co. Physiograph. During the measurement period, animals were constrained by a plastic animal holder and electrical contact maintained by insertion of hypodermic electrodes. Five minutes following intubation with ethanol, a sharp elevation in respiratory rate was observed (100/min.). The rate then decreased abruptly within thirty minutes and returned to the normal value (84/min.). However, the normal animal, when handled and traumatized by the intubation of water exhibited a similar transitory increase in respiration rate (109/min.). This increase in respiration rate, following intubation and insertion of the measuring electrodes, could be attributed to trauma and excitement of the animal caused by handling. During the six hour measurement period, no significant differences in respiration rate, heart rate, or EKG were

observed between ethanol treated and control animals. Complete respiratory function could not be assessed, however, since measurement of pulmonary capacity could not be estimated.

BODY TEMPERATURE FOLLOWING ETHANOL ADMINISTRATION

Variation in body temperature following an oral dose of 5 ml/kg ethanol was recorded by rectal insertion of a sensing thermocouple. A similar group of untreated animals served as controls. Within three hours of administration of the ethanol, body temperature dropped slightly to a mean value of 35.12 $\pm$ 0.12°C then returned gradually to the normal value (Table 29). This decrease in body temperature was significant (95% confidence) three hours following ingestion of the ethanol. Although mean values of body temperature were comparably lowered 6 and 9 hours after administration of alcohol, variation in individual values does not permit assignment of statistical significance at these later time periods. Though not statistically significant, mean value of rectal temperature in adrenalectomized rats was lower than that of intact animals.

TABLE 29

VARIATION IN BODY TEMPERATURE FOLLOWING INGESTION OF ETHANOL

Time After Ethanol Dosage	Ethanol Treated 5 ml/kg Oral (± S. E.)	Control No Ethanol ± S. E.
0	35. 92 ± 0. 05	35. 85 ± 0. 16
3	35. 12 ± 0. 12[a]	36. 22 ± 0. 19
6	35. 22 ± 0. 26	36. 13 ± 0. 10
9	35. 32 ± 0. 20	35. 88 ± 0. 15
12	36. 22 + 0. 19	36. 15 ± 0. 30
15	36. 16 ± 0. 21	36. 03 ± 0. 18

Five rats per group.

[a] $P < 0.05$

SERUM IRON CONCENTRATIONS IN ADRENALECTOMIZED ANIMALS FOLLOWING CARBON TETRACHLORIDE INHALATION

Examination of serum iron concentrations showed that animals given ethanol eighteen hours prior to exposure to 1000 ppm carbon tetrachloride exhibited higher values than those which were exposed eight hours after ethanol treatment. (Table 30). All animals were sacrificed twenty hours after carbon tetrachloride administration.

The increased serum iron concentrations found in the group of animals which received ethanol prior to exposure is suggestive of greater hepatic changes than that observed in the control groups. Histological examination was made to determine qualitative morphological differences between these groups of animals.

HISTOPATHOLOGICAL STUDIES OF INTACT AND ADRENALECTOMIZED RATS EXPOSED TO CARBON TETRACHLORIDE AT VARIOUS TIMES AFTER ETHANOL INGESTION

Within twenty hours after exposure to 1000 ppm carbon tetrachloride, minimal hepatic morphological changes were observed in intact or adrenalectomized rats (Figures 9 and 50).

TABLE 30

SERUM IRON CONCENTRATION IN ADRENALECTOMIZED ANIMALS EXPOSED TO CARBON TETRACHLORIDE AT VARIOUS TIMES AFTER ETHANOL INGESTION

Time Interval Between Ethanol Ingestion and CCl_4 Exposure (Hours)	Serum Iron Concentration (ppm $\pm$ S. E.)
0	10.9 ± 0.5
8	14.1 ± 1.1
12	20.2 ± 1.0[a,b]
18	20.3 ± 1.0[b]
Normal	10.0 ± 1.0

1000 ppm carbon tetrachloride for two hours.

5 ml/kg ethanol administered orally.

Four animals per group.

[a]Mean of three animals. [b]$P < 0.5$ versus normal.

Animals sacrificed twenty hours after carbon tetrachloride exposure.

When adrenalectomized rats were given 5.0 ml/kg ethanol immediately preceding this exposure to carbon tetrachloride, necrosis of individual and small groups of hepatic cells could be detected twenty hours later (Figure 51). Cells in the central lobular region appeared somewhat larger and less eosinophilic than neighboring cells. Only minimal fatty change could be observed. Similarly treated intact animals exhibited focal necrosis, moderate fatty infiltration and hydropic degeneration in the paracentral areas (Figure 52).

When the time interval of ethanol-carbon tetrachloride administration was increased to four hours, marked early central lobular necrosis with hydropic degeneration could be observed in adrenalectomized rats (Figure 53). Minimal degenerative fatty infiltration could be detected in the degenerating areas. In similarly treated intact animals, central necrosis and mid-zonal fatty accumulation was observed. Figure 54 illustrates the greatest amount of hepatic change observed in this group of animals. The hepatic changes in this group of animals were minimal. No disruption of hepatic architecture was observed in three of the animals. However, a loss of basophilia was observed in cells surrounding the central vein.

When adrenalectomized animals were exposed to carbon tetrachloride eight hours after ethanol ingestion, early central

lobular necrosis with acute inflammatory infiltration can be observed. Minimal fatty accumulation could be observed in degenerating areas (Figure 55). In similarly treated intact animals, hydropic degeneration, necrosis, and inflammatory infiltrate were observed (Figure 56). Moderate fatty infiltration in para-central areas were evident. The extent of morphological changes were qualitatively more severe than those illustrated in Figure 52.

Progressive pathological hepatic changes were observed in adrenalectomized animals which were given ethanol twelve hours prior to carbon tetrachloride exposure. As illustrated in Figure 57, central lobular necrosis with infiltration of inflammatory cells was evident. Degenerative fatty infiltration in cells adjacent to necrotic foci was observed. The pathological changes observed in these animals were more severe than those which received simultaneous administration of ethanol and carbon tetrachloride. (See Figure 51). Similarly, intact animals given the same treatment showed central lobular necrosis and fatty atrophy in central areas and prominent fatty accumulation in mid-zonal areas (Figure 52).

The pathological changes observed in intact and adrenalectomized animals exposed to 1000 ppm carbon tetrachloride

Figure 51

Liver from an adrenalectomized rat given an oral dose of 5.0 ml/kg ethanol immediately preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Necrosis of individual and small groups of cells may be detected. Cells in the central lobular regions appear somewhat less eosinophilic and slightly larger than neighboring cells. Very minimal degenerative fatty infiltration can be observed.

Figure 52

Liver from an intact rat given an oral dose of 5.0 ml/kg ethanol immediately preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Moderate para-central degenerative fatty infiltration and focal necrosis may be detected. Hydropic degeneration may be observed in central areas.

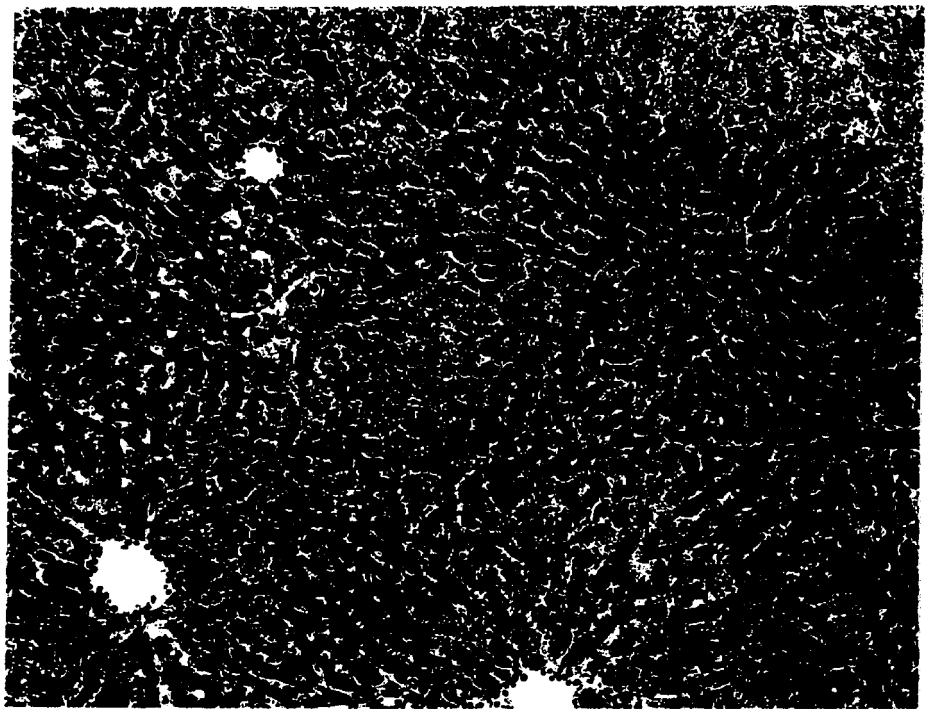


Figure 51

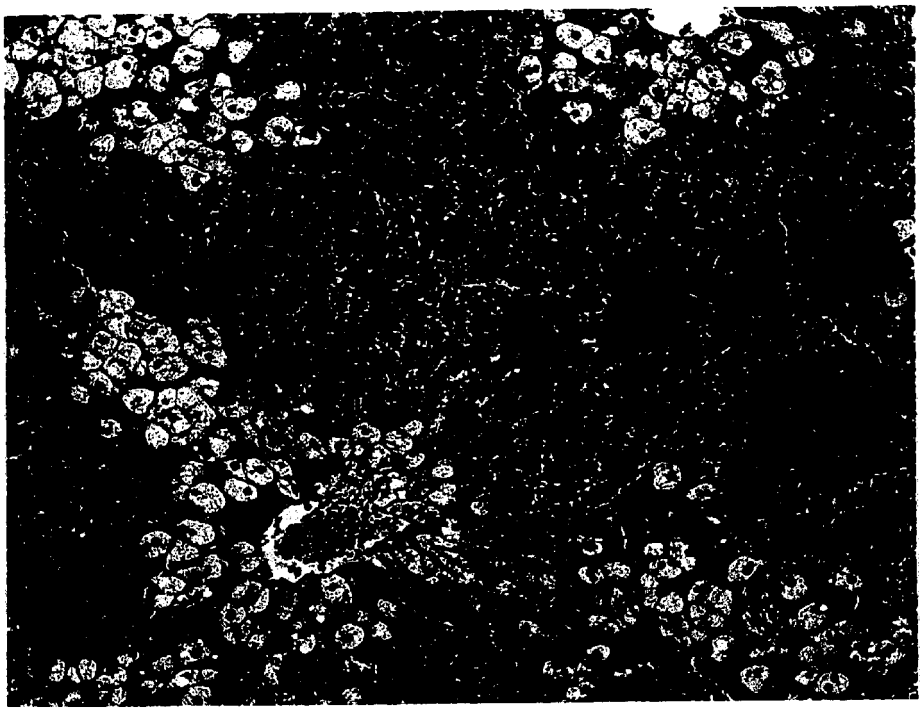


Figure 52

Figure 53

Liver from an adrenalectomized rat given an oral dose of 5.0 ml/kg ethanol four hours preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Necrosis of small groups of cells with round cell infiltration in relation to these cells can be observed. Degenerative changes are most marked in the central lobular areas. Minimal degenerative fatty infiltration can be detected in the degenerating area.

Figure 54

Liver from an intact rat given an oral dose of 5.0 ml/kg ethanol four hours preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

The liver exhibits paracentral fatty infiltration. Necrosis may be observed in groups of cells surrounding the central vein. This is characteristic of the most severe hepatic damage observed in this group of animals.

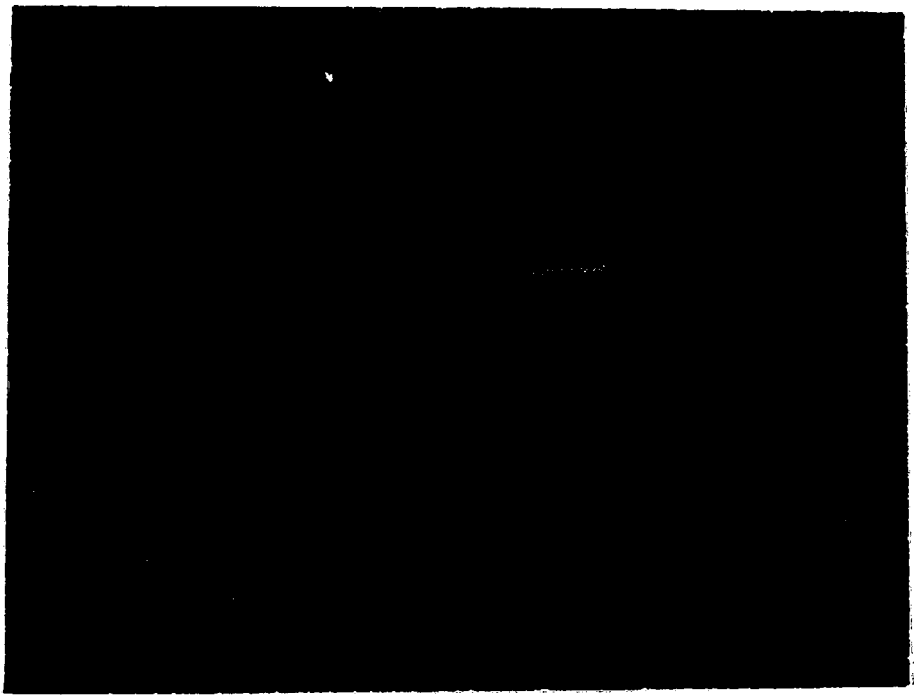


Figure 53

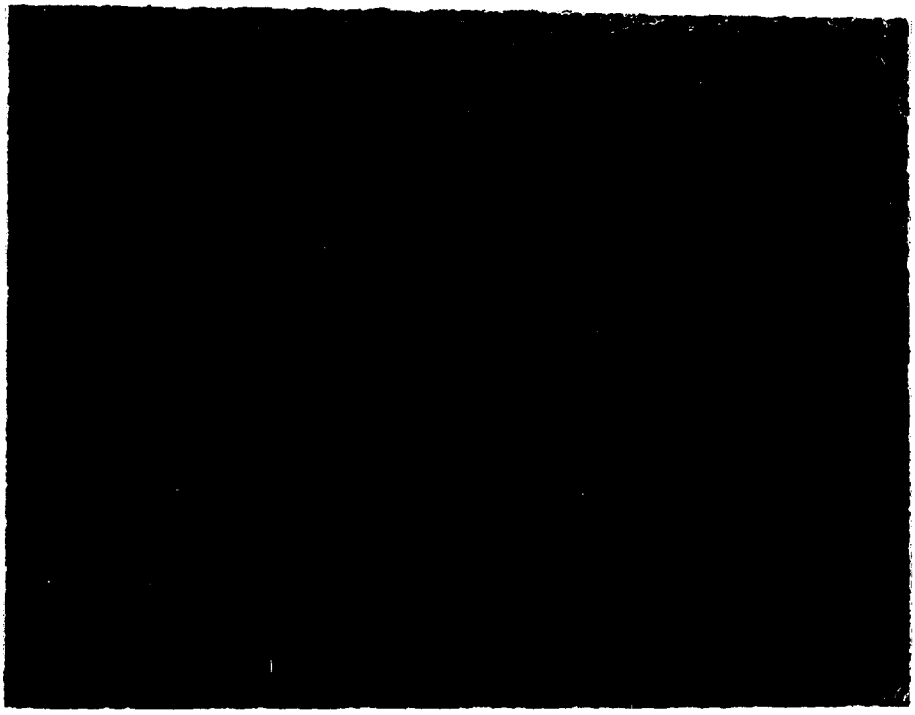


Figure 54

Figure 55

Liver from an adrenalectomized rat given an oral dose of 5.0 ml/kg ethanol eight hours preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Early central lobular necrosis and minimal fatty infiltration can be observed. Acute inflammatory cellular infiltrates can be detected.

Figure 56

Liver from an intact rat given an oral dose of 5.0 ml/kg ethanol eight hours preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Focal early central necrosis and inflammatory infiltrate can be observed. Hydropic degeneration and moderate fatty infiltration in para-central areas is evident.

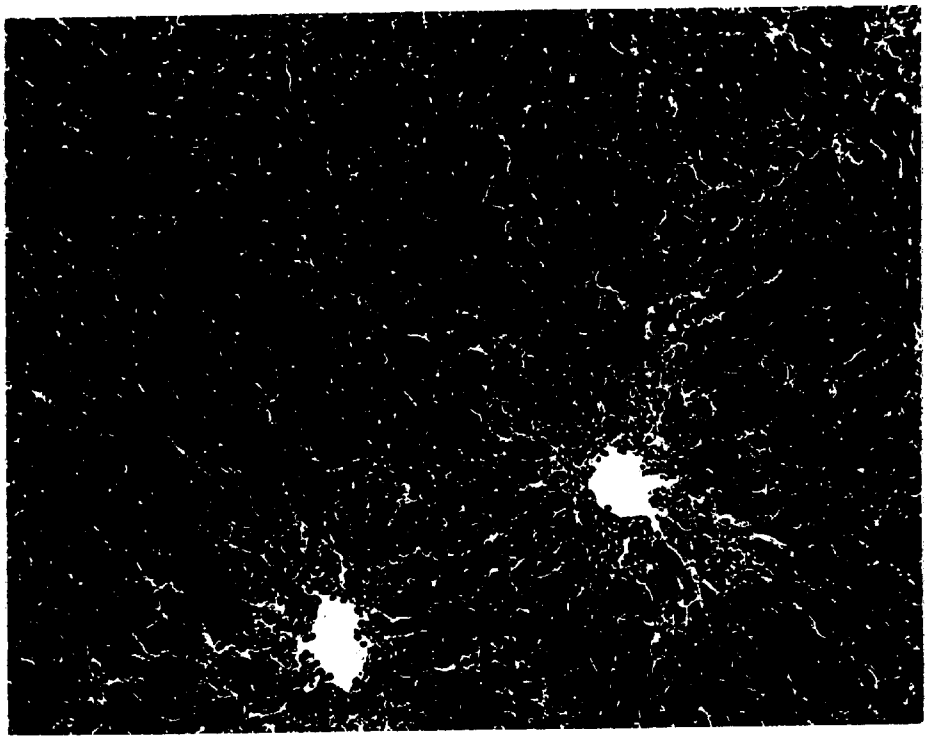


Figure 55

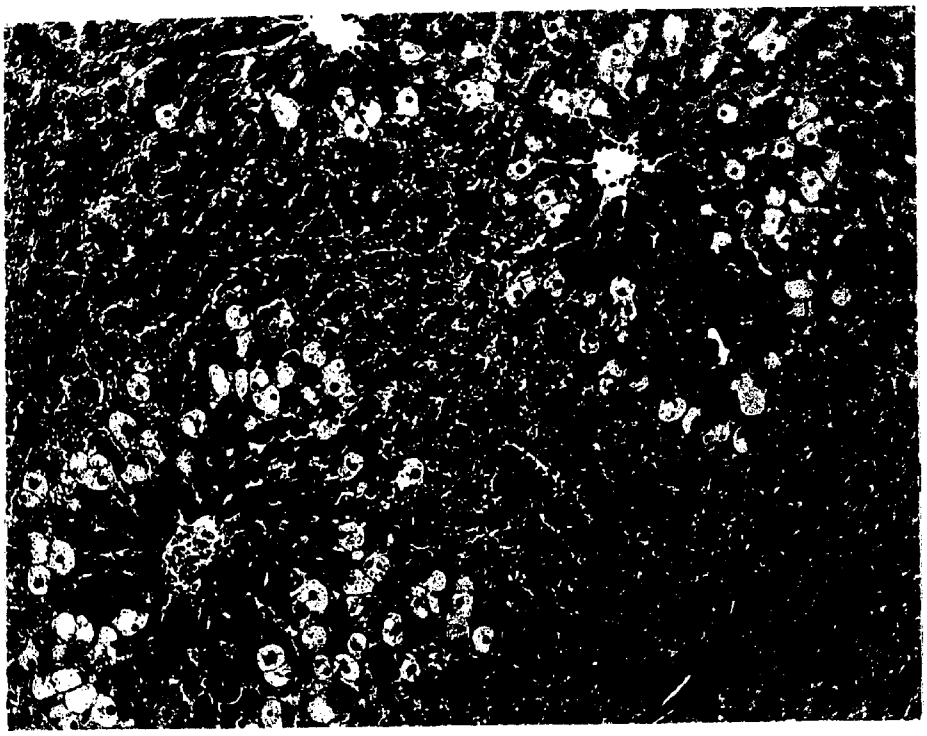


Figure 56

Figure 57

Liver from an adrenalectomized rat given an oral dose of 5.0 ml/kg ethanol twelve hours preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Central lobular necrosis with infiltration of inflammatory cells is noted. Degenerative fatty infiltration in cells adjacent to necrotic foci is evident.

Figure 58

Liver from an intact rat given an oral dose of 5.0 ml/kg ethanol twelve hours preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

The liver exhibits central lobular necrosis and fatty atrophy. Fatty accumulation is prominent in mid-zonal areas. A diffuse inflammatory cell infiltrate is present.

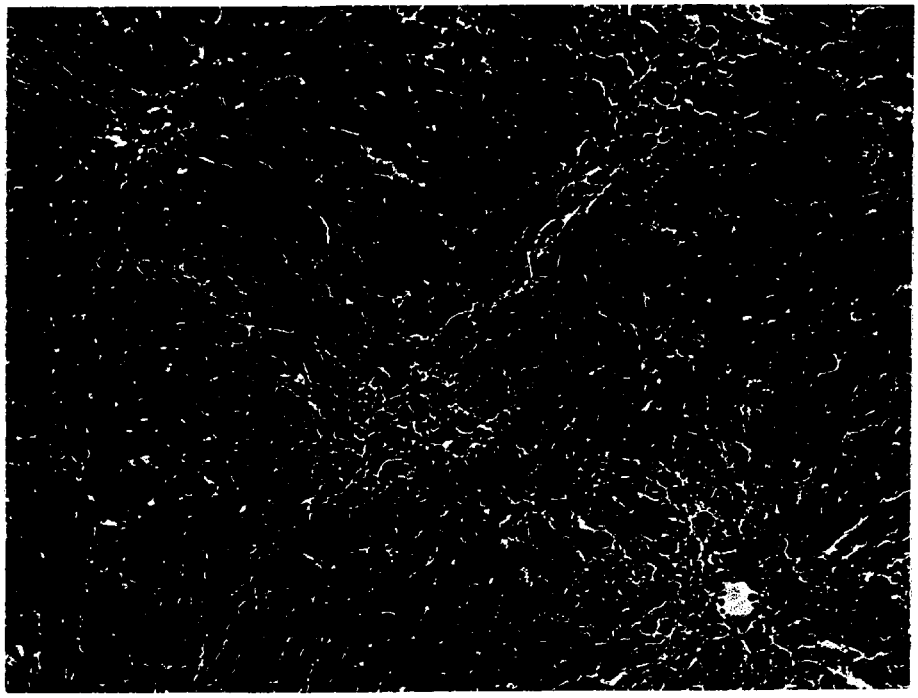


Figure 57

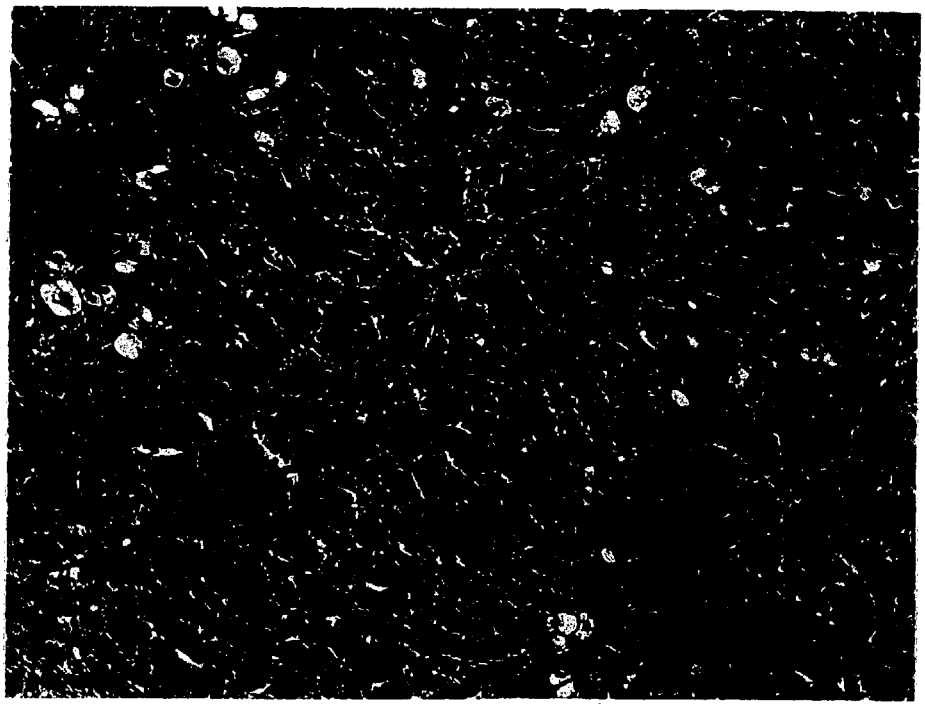


Figure 58

Figure 59

Liver from an adrenalectomized rat given an oral dose of 5.0 ml/kg ethanol twenty four hours preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

Hydropic degeneration and foci of necrosis are observed in the central lobular area. No lipidic infiltration is noted.

Figure 60

Liver from an intact rat given an oral dose of 5.0 ml/kg ethanol twenty four hours preceding exposure to 1000 ppm carbon tetrachloride for two hours. The animal was sacrificed twenty hours after exposure. H & E, 145X.

The liver exhibits marked degenerative fatty infiltration, most prominent in the central lobular area. Inflammatory cell infiltrate is present and hydropic degeneration is noted.

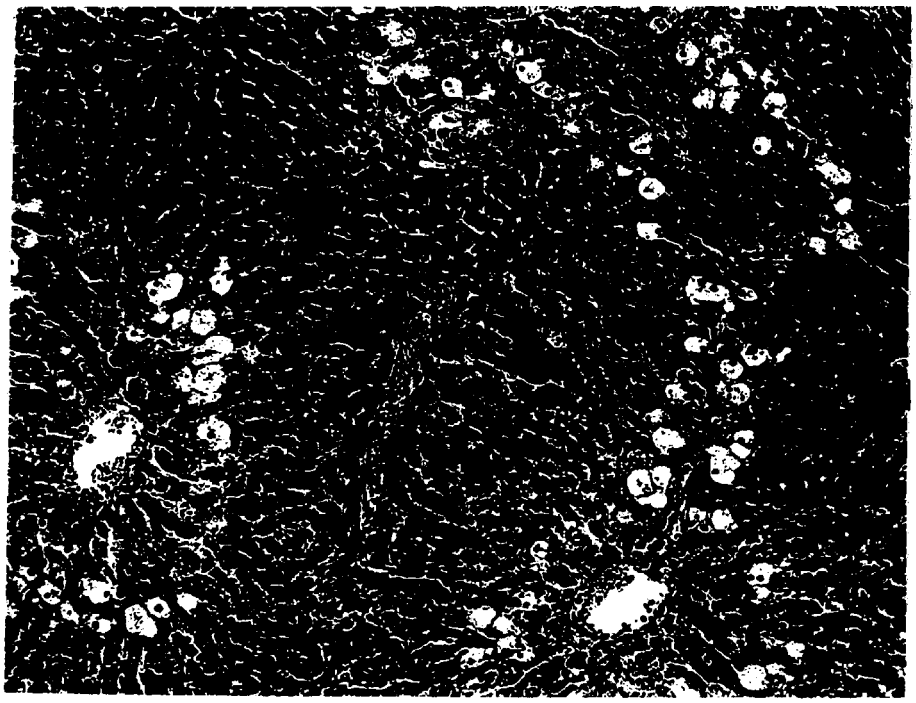


Figure 59

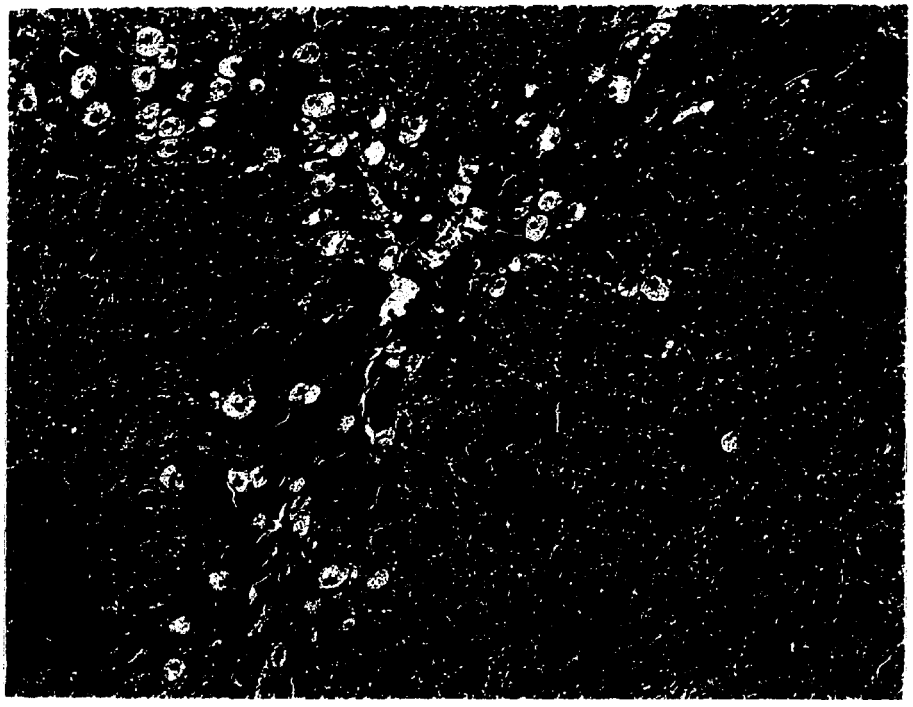


Figure 60

eighteen hours following ethanol ingestion have been described earlier (See Figures 10 and 49). Central lobular necrosis was observed in both intact and adrenalectomized animals. Qualitative estimation of pathological changes showed the greatest extent of morphological changes in animals given ethanol twelve to eighteen hours prior to exposure.

When a twenty four hour time interval elapsed between ethanol ingestion and carbon tetrachloride exposure, pathological hepatic changes were observed in adrenalectomized animals (twenty hours after exposure). Hydropic degeneration and foci of necrosis were observed in the central hepatic areas. No lipidic infiltration, however, was observed (Figure 59). Similarly treated intact animals showed marked degenerative fatty infiltration which was most prominent in the central lobular area (Figure 60). Inflammatory cell infiltrates and hydropic degeneration were noted.

DISCUSSION

The efflux of enzymes in response to carbon tetrachloride exposure as a function of time after ethanol administration coincides to some degree with the development of lipid accumulation due to the alcohol. Serum enzyme levels are elevated over the same time interval, approximately 6 to 20

hours, that ethanol induced fatty accumulation of the liver can be observed. However, liver lipid accumulation peaks at about 9 hours, while the intensity of the serum enzyme response reaches a maximum when carbon tetrachloride exposure occurs 16 to 18 hours after ethanol ingestion. Although adrenalectomized animals did not develop fatty liver following ethanol administration, the marked serum enzyme response of these animals to carbon tetrachloride was similar to the response of the intact ethanol treated animals given carbon tetrachloride. The elevated enzyme response was observed in adrenalectomized animals given this dose of carbon tetrachloride eight to eighteen hours after administration of the alcohol. During this time period, no increase in liver lipid was observed in adrenalectomized animals given this dose of ethanol. At the time of exposure to carbon tetrachloride, all adrenalectomized animals pretreated with ethanol showed normal liver lipid values. Examination of liver sections stained for lipid also failed to reveal the presence of increased lipid droplets within the parenchyma. These observations would indicate that the increased serum enzyme levels measured after ethanol-carbon tetrachloride administration, 1) occurred during the period

of ethanol-induced fatty accumulation of the liver, 2) but were not dependent upon the presence of the lipid. Bilateral adrenalectomy was effective in preventing the fatty accumulation of the liver following exposure to carbon tetrachloride. Stern and Brody (170) have reported similar results that adrenalectomy inhibited liver lipid accumulation following a large oral dose of carbon tetrachloride. Mallov (109, 110) suggested that increased lipid metabolism may be effective in preventing ethanol induced fatty liver in the adrenalectomized rat. Analysis of total liver lipid in ethanol treated animals twenty hours after exposure to carbon tetrachloride showed no abnormal increase in lipid values. The slight decrease of total liver lipid in animals when ethanol-carbon tetrachloride administration was separated by a time interval of 18 or 24 hours, may be explained by differences in dietary uptake. Administration of this dose of ethanol or carbon tetrachloride causes a loss of appetite. When carbon tetrachloride was administered 18 or 24 hours after this intoxicating dose of ethanol, the period of stress was longer than in those groups of animals which received the combination of agents at a shorter time interval. This longer time interval of depressed appetite may account for the slightly decreased liver lipid levels in

the last two groups of animals.

When the time interval between ethanol-carbon tetrachloride administration was short, 0-4 hours, serum enzyme activity was not greatly elevated twenty hours after exposure. (All animals were sacrificed twenty hours after administration of carbon tetrachloride to measure comparable serum enzyme activity changes at this time). This apparent latent period was observed in both intact and adrenalectomized animals. During this period, a potentiation index of 0.8 to 2.4 was measured. To determine whether alcohol intoxication would depress respiration sufficiently to decrease the apparent dose of inhaled carbon tetrachloride, respiration rate, heart rate, and EKG were measured. Respiration rate was not significantly altered following this dose of ethanol and does not suggest marked decrease in inhalation of carbon tetrachloride. When the time interval of ethanol-carbon tetrachloride exposure was increased to eight hours, marked serum enzyme elevation was observed in both adrenalectomized and intact animals. When the time interval between administration of the agents was increased to twenty-four hours, the serum enzyme changes were minimal. Apparently the effect of a single ingestion of ethanol on the enzyme response following carbon tetrachloride

exposure does not extend over a much greater period than 24 hours.

The mechanism involved in this latent period, between ethanol ingestion and carbon tetrachloride exposure, suggests many interesting possibilities for further investigation. Measurement of internal body temperature following intubation of this dose of ethanol showed a slight decrease within three hours of ingestion of the alcohol. Some evidence exists that decreased body temperature is associated with decreased toxic response to carbon tetrachloride. Larson and Plaa (95) lowered body temperature of rats by immersion in ice water and maintained this decreased temperature in a thermostatic bath. Following administration of carbon tetrachloride, fatty infiltration and necrosis of the liver were markedly inhibited. During this period of hypothermia, oxygen consumption was reduced by 50% of the normal value. These investigators suggested an inhibition of degenerative processes induced by carbon tetrachloride by the decreased body temperature and accompanying decrease in metabolic activity.

Brody and co-workers (19, 20, 26, 27, 170) reported protection against hepatotoxic effects produced by carbon

tetrachloride by administration of sympathetic blocking agents and cord section. These treatments also result in a decrease in temperature which may be associated with the protective effects observed. Calvert and Brody (20, 26) reported an increase in hepatotoxic effects of carbon tetrachloride in rats treated with thyroxin. The increased body temperature and metabolic demands of the hepatocytes may have contributed to the results observed.

When animals were exposed to carbon tetrachloride shortly after dosage with ethanol, the alcohol induced hypothermia may have prevented some hepatotoxic changes. Minimal increases in serum enzyme activity were noted when the time interval between alcohol-carbon tetrachloride administration was short. The rapid excretion of carbon tetrachloride at this low dosage may have reduced the amount of carbon tetrachloride to a level which did not produce extensive necrosis when the hypothermic period elapsed. This small decrease in temperature however was not comparable to the severe lowering of body temperature reported by Larson and Plaa. If the ethanol induced hypothermia exerted some pro-

tective effect against carbon tetrachloride damage, this action was accomplished by only a slight transient decrease in temperature. The decrease in temperature induced by ethanol did not extend beyond the latent period observed in serum enzyme measurements. The presence of alcohol in the body at the time of carbon tetrachloride exposure did not cause an increased serum enzyme activity. The simultaneous administration of alcohol and carbon tetrachloride was not necessary to produce the elevated serum enzyme activity indicative of increased tissue damage. In fact, the highest serum enzyme activity was observed only when the greater part of the alcohol had been metabolized before carbon tetrachloride administration. This observation was not compatible with the hypothesis of Stewart et al (171) with respect to the potentiation of toxicity of carbon tetrachloride by ethanol. These authors suggested that the increased toxicity was provoked by increased hydrocarbon solubility caused by the presence of alcohol in the tissue.

From histopathological examination of liver, measurement of serum enzyme activity and serum iron levels, the greatest tissue damage occurred after the major portion of the ethanol dosage had been metabolized. The more severe histological changes were observed when ethanol was administered

eight to eighteen hours prior to carbon tetrachloride exposure, in both intact and adrenalectomized animals. Although adrenalectomy did not prevent necrosis following carbon tetrachloride administration, it effectively inhibited fatty changes occurring after ethanol or carbon tetrachloride administration. These results are not compatible with the statement by Browning (22) in her recent review of carbon tetrachloride. This author states that adrenalectomy, similar to Phenergan, prevents necrosis, but not fatty changes of the liver produced by carbon tetrachloride. However, these results confirm the findings of Mallov (109, 110) and Brody and co-workers. Brody, Calvert and Schneider (20) reported that adrenalectomy prevented the fatty accumulation, but not necrosis, following carbon tetrachloride exposure. These authors noted only minimal protective effects by adrenalectomy in the extent of necrosis produced by the organic halide (++++ in intact animals vs. +++ in adrenalectomized animals given the same dose of carbon tetrachloride). Similar results may be observed by comparison of similarly treated intact and adrenalectomized animals. The hepatic changes observed in Figure 35 were somewhat less severe than those observed in Figure 36, when ethanol and carbon tetrachloride were given simultaneously. When ethanol was given twelve or eighteen hours prior to carbon tetrachloride exposure, differences in the extent of the necrotic

changes cannot be observed between intact and adrenalectomized animals.

This observation could be related to the mediation of catacholamines in the toxicity of carbon tetrachloride, as suggested by Brody and co-workers. Abelin (1), Perman (127-129), and Wartburg (181) have demonstrated an increase in catecholamine excretion in rats following a single acute dose of ethanol. This increased excretion (200% increase in epenephrine) was observed twenty four hours after ethanol ingestion. Since a relationship between catecholamine excretion and anoxic damage to hepatic parenchyma following carbon tetrachloride exposure has been demonstrated by Brody and co-workers, increased hepatic damage could be expected in intact rats treated with ethanol and carbon tetrachloride.

In addition, slightly depressed body temperatures have been measured in a number of adrenalectomized rats. A depressed body temperature could effect the toxic action of carbon tetrachloride, as mentioned earlier. Mallov (109, 110) has suggested that the inhibition of hepatic steatosis by adrenalectomy may be due in part to an increased hepatic lipid oxidation. Since reduced pyridine nucleotides are produced in fatty acid oxidation, these cellular constitutents may play a role in the

mediation of the toxic response to carbon tetrachloride. As Gibb (55) has demonstrated, hepatic levels of pyridine nucleotides may play an important part in the toxicity of carbon tetrachloride. A mechanism, such as increased fatty acid oxidation, which increases the availability of reduced pyridine nucleotides, may be effective in preventing imbalances which are incompatible with normal function.

When twenty four hours elapsed between administration of ethanol and carbon tetrachloride, serum enzyme activity approached normal values. The potentiation index for this time interval was 3.2 and 1.5 for adrenalectomized and intact rats, respectively. The residual effect of a single oral dose of ethanol after this period of time apparently did not exert a profound effect upon the serum enzyme levels. The greatest effect of a single oral dose of ethanol upon carbon tetrachloride toxicity was observed twelve to eighteen hours following ingestion of the alcohol.

Several mechanisms have been postulated for the potentiating action of ethanol upon halogenated hydrocarbon solvent toxicity. The hypothesis that ethanol increases the solubility of solvent in the blood stream is untenable since potentiation does not occur shortly after ethanol ingestion when blood levels are highest. The more recent hypothesis that ethanol induced fatty liver allows for tissue accumulation of carbon tetrachloride also

appears untenable in view of the present data. In the adrenalectomized rat, ethanol potentiation of carbon tetrachloride toxicity still occurred even though liver lipids were not elevated in these animals.

V-C -- BIOLOGICAL RESPONSE TO CARBON TETRACHLORIDE AND ETHANOL WHEN HEPATIC STEATOSIS WAS INHIBITED BY CORDOTOMY

Recently, Reboucas and Isselbacher (132) and Levy and Ramey (99) have shown that spinal transection, which interferes with the mobilization of fatty acids from adipose tissue was effective in preventing alcohol induced fatty liver. This surgical manipulation was employed to investigate the effect of alcohol potentiation of carbon tetrachloride. If ethanol induced fatty liver is causally related to the increase in carbon tetrachloride toxicity, prevention of the fatty accumulation in liver by cordotomy should exert a protective effect and diminish the toxic action. If, however, the increased toxicity of the hydrocarbon is not due to its increased solubility in the fatty liver, the usual increased toxic response would be observed in ethanol-carbon tetrachloride treated rats with or without cordotomy. Brody and co-workers (19, 170) have demonstrated the effectiveness of cordotomy in diminishing the toxicity of carbon tetrachloride itself. Following administration of carbon tetrachloride to cord sectioned rats, fatty accumulation and necrosis of the liver were greatly reduced. These workers postulated that carbon tetrachloride does not exert its toxic action directly upon the liver parenchyma, but rather, induces a massive adrenergic discharge which causes decreased hepatic blood flow. The resulting liver damage is associated with hepatic anoxia, mediated by the sympathetic nervous system. The protective effect

of cord section is apparently related to the prevention of this adrenergic discharge following administration of the hydrocarbon. A preliminary study was made to determine the biological response to carbon tetrachloride and ethanol when hepatic steatosis was inhibited by cord section.

PROCEDURE

Male rats, 200-250 grams of the Sprague-Dawley strain were used in this experiment. Spinal transection at the C-7 level was performed under ether anesthesia. Animals were allowed to recover from surgical stress for one day before administration of alcohol. Animals were maintained in a heated exposure chamber at 88-92°F and periodic intraperitoneal or subcutaneous injections of physiological saline were given to prevent dehydration. Bladders of the rats were emptied by pressure at short (3 hours) intervals. Animals were given crushed Rockland Rat Chow pellets ad libitum. Ethanol was administered by intubation at a dose of 5 ml/kg B.W. as a 50% aqueous solution to a group of the animals. Eighteen hours following administration of the ethanol, groups of the cord sectioned animals were exposed to 2000 ppm carbon tetrachloride for four hours. Twenty hours following the carbon tetrachloride exposure, animals were sacrificed and blood was collected for analysis of serum enzyme activity. Sections of organs were reserved for histopathological examination. Liver lipid levels were determined as described in Section II.

RESULTS

Liver lipid levels following administration of ethanol, carbon tetrachloride, or their combination, were not significantly elevated above values of untreated cord sectioned animals (Table 31). The rise in total liver lipid associated with administration of ethanol or carbon tetrachloride was effectively inhibited by spinal transection of the animal.

Serum enzyme activity of glutamic oxaloacetic transaminase, isocitric dehydrogenase, and glutamic pyruvic transaminase of cord sectioned control rats (Table 32) was similar to that of intact normal animals. Cord section did not significantly alter the serum enzyme levels measured. Serum enzyme levels of cord sectioned rats dosed with ethanol were comparable to those of similarly treated intact animals and to the cord sectioned controls. Twenty hours after exposure to 2000 ppm carbon tetrachloride for four hours, cord sectioned animals exhibited levels of serum GOT, GPT, and ICD that were elevated 5.3, 5.8, and 3.8 times that of control values, respectively. In contrast, animals pretreated with ethanol eighteen hours prior to exposure to this concentration of carbon tetrachloride exhibited serum enzyme levels of GOT, GPT, and ICD which were elevated 7.7, 12.3, and 10.0 times

TABLE 31

TOTAL LIVER LIPID IN CORD SECTIONED RATS TREATED WITH CARBON TETRACHLORIDE AND/OR ETHANOL

Treatment	Percent Total Liver Lipid (± SE)
EC	4.36 ± 0.07
C	4.23 ± 0.21
E	4.05 ± 0.27
Control	4.35 ± 0.02

All animals cord sectioned. Four animals per group.

EC -- 5 ml/kg ethanol 18 hours prior to CCl_4 administration.

C -- 2000 ppm CCl_4 for four hours.

E -- 5 ml/kg ethanol, 18 hours prior to sacrifice.

TABLE 32

SERUM ENZYME ACTIVITY IN CORDOTOMIZED ANIMALS TREATED WITH CARBON TETRACHLORIDE AND/OR ETHANOL

	Serum Enzyme Activity (± SE)			Potentiation Index		
Treatment	S-GOT	S-GPT	S-ICD	GOT	GPT	ICD
EC	2377 ± 125[b]	515 ± 103	6216 ± 130[b]	7.7[b]	12.3	10.0[b]
C	1654 ± 35[a]	246 ± 86[a]	2325 ± 75[a]	5.3[a]	5.8[a]	3.8[a]
E	210 ± 10	36.5 ± 3.5	550 ± 50	0.7	0.9	0.9
Control	310 ± 30	42.5 ± 7.5	620 ± 60	1.0	1.0	1.0

All animals cord sectioned at C-7 level. Four animals per group.

EC -- 5 ml/kg ethanol 18 hours prior to CCl_4 exposure.

C -- 2000 ppm CCl_4 for four hours.

E -- 5 ml/kg ethanol 18 hours prior to sacrifice.

[a] $P < 0.05$ versus normal.

[b] $P < 0.05$ versus C.

Figure 61.

Liver from a cord sectioned rat given 5.0 ml/kg ethanol eighteen hours prior to exposure to 2000 ppm carbon tetrachloride for four hours. Animal was sacrificed twenty hours after exposure. H & E, 145X.

Minimal focal cloudy swelling. No accumulation of hepatic lipid is evident. Degenerative cytoplasmic changes and congestion are present.

Figure 62

Liver from a cord sectioned rat exposed to 2000 ppm carbon tetrachloride for four hours. Animal was sacrificed twenty hours after exposure. H & E, 145X.

Focal cloudy swelling and congestion can be observed. Degenerative changes are present.

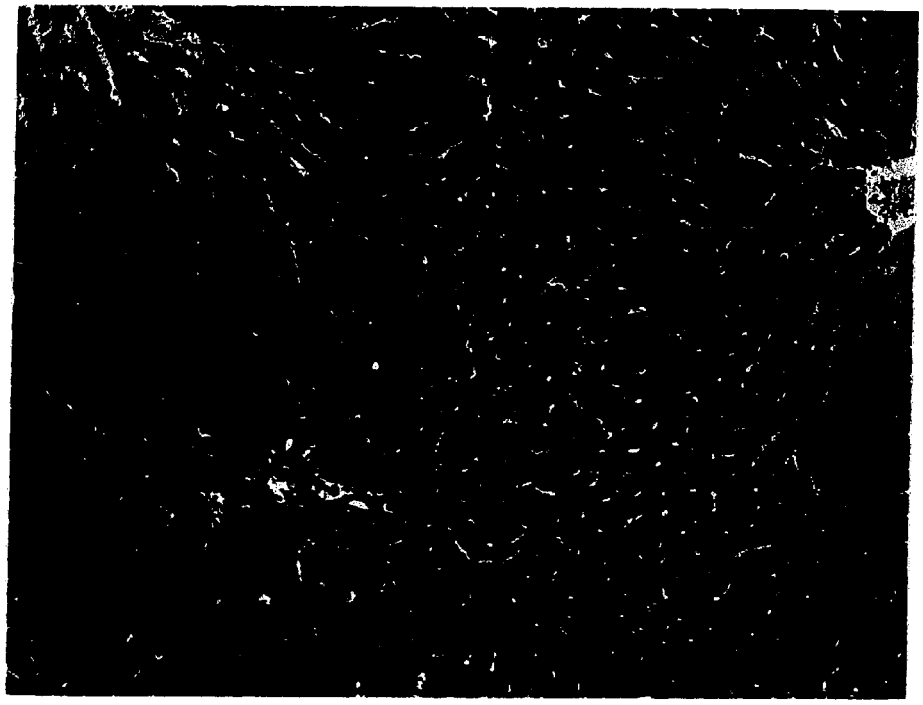


Figure 61

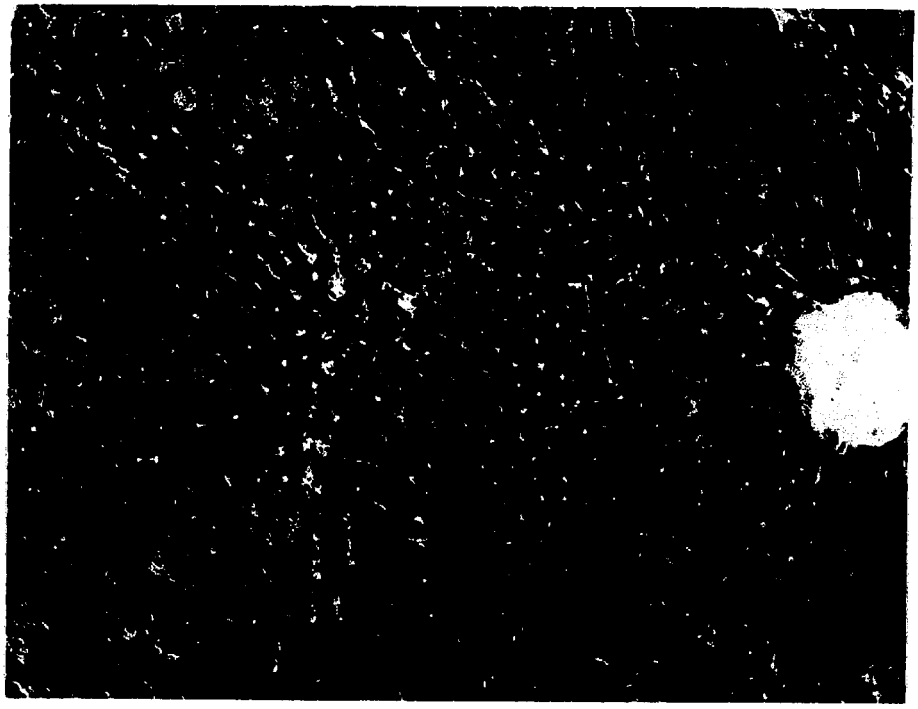


Figure 62

Figure 63

Liver from a cord sectioned rat given 5.0 ml/kg ethanol eighteen hours prior to sacrifice. H & E, 145X.

Hepatic architecture is intact. Degenerative changes can be observed. Little inflammatory infiltration is present. No accumulation of lipid is evident.

Figure 64

Liver from a rat cord sectioned at C-7 thirty eight hours prior to sacrifice.

The liver exhibits moderate congestion. Focal degenerative changes can be seen distributed throughout the lobule. Focal and periportal infiltration of inflammatory cells can be observed.

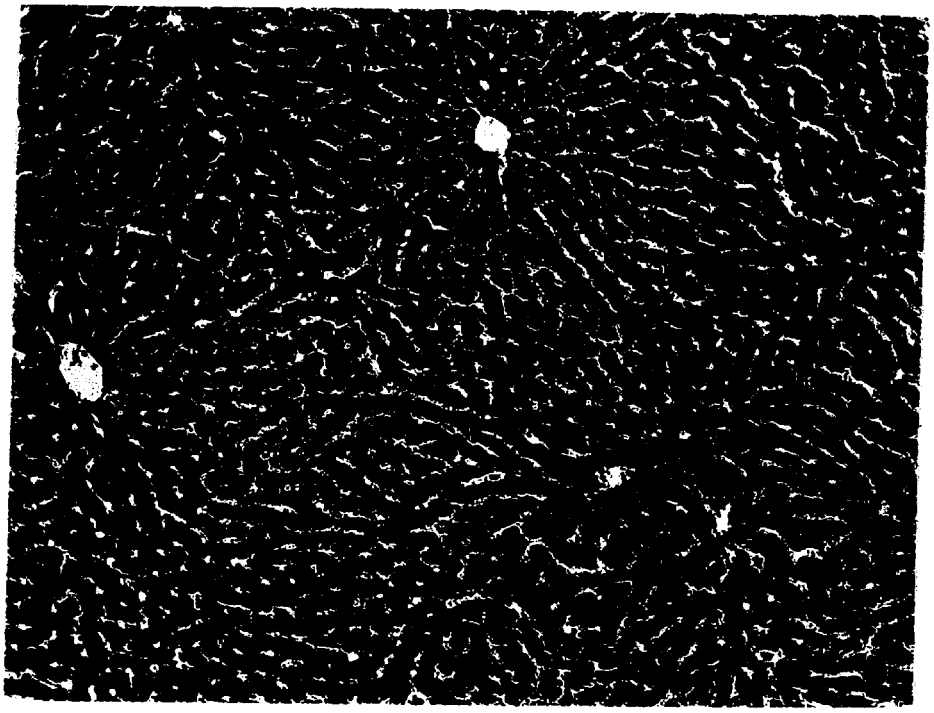


Figure 63

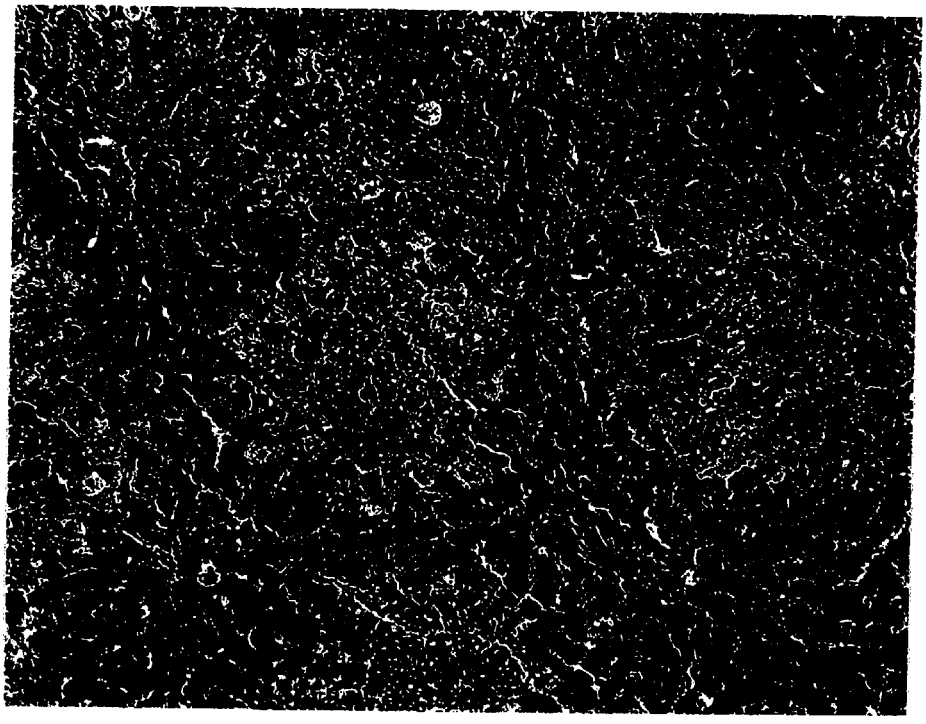


Figure 64

that of the control value. Although cord section inhibited the ethanol induced fatty liver, serum enzyme levels of GOT and ICD were significantly elevated in the alcohol pretreated animals.

Histopathological examination of liver stained with H & E and Oil Red O confirmed the results of liver lipid analysis. Fatty accumulation of the liver was not observed in cord sectioned animals treated with ethanol. Cord section exerted a marked protective effect upon the liver of carbon tetrachloride treated animals. No necrosis or fatty infiltration was observed following inhalation of this dose of carbon tetrachloride by the cord sectioned animals. A minimal focal cloudy swelling was the only generalized pathological change in the liver observed in all cord sectioned animals. (See Figures 61 through 64).

DISCUSSION

Analysis of total liver lipid demonstrated that when cordotomy was performed in the rat, ethanol induced fatty liver was prevented. This confirms the observation of Reboucas and Isselbacher (132) who noted a decrease in liver lipid content in similarly treated cordotomized rats. Although this ethanol induced fatty accumulation of the liver was inhibited, significant differences in potentiation index were observed between ethanol pretreated and untreated rats exposed to carbon tetrachloride. Animals pretreated with 5 ml/kg ethanol eighteen hours prior

to carbon tetrachloride exposure showed significantly greater increases in serum GOT and ICD than similarly treated animals which were not given ethanol. Although liver lipid levels of both groups of animals were not significantly different, the animals pretreated with ethanol showed serum enzyme values which were characteristic of greater tissue damage or alteration in cellular permeability.

The results of Brody and co-workers were confirmed in this study, fatty accumulation and necrosis were effectively inhibited following administration of carbon tetrachloride to the cord sectioned animal. Although the fatty infiltration of the liver following ethanol intoxication was prevented by this surgical manipulation, alcohol pretreated animals exhibited elevated serum enzyme levels following administration of carbon tetrachloride. It appears that the increased tissue damage caused by ethanol-carbon tetrachloride, is not causally related to the ethanol induced fatty liver produced by alcohol.

Serum enzyme levels in carbon tetrachloride exposed cordotomized animals were lower than those observed in intact animals exposed to similar concentrations of carbon tetrachloride.

This difference may be due to the prevention of a massive adrenergic discharge following carbon tetrachloride inhalation which results in vasoconstriction and anoxic changes in the liver, or it may be ascribed to impaired respiratory function of the cordotomized animal. Although animals were maintained in a heated chamber, the animals were exposed to carbon tetrachloride in a small exposure chamber at room temperature. Since body temperature has been related to the apparent toxicity of carbon tetrachloride, variation in body temperature could appreciably affect the response observed. Lewis and Page (102) have demonstrated marked changes in plasma free fatty acids in cord sectioned dogs subjected to variation in temperature. A decrease of 2°C in cordotomized dogs was accompanied by a 60% decrease in plasma free fatty acids and a concomitant decrease of 5-20% total serum protein. As suggested by von Oettingen, depressed plasma lipid levels could possibly have altered the absorption of an inhaled dose of carbon tetrachloride. Because of the generalized physical and biochemical changes which occur, cordotomy was not further employed in the investigation of hepatic steatosis and its relation to potentiation of carbon tetrachloride toxicity.

V-D -- BIOLOGICAL RESPONSE TO CARBON TETRACHLORIDE AND/OR ETHANOL IN HYPOTHERMIC RATS

INTRODUCTION

Recently, Larson and Plaa (95) have reported the effect of hypothermia upon the production of hepatic lesions by carbon tetrachloride. Their studies indicated that hypothermia in rats was effective in preventing necrosis and fatty changes following carbon tetrachloride administration. These authors suggested that the lowered metabolic rate produced by hypothermia, reduced the hepatotoxicity of carbon tetrachloride. Similarly, hypothermia has been shown to cause changes in serum free fatty acids and proteins (102). It would be pertinent, therefore, to investigate the biological response to carbon tetrachloride and ethanol in hypothermic animals. If the mechanism of potentiation is related to the metabolic activity following carbon tetrachloride administration, it would be of interest to observe if alcohol was capable of exacerbating carbon tetrachloride hepatotoxicity in hypothermic rats. A preliminary investigation was made to examine biological changes in hypothermic rats following carbon tetrachloride and/or ethanol administration.

PROCEDURE

Hypothermia was induced in male Sprague-Dawley rats, 175-200 gm, in the following manner. Animals were secured

in bottomless plastic infant feeding bottles and immersed up to the neck in an ice bath. Rectal temperature was monitored until depressed to 22-23°C (within 10-20 minutes). At this time, animals were removed from the bath and body temperature continued to decrease slightly to 20-21°C. Animals were then placed in a thermostatic bath maintained at 24°C, by a Beckman circulating thermostatic heater. Ethanol was administered one hour later at the rate of 5.0 ml/kg by oral intubation. Eighteen hours later a group of these animals was given an oral dose of 0.6 ml/kg carbon tetrachloride. This dose of carbon tetrachloride was sufficient to produce necrotic hepatic lesions in normal animals. Twenty hours after administration of carbon tetrachloride, animals were sacrificed and blood collected for determination of serum enzyme activity. Sections of heart, liver, lung, kidney, adrenal, and spleen were reserved for histopathological examination.

RESULTS

SERUM ENZYME ACTIVITY IN HYPOTHERMIC RATS TREATED WITH ETHANOL AND/OR CARBON TETRACHLORIDE

In the hypothermic rats, insufficient serum was collected to determine activity of ICD. However, values of S-GOT and S-GPT were markedly elevated in all hypothermic animals as compared to normal values (Table 33). No increase in serum enzyme activity

TABLE 33

SERUM ENZYME ACTIVITY IN HYPOTHERMIC RATS FOLLOWING ADMINISTRATION OF CARBON TETRACHLORIDE AND/OR ETHANOL

Treatment	Body Temperature At Sacrifice (°C)[1]	Serum Enzyme Activity (± SE) S-GOT	S-GPT	Potentiation Index S-GOT	S-GPT
T	22.5	2267 ± 130	104 ± 13	1.0	1.0
TE	22.3	1570 ± 236	109 ± 16	0.7	1.0
TC	22.3	1808 ± 256	141 ± 28	0.8	1.4
TCE	21.9	1705 ± 290	141 ± 33	0.8	1.4

T -- Hypothermia

TE -- 5.0 ml/kg ethanol eighteen hours prior to sacrifice

TC -- 0.6 ml/kg carbon tetrachloride, oral, twenty hours prior to sacrifice

TCE -- 5.0 ml/kg ethanol eighteen hours prior to carbon tetrachloride
Sacrificed twenty hours after carbon tetrachloride administration

[1]Mean of four animals

could be related to treatment with carbon tetrachloride and/or ethanol in the hypothermic rat.

HISTOLOGICAL STUDIES

Examination of liver sections of hypothermic rats revealed congestion, but no necrosis, after thirty eight hours of hypothermia (Figure 65). Hypothermic rats which were given an oral dose of 5.0 ml/kg ethanol eighteen hours prior to sacrifice showed no abnormalities of the liver. Fatty changes were minimal in the livers of these animals (Figure 66).

Twenty hours after the intubation of 0.6 ml/kg carbon tetrachloride, hypothermic rats exhibited minimal focal degenerative infiltration of the liver (Figure 67). In one animal, minimal focal vacuolar degeneration of proximal tubular epithelium was observed.

Hypothermic rats which received 5.0 ml/kg ethanol prior to carbon tetrachloride exhibited a moderate lipidic infiltration in the liver at the time of sacrifice (Figure 68). Hepatic architecture at this time, twenty hours after carbon tetrachloride administration, was intact.

Figure 65

Liver from a hypothermic rat after thirty eight hours of reduced body temperature. H & E, 145X.

Congestion, but no necrosis, can be observed. Hepatic architecture is intact.

Figure 66

Liver from a hypothermic rat given 5.0 ml/kg ethanol eighteen hours prior to sacrifice. H & E, 145X.

Hepatic architecture is intact. Minimal lipidic changes and congestion can be observed.



Figure 65

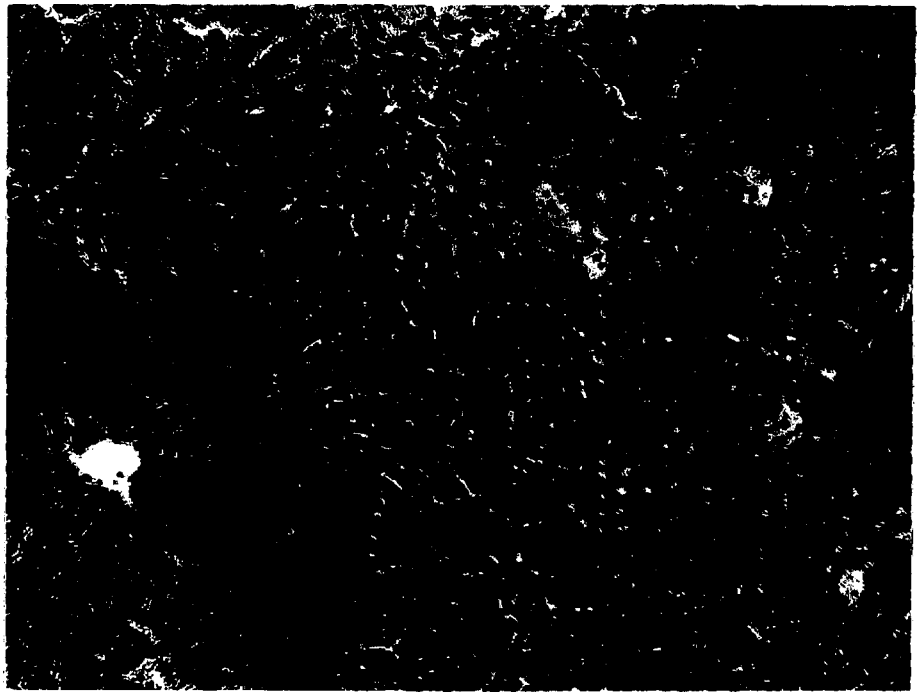


Figure 66

Figure 67

Liver from a hypothermic rat given 0.6 ml/kg carbon tetrachloride twenty hours prior to sacrifice. H & E, 145X.

Minimal focal degenerative infiltration can be observed. Hepatic architecture is intact.

Figure 68

Liver from a hypothermic rat given 5.0 ml/kg ethanol eighteen hours prior to administration of 0.6 ml/kg, oral, carbon tetrachloride. The animal was sacrificed twenty hours after carbon tetrachloride administration. H & E, 145X.

Moderate lipidic infiltration of the hepatic parenchyma can be observed. Hepatic architecture is intact.

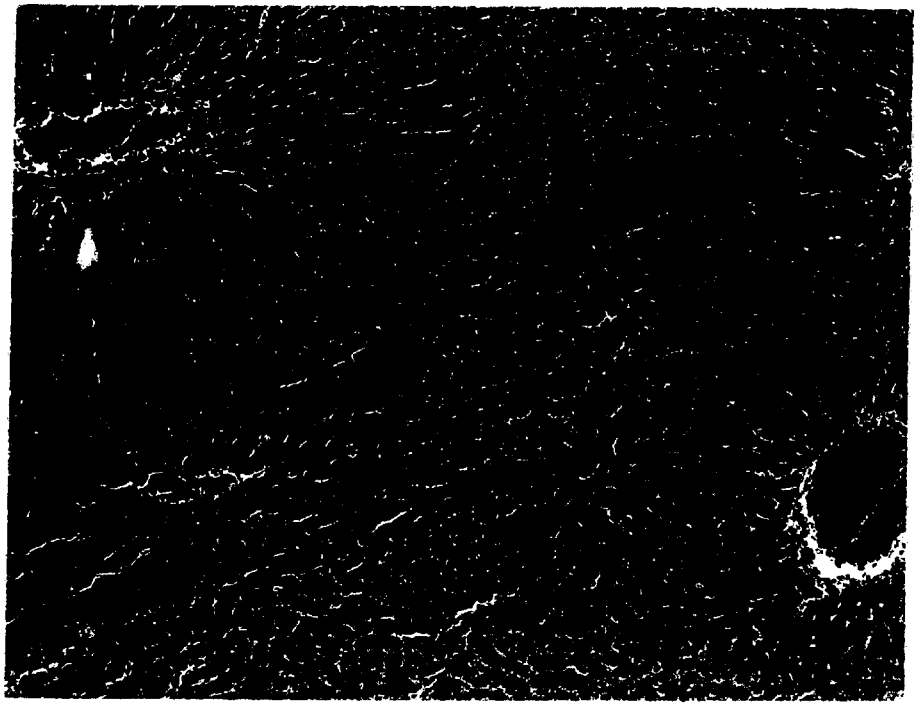


Figure 67



Figure 68

DISCUSSION

Elevation of serum enzyme activity in the hypothermic rat could not be ascribed to morphological changes produced by carbon tetrachloride and/or ethanol administration. No significant increase in serum enzyme activity was produced by these agents in comparison with control hypothermic animals. In relation to normothermic animals, serum GOT and GPT were significantly elevated in the hypothermic animals. This elevation in activity could be related to the anoxic changes and stress of hypothermia resulting in anaerobiosis. Zierler (190) demonstrated the loss of lactic dehydrogenase and aldolase from isolated rat diaphragm under conditions of depressed temperature. This efflux of enzyme into the incubating medium was increased by high potassium or insufficient glucose concentration, or such metabolic antagonists as nitrophenol or iodoacetate. Similarly, Wu (189) was able to demonstrate leakage of enzymes from ascites tumor cells under the influence of pH changes, nitrophenol, and glycolyzable substrates. It would be reasonable, therefore, to relate the increased serum enzyme activity observed to the decrease in metabolism and body temperature. Larson and Plaa (95) have shown that in such hypothermic rats, total body oxygen consumption was reduced by one half at these temperatures. As these authors stated, the lack of hepatic necrosis produced by carbon tetrachloride in hypothermic animals may be related to the decrease in metabolic activity. Similarly, Brody and co-workers (20, 26) have shown an increased hepatotoxicity of carbon tetrachloride in rats whose

metabolic rate was increased by thyroid hormones.

Butler (24) and Reynolds (139) and others (125) have suggested that carbon tetrachloride exerts its toxic effect by free radical products formed during its metabolism. These highly reactive free radicals would be capable of combining with protein or other cellular constituents to produce a toxic effect. As Butler stated, the C-Cl dipole moment is reduced with increased halogenation to lower the activation energy required for homolytic cleavage of carbon tetrachloride to form the free radical. That is to say, carbon tetrachloride is capable of producing a free radical with relative ease as compared to chloroform, for example (180). This relative ease of free radical formation of carbon tetrachloride could account for the identification of chloroform as one of its metabolites. Similarly, Butler was unable to show the in vivo production of dichloromethane following chloroform administration. Chloroform, which has a higher dipole moment than carbon tetrachloride, would be able to form analogous free radicals only with greater difficulty.

Free radicals in biological material have been demonstrated by Commoner and others (30). The highest free radical content was observed in metabolically active material such as green leaves, frog eggs, kidney, and liver. This observation suggests that carbon tetra-

chloride could react with endogenous free radicals in tissue with the formation of a chloride free radical. As pointed out by Wirtshafter (186) the transfer constant of carbon tetrachloride in alkene polymerization is favorable for the production of a free radical by reaction with existing species.

In spite of the greater bond strength of C-H bonds as compared to C-Cl bonds, the latter are preferentially cleaved by free radical reactions. This behavior can be explained by examination of the energy requirements for bond cleavage or formation. A coupling of these processes occurs in the reaction of free radicals so that the energy required to cleave a bond is influenced by the energy of bond formation. The energy of activation required for reaction is decreased by partial bond formation and any favorable electronic configurational interactions of the radical formed and the radical source. Similarly, the transfer constant for carbon tetrachloride is more favorable to free radical formation by this reaction than that of chloroform. This would account for the primary step of oxidation of carbon tetrachloride to carbon dioxide as observed by McCollister et al. (113). The chloride free radicals derived from the organic halide could react with sulfhydryl groups in essential cellular constituents. The unidentified urinary metabolite of carbon tetrachloride found

by McCollister could possibly be a mercapturic acid formed by such a mechanism. The alkylation of sulfhydryl compounds by organic halides has been demonstrated by several investigators. Lewis (100) has shown that methyl bromide, methylated the sulfhydryl groups of glutathione, cysteine, and sulfhydryl enzymes. Similarly, Bray (13, 14) has demonstrated a liberation of chloride ion from aliphatic chlorine compounds with concomitant decrease of free sulfhydryl groups in liver homogenates. Likewise, Butler (24) has demonstrated the in vitro reaction of carbon tetrachloride with cysteine, cytochrome C, and ascorbic acid. Deficiency of cysteine has been recognized as a factor leading to liver necrosis by Glynn and co-workers (57). This dietary deficiency of cysteine has been shown to produce greatly depressed hepatic levels of glutathione (Leaf and Neuberger, 97). In this respect, Binet et al (9) reported decreased hepatic levels of glutathione following chloroform administration to dogs and rabbits. However, Eden and Harrison (39) measured increased levels of glutathione in rats following carbon tetrachloride administration. These authors attributed the increase observed to regeneration of damaged liver. Other workers (40, 114) have suggested that conjugation of organic halides with methionine produces liver injury by depletion of available stores of this essential amino acid.

Following exposure to carbon tetrachloride, administration of methionine has been used to alleviate symptoms of intoxication in human cases (153). In experimental studies, Asagoe (5) and Kobayashi (86) have reported mollification of hepatic lipid accumulation, glycogen depletion, and collagen formation in rats treated with methionine following carbon tetrachloride administration. Similarly, glutathione (111) and thioctic acid (12, 106) have been investigated because of their protective action in liver disease.

In addition to the formation of the carbon-sulphur bond, carbon tetrachloride can react with other cellular constituents. If the chlorine released by free radical formation ultimately yields the chloride ion, two electrons must be obtained from cellular sources. Cysteine glutathione, cytochrome, and ascorbic acid have been shown by Butler (24) to furnish these electrons. Vitamin B_{12}, which has been studied by Vaisler (176), Kasbekar et al (83), and Bengmark and others (8), decreases in content in the liver following administration of carbon tetrachloride in the rat. This decrease in hepatic levels of vitamin B_{12} which is associated with neogenesis of methyl groups and transmethylation is accompanied by an impairment in the metabolism of sulfhydryl compounds in the liver. Administration of vitamin B_{12} prior to

carbon tetrachloride administration markedly alleviates the hepatic lesions produced as observed by these authors. This compound may exert its protective effect either by maintenance of mitochondrial integrity as suggested by Kasbekar et al (83) or it may serve as an acceptor for chloride free radicals. In addition, these authors noted decreased hepatic glutathione levels following carbon tetrachloride administration.

The protective action of antioxidants such as tocopherol acetate, sodium selenite, or n, n-diphenyl-p-phenylenediamine when given prior to carbon tetrachloride administration has been described by Gallagher (50). In a study by Tamara, 34% of the vitamin E content of the liver was lost within forty eight hours after administration of carbon tetrachloride. Because of their antioxidant properties, these compounds could react readily with free radical products of carbon tetrachloride metabolism. In this respect, Gallagher (50-53), Gibb (55), Slater et al (158) have demonstrated marked changes in pyridine nucleotide concentrations following carbon tetrachloride administration. The reduced forms of pyridine nucleotides act readily as hydrogen donors, and could reasonably react with chloride free radicals derived from carbon tetrachloride metabolism. This suggestion is compatible with the findings of Slater et al (158). Following the administration of carbon

tetrachloride, a rapid decrease in liver $NADPH_2$ was observed by these authors. This produced marked changes in the NADP: $NADPH_2$ ratio in rat liver. Similarly, Gibb (55) observed a marked protection of hepatic structure when total liver pyridine levels were elevated by nicotinamide prior to carbon tetrachloride administration. In thyrotoxic rats, this author noted a decrease in reduced pyridine nucleotides. Such animals have been shown to be more susceptible to hepatotoxic effects of carbon tetrachloride by Brody and co-workers.

Increased hepatotoxicity of carbon tetrachloride may be associated with decreased levels of pyridine nucleotides. In addition, it may be suggested that a deficit in triphosopyridine nucleotide could allow chloride free radicals to react with other cellular components which could lead to irreversible cellular changes. Or conversely, free radicals formed by carbon tetrachloride metabolism could deplete available pyridine nucleotides to a critical level leading to cell death. Alterations of oxidation reduction potentials by carbon tetrachloride could interfere with sulfhydryl enzymes by direct removal of electrons or by depletion of such compounds as glutathione or ascorbic acid.

Since the highest concentration of endogenous free radicals was found in actively metabolizing tissue, it is reasonable that

inhibition of metabolism could decrease the number of these species present. Kalant, Hawkins, and Watkin (82) have demonstrated that ethanol causes an increase in metabolic rate which was accounted for by the preferential oxidation of ethanol in place of its caloric equivalent of glucose. This report confirmed the findings of Perman (127-129) who noted an increase in oxygen consumption following administration of 0.4 gm/kg ethanol. The release of catecholamines was found to be independent of this observed metabolic increase. Ganglionic blockade with hexamethonium did not influence the increased oxygen consumption. This author suggested that acetaldehyde, formed in the metabolism of ethanol might be responsible for the calorigenic effect. Methanol and n-propanol, which do not produce acetaldehyde as a metabolite, did not cause this effect. Acetaldehyde, however, exhibited a transient effect similar to ethanol. With large doses of ethanol, a marked decrease in metabolic activity was noted which coincided with the onset of maximal narcotic effect of the ethanol. This depression of metabolic rate persisted for at least an hour after the administration of 4.0 gm/kg ethanol. It is of parenthetical interest to note that the amount of unmetabolized ethanol 10 hours after administration of 5.0 ml/kg, would be equivalent to the small dose which produced a stimulation of

metabolic rate. (Mean rate of ethanol disappearance, Nelson and Abbenhaus, 117). It can be suggested that an increase in metabolic rate could increase the quantity of endogenous free radicals, or the production of chloride free radicals produced by homolytic cleavage in carbon tetrachloride metabolism. By either of these mechanisms, the quantity of free radicals would be increased and available for reaction with essential cellular constituents.

Among the metabolic effects of ethanol, which lead to hepatic lipid accumulation, Lieber and Schmid (103) and Reboucas and Isselbacher (132) have demonstrated an increased fatty acid synthesis following ethanol ingestion. Similarly, a decreased fatty acid oxidation has been demonstrated following ethanol administration (103). It has been demonstrated by Wakil (179), Matthes (112), and Bloomfield (11) that $NADPH_2$ serves as the primary hydrogen donor in fatty acid synthesis and elongation of fatty acid chains. Conversely, oxidation of fatty acids, which results in the production of reduced pyridine nucleotides, is inhibited following ethanol administration. In confirmation of these findings, Slater, Sawyer and Strauli (159) reported a decrease in the $NADPH_2$:NADP ratio thirteen to twenty four hours after administration of small doses of ethanol. Although the depressed ratio was not examined critically by these authors, at these times, the depressed

ratios reported could reflect changes due to enhanced fatty acid synthesis or depressed oxidation.

If reduced pyridine nucleotides are utilized by increased fatty acid synthesis, or if inhibited fatty acid oxidation decreases their production, a deficit of these cellular constituents may occur at the time of carbon tetrachloride administration. If insufficient quantities of reduced pyridine nucleotides are available as electron donors, free radical products would be able to react directly with sulfhydryl enzymes, or other cellular constituents. Conversely, reaction of reduced pyridine nucleotides with free radical products of carbon tetrachloride could decrease their concentration to a critical level which would be incompatible with cellular integrity. This series of biochemical events involving free radical formation would serve to explain some of the relationships observed in the potentiation of toxicity of carbon tetrachloride as it relates to ethanol metabolism.

V - E -- BIOLOGICAL EFFECTS OF CARBON TETRACHLORIDE AND SORBITOL IN RATS

INTRODUCTION

In the investigation of the pathogenesis of fatty accumulation of the liver, Reboucas and Isselbacher (132) were able to differentiate the increased synthesis of fatty acids from hepatic steatosis induced by ethanol. Following the administration of sorbitol, an increase in fatty acid synthesis was observed similar to that produced by ethanol. However, despite increased synthesis, fatty accumulation of the liver was not produced following sorbitol administration. Isselbacher and Krane (76) reported a similar action of sorbitol and ethanol upon the inhibition of galactose oxidation. In addition, these authors noted a marked increase in liver $NADH_2$ after administration of sorbitol or ethanol. Because of similar metabolic effects of sorbitol and ethanol, it would be pertinent to examine the biological effects of carbon tetrachloride in animals which were treated with sorbitol.

PROCEDURE

Male Sprague Dawley rats, 200-225 gms were employed in this preliminary study. Eighteen hours after the oral administration of 9.0 or 18.0 gms/kg sorbitol, animals were exposed to 1000 or

2000 ppm carbon tetrachloride for two hours. Twenty hours after exposure, or thirty eight hours after sorbitol administration, animals were sacrificed and blood collected for determination of serum enzyme activity. Sections of liver were reserved for lipid determination.

RESULTS

HEPATIC LIPID CONTENT OF ANIMALS TREATED WITH CARBON TETRACHLORIDE AND/OR SORBITOL

Following the administration of sorbitol, a significant increase in total hepatic lipid was not observed (Tables 35 and 37). Animals, which were treated with sorbitol eighteen hours prior to exposure to carbon tetrachloride, showed moderate increases in total hepatic lipid. These increases were significant ($P<0.05$) twenty hours after exposure.

SERUM ENZYME ACTIVITY IN ANIMALS TREATED WITH CARBON TETRACHLORIDE AND/OR SORBITOL

Animals which were treated with 18.0 gm/kg sorbitol eighteen hours preceding exposure to 2000 ppm carbon tetrachloride for two hours showed marked increases in serum GOT, GPT, and ICD. Potentiation indexes of 30.0, 21.0, and 14.3 were observed in these animals (Table 34). In contrast, normal animals exposed to carbon

TABLE 34

SERUM ENZYME ACTIVITY IN RATS FOLLOWING CARBON TETRACHLORIDE AND/OR SORBITOL ADMINISTRATION

	SERUM ENZYME ACTIVITY (± SE)			POTENTIATION INDEX		
TREATMENT	S-GOT	S-GPT	S-ICD	S-GOT	S-GPT	S-ICD
S	239 ± 20	20 ± 6	555 ± 67	1.0	1.0	1.0
C	2,422 ± 900	159 ± 54	4,650 ± 2,199	10.0	8.0	8.1
SC	7,493 ± 1,349[a]	415 ± 50[a]	8,247 ± 1,273[a]	30.0[a]	21.0[a]	14.3[a]
Normal	248 ± 44	20 ± 1	575 ± 70	1.0	1.0	1.0

TABLE 35

HEPATIC LIPID LEVELS IN RATS TREATED WITH CARBON TETRACHLORIDE AND/OR SORBITOL

TREATMENT	PERCENT TOTAL LIPID (± SE)
S	4.71 ± 0.06
C	5.19 ± 0.23
SC	5.56 ± 0.10[a]
Normal	4.60 ± 0.14

S - 18 gms/kg Sorbitol, oral.

C - 2000 ppm carbon tetrachloride for two hours. Five animals per group. All animals sacrificed 20 hours after carbon tetrachloride, or 38 hours after sorbitol administration.

[a] $P < 0.05$ versus normal.

TABLE 36

SERUM ENZYME ACTIVITY IN RATS TREATED WITH CARBON TETRACHLORIDE AND/OR SORBITOL

	SERUM ENZYME ACTIVITY (± SE)		POTENTIATION INDEX	
TREATMENT	S-GOT	S-ICD	S-GOT	S-ICD
S	183 ± 26	639 ± 83	0.7	1.1
C	261 ± 70	850 ± 149	1.0	1.5
SC	818 ± 197[a]	1,381 ± 165[a]	3.3[a]	2.5[a]
Normal	248 ± 44	550 ± 123	1.0	1.0

TABLE 37

HEPATIC LIPID LEVELS IN RATS TREATED WITH CARBON TETRACHLORIDE AND/OR SORBITOL

TREATMENT	PERCENT TOTAL LIPID (± SE)
S	4.56 ± 0.32
C	4.80 ± 0.09
SC	7.09 ± 0.38[a]
Normal	4.60 ± 0.14

S - 9.0 gm/kg Sorbitol, oral

C - 1000 ppm carbon tetrachloride for two hours. Six animals per group. All animals sacrificed 20 hours after carbon tetrachloride or 18 hours after sorbitol administration.

[a] $P < 0.05$ versus normal.

tetrachloride had potentiation indexes of 10.0, 8.0, and 8.1 for the corresponding enzymes. The administration of sorbitol did not affect the serum enzyme levels measured thrity eight hours after dosage.

Because of the toxic symptoms of weight loss and severe diarrhea in animals fed 18 gms sorbitol/kg, the dose of sorbitol was decreased to 9.0 gms/kg, and the animals were exposed to 1000 ppm carbon tetrachloride for two hours. This dose of carbon tetrachloride was sufficient to produce marked morphological changes in ethanol pretreated animals.

Eighteen hours after administration of this dose of sorbitol, enzyme levels of serum GOT and ICD were within normal limits (Table 36). Similarly, animals exposed to carbon tetrachloride exhibited serum enzyme activity which was within normal limits twenty hours after exposure. Animals which were given 9.0 gms/kg sorbitol eighteen hours prior to exposure showed levels of serum GOT and ICD which were elevated 3.3 and 2.5 times the normal value. It can be noted, however, that the serum enzyme response was less than that observed in ethanol treated animals exposed to the same concentration of carbon tetrachloride. On a molar basis, this dose of sorbitol was equivalent to one-half the dose of ethanol administered in this study.

DISCUSSION

Sorbitol, which has several metabolic properties in common with ethanol, differs in its action by not producing fatty accumulation of the liver. Although increased fatty acid synthesis following sorbitol administration has been demonstrated by Reboucas and Isselbacher (132), hepatic steatosis was not observed. Serum enzyme activity in animals pretreated with sorbitol prior to carbon tetrachloride exposure showed marked increases following administration of this combination of agents. This observation indicates that a potentiating action is produced by both ethanol and this alcoholic carbohydrate.

However, these data do not necessarily permit the assignment of similar mechanisms of potentiating action for ethanol and sorbitol. Although some similarities in metabolic action are common to both these compounds, sorbitol possesses certain toxic properties. This increase in serum enzyme activity following carbon tetrachloride and sorbitol administration could be explained as a direct action upon cellular permeability. Brody (18) has shown that sorbitol possesses surfactant properties. These detergent properties may explain the lack of accumulation of hepatic lipid following sorbitol ingestion although fatty acid synthesis is enhanced. Surfactants, such as Triton, have been shown (Recknagel et al.) to enhance release of

triglycerides into the serum. Ethanol, which has little or no surfactant effects, may produce fatty accumulation of the liver by increased fatty acid synthesis without concomitant enhanced triglyceride release. In addition, ethanol has been shown to stimulate the secretion of catecholamines. The increase in metabolic functions caused by these hormones may also serve to influence the degree of lipid accumulation.

In this respect, the liver lipid values (Tables 35 and 37) could be examined. Following administration of carbon tetrachloride, small increases in mean liver lipid values were observed. However, animals treated with both sorbitol and carbon tetrachloride showed marked increases in hepatic lipid. Recknagel and others (135) have suggested that carbon tetrachloride exerts an inhibitory action upon a triglyceride secreting mechanism which results in hepatic steatosis. After the administration of Triton, a non-ionic detergent which enhances triglyceride release into the serum, increased serum triglyceride levels were not observed in carbon tetrachloride intoxicated rats by these workers.

A similar mechanism may be suggested by these data. If sorbitol increased fatty acid synthesis, the administration of carbon tetrachloride, which inhibits release of hepatic triglyceride, could

account for the markedly elevated lipid levels. Since animals treated with sorbitol exhibited normal serum enzyme levels, it may be presumed that this agent did not affect GOT, GPT, or ICD enzyme release. The increase in serum enzyme activity observed following carbon tetrachloride administration can be attributed largely to changes produced by the organic halide. Although hepatic lipid accumulation was not observed at the time of carbon tetrachloride exposure of the sorbitol treated animals, serum enzyme activity was elevated twenty hours later. That the accumulation of hepatic lipid favored the partition of carbon tetrachloride into the hepatocytes can only be assessed by serial examination of hepatic lipid and carbon tetrachloride concentrations which develop concomitantly. From earlier observations, however, it was noted that carbon tetrachloride concentrations diminished rapidly following exposure. Rechnagel and co-workers (134) have shown that significant lipidic changes in the liver occur three hours after carbon tetrachloride administration (133). At this time it has been shown that an inhaled dose of carbon tetrachloride has been largely excreted (33). Increased fatty acid synthesis may be related to enhanced toxicity of carbon tetrachloride by several mediating factors and not directly to the increase in liver lipid. These factors have been previously discussed. Since sorbitol treated animals did not have a fatty liver at the time of exposure, a causal relation be-

tween the amount of hepatic lipid present at the time of exposure and the mechanism of potentiating of carbon tetrachloride toxicity cannot be assumed. These observations suggest that other mechanisms are operating which result in potentiation of toxicity.

VI -- DISCUSSION

In this overall discussion of the results obtained in the present study, no attempt will be made to duplicate the rather extensive annotated discussion included at the end of each section. Rather, this will be an attempt to correlate all of the findings and to set forth some general conclusions.

Although many other ramifications of the problem of the potentiating action of ethanol upon carbon tetrachloride toxicity have intruded into this study, the major emphasis has been to assess the role of ethanol induced fatty liver as a potentiating mechanism.

The relationship of fatty accumulation of the liver to the toxicity of carbon tetrachloride has been investigated utilizing several experimental approaches. Employing a number of parameters of toxicity to evaluate the severity of toxic changes, it was observed that ethanol markedly enhanced the toxic effects of carbon tetrachloride. This enhanced toxicity of carbon tetrachloride occurred eighteen hours after ethanol ingestion when the alcohol had largely been metabolized or excreted. Since this potentiation did not occur when ethanol was given two hours prior to exposure, it was presumed that the enhanced toxicity was not related to a direct interaction between ethanol and carbon tetrachloride.

It has been suggested that the increase in total hepatic lipid following ethanol ingestion is the causative mechanism of potentiation of toxicity of the halogenated hydrocarbons. Because of their lipid solubility, it was suggested that the chlorinated hydrocarbons would be preferentially absorbed into the fatty liver and being present in greater concentration would exert a more severe toxic action. To examine this hypothesis, the toxic response to carbon tetrachloride was examined in animals with hepatic steatosis induced by agents other than ethanol. In related studies, the ability of ethanol to potentiate carbon tetrachloride toxicity was examined in animals who were treated so that they did not respond to ethanol by the production of a fatty liver.

If the presence of increased amounts of hepatic lipid were causally related to the potentiation of toxicity of carbon tetrachloride, an enhanced toxic response to the organic halide could be expected when fatty accumulation of the liver was induced by any mechanism. Comparison of serum enzyme, iron and morphological effects showed similar responses to a given dose of carbon tetrachloride in normal animals and those with fatty liver accumulation produced by choline deficiency or by orotic acid. Increased toxicity was observed in normal animals and those with fatty livers only when ethanol was

ingested prior to carbon tetrachloride administration. Similarly, determination of carbon tetrachloride in blood or liver failed to reveal an increased concentration of the organic halide in animals with fatty livers. From these observations, it was concluded that potentiation of toxicity of carbon tetrachloride may be associated with ethanol induced fatty liver but is not causally related to elevated liver lipid levels produced by other means.

Similarly, the response to carbon tetrachloride was determined in animals in which hepatic steatosis produced by ethanol was inhibited. In the adrenalectomized animal, hepatic steatosis was effectively inhibited following ethanol administration. Although fatty accumulation was prevented in these animals, an enhanced toxicity to carbon tetrachloride was observed when they had received a prior oral dose of ethanol. Maximal lipidic changes were observed in normal animals nine hours after ethanol administration, yet the greatest morphological and serum enzyme changes were observed when carbon tetrachloride was administered twelve to eighteen hours following ethanol ingestion. In adrenalectomized animals, the most severe changes also occurred when carbon tetrachloride was administered twelve to eighteen hours after ethanol,

although hepatic lipid accumulation was inhibited. In addition, determination of carbon tetrachloride concentrations in blood and liver did not reveal marked differences as a result of prior ethanol ingestion. It was concluded from these observations that fatty liver is not essential to ethanol potentiation of carbon tetrachloride toxicity. Metabolic or physiological changes induced by ethanol and not necessarily related to lipid accumulation, may mediate the toxicity of carbon tetrachloride.

In the cordotomized animal, hepatic accumulation of lipid following ethanol administration was again inhibited. However, enhanced serum enzyme activity following carbon tetrachloride administration was observed in the ethanol pretreated cord sectioned animals. Although hepatic levels were not elevated at the time of carbon tetrachloride exposure, an increased toxic response was observed. This response may have been mediated by hypothermia, and an associated decrease in metabolic rate. In addition, catecholamine release and the resulting stimulation of metabolism has been reported to be inhibited in cordotomized animals. These factors may have contributed to the minimal morphological hepatic changes which were observed.

When the metabolic rate was depressed by hypothermia, the administration of carbon tetrachloride and ethanol produced minimal histological changes. It is possible that the characteristic hepatic lesions produced by carbon tetrachloride may develop more slowly at depressed temperatures.

Ethanol has been shown to exert many effects upon metabolic and hormonal relationships, some of which may be related to the potentiating mechanism. For example, as reported by Stenstam (168), Tygstrup (175) and others (76), ethanol exerts a prolonged inhibitory effect on galactose metabolism. In the normal metabolism of galactose, the liver converts uridine diphosphate galactose to the corresponding glucose analog through the action of UDP-galactose-4-epimerase. This reaction requires NAD and is inhibited by $NADH_2$ which is a major product of ethanol oxidation. This enzyme (UDP galactose-4-epimerase) is not inhibited directly by the presence of alcohol but rather by the increased concentration of $NADH_2$ produced in the metabolism of the ethanol. From this example, the possibility exists that ethanol may influence other enzyme systems essential to metabolism of carbon tetrachloride. Although carbon tetrachloride is excreted largely unchanged through the lungs, McCollister and co-workers (113) have reported as much

as 20% of the administered dose was excreted as carbon dioxide. Residues of carbon tetrachloride were recovered as urinary carbonate and urea, and a metabolite, possibly a glucuronide, was found in the urine. Paul (125) using gas chromatographic techniques, reported the presence of chloroform in blood following administration of carbon tetrachloride. The latter observation has been verified in our laboratories, and the additional appearance of methylene chloride as a metabolite has been suggested (33). Although the metabolism of carbon tetrachloride has been verified, the biochemical pathway of these reactions has not been determined. If the metabolism of carbon tetrachloride is involved in the production of the characteristic toxicity observed, a change in enzyme function in some part of the metabolic pathway could alter the toxic response. It has been suggested that the metabolites of carbon tetrachloride are more toxic than the parent compound itself and that the hydrocarbon nucleus acts as a potent alkylating agent (139). If this were so, a stimulation of metabolism would result in the formation of greater quantities of these toxic by-products. If, however, carbon tetrachloride exerts its toxic action directly, the inhibition of an enzyme system involved in its metabolism would allow higher concentrations of the hydrocarbon to remain in the tissues for longer periods of time.

As suggested in Section V-D, not only pyridine nucleotides, but other cellular electron donating groups may be affected by free radicals formed during carbon tetrachloride metabolism. Alterations in glutathione, α-tocopherol and ascorbic acid levels, and sulfhydryl activity have been reported following carbon tetrachloride administration. All of these cellular constituents are capable of reaction with free radicals which may be formed during carbon tetrachloride metabolism. Similarly, thyroid hormones which stimulate metabolic rate has been shown to exacerbate carbon tetrachloride toxicity. Ethanol, which exerts a stimulating effect upon metabolism, causes altered pyridine nucleotide levels and increases catecholamine release, could conceivably influence the toxicity of carbon tetrachloride through the mediation of one or more of these actions.

Although these reports indicate that the administration of ethanol brings about a multitude of biochemical and physiological changes, no causal relationship can be assumed between these changes and the potentiated response to carbon tetrachloride. The development of ethanol induced fatty liver is one of the most interesting of these effects and a number of suggestions have been made that this may well be a factor directly related to potentiation of carbon tetrachloride toxicity.

The present study makes it quite apparent that fatty liver, of itself, does not potentiate carbon tetrachloride toxicity. In addition, potentiation of carbon tetrachloride toxicity occurs in ethanol treated animals even though the development of fatty liver is prevented by a number of techniques. Although liver lipid accumulation occurs following ethanol ingestion, the present data indicate that no causal relationship exists between fatty liver development and potentiation of carbon tetrachloride toxicity.

SUMMARY

The increase in hepatic lipid observed following the ingestion of ethanol has been implicated as a factor in the potentiation of toxicity of carbon tetrachloride. It has been suggested that increased amounts of liver lipid would favor the partition of the hydrocarbon into the liver which would result in increased tissue damage. This investigation was initiated to determine the relationship of lipid to the hepatotoxicity of carbon tetrachloride.

To determine whether the enhanced toxicity of carbon tetrachloride is manifested only in animals with elevated hepatic lipid levels, the effects of various doses of carbon tetrachloride were measured in rats with hepatic steatosis induced by high fat or choline deficient diets, ethionine, and orotic acid. In other studies, the toxic effects of carbon tetrachloride were investigated in ethanol treated rats whose hepatic steatosis was inhibited by adrenalectomy, cord section, or hypothermia.

The toxic effects of carbon tetrachloride were measured in various ways. Changes in serum levels of glutamic-oxaloacetic transaminase, glutamic-pyruvic transaminase, isocitric dehydrogenase, and iron served as indirect indices of hepatic alteration.

Hepatic concentrations of pyridine nucleotides, total lipids, and carbon tetrachloride were determined following administration of carbon tetrachloride and/or ethanol. Morphological changes produced by various treatments were estimated from histopathological examination of various tissues.

Nine hours after ingestion of ethanol (5ml/kg) an increase in total hepatic lipid was observed which disappeared within twenty-four hours after dosing. Ingestion of ethanol eighteen hours prior to administration of various doses of carbon tetrachloride resulted in markedly elevated levels of serum enzyme activity and serum iron concentration. Carbon tetrachloride also caused more extensive morphological changes in the liver of animals pretreated with ethanol.

Morphological and serum enzyme changes produced by carbon tetrachloride were similar in control rats and those with hepatic steatosis induced by choline deficiency or orotic acid. Prior ingestion of ethanol rather than total lipidic changes appeared to be necessary for enhanced toxicity of carbon tetrachloride. Only animals pretreated with ethanol showed marked alterations in serum enzymes, iron,or liver pyridine nucleotides following carbon tetrachloride administration. Increased blood or liver concentrations

of carbon tetrachloride could not be detected in animals treated with ethanol or the other steatogenic agents.

Hepatic lipid levels remained normal following ethanol ingestion by adrenalectomized or cord sectioned rats, but serum enzyme and iron levels were greatly increased after carbon tetrachloride administration to those animals. The concentration of carbon tetrachloride in blood and liver of intact, adrenalectomized or cord sectioned animals given the solvent was independent of prior treatment with ethanol or amount of hepatic lipid present. Morphological changes were more extensive in the animals treated with both carbon tetrachloride and ethanol than in those given carbon tetrachloride alone. Since no protective effect against the enhanced toxicity of carbon tetrachloride was observed when ethanol induced fatty liver was prevented, hepatic lipid accumulation cannot be presumed to be the causative mechanism of potentiation.

Sorbitol, which is metabolized similarly to ethanol, exacerbated the toxicity of carbon tetrachloride. Serum enzyme activities were elevated in animals given sorbitol eighteen hours prior to carbon tetrachloride exposure. Fatty accumulation of the liver was not produced following sorbitol ingestion. When total metabolic activity was reduced by hypothermia, liver damage did not occur following administration of carbon tetrachloride or ethanol and carbon tetrachloride.

In summary, enhanced toxicity of carbon tetrachloride was not observed when hepatic steatosis was induced by agents other than ethanol. Conversely when hepatic steatosis following ethanol ingestion was inhibited, enhanced toxicity of carbon tetrachloride still was observed. It was concluded that ethanol induced fatty change in the liver is not necessary for potentiation of toxicity of carbon tetrachloride.

Appendix I

Recovery of carbon tetrachloride and chloroform using the microdiffusion technique.

Appendix II

Gas chromatogram of carbon tetrachloride and chloroform.

Appendix III

Calibration curve of Fujiwara chromogen.

Appendix IV

Calibration curve of β-NAD.

Appendix V

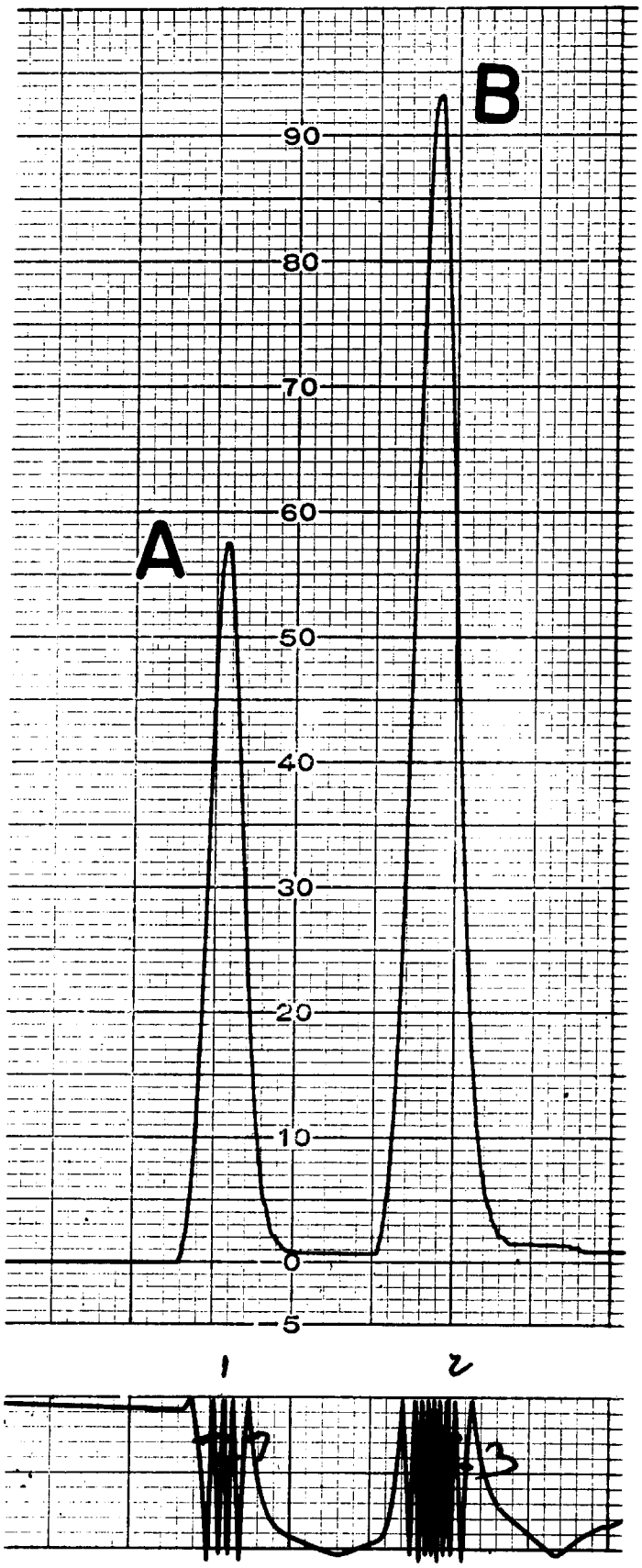
Calibration curve of β-$NADH_2$.

Tissue sections were prepared by the University of Michigan Pathology Department. Serum iron measurements were made by Fred Grunder, Department of Industrial Health.

RECOVERY OF CARBON TETRACHLORIDE EMPLOYING THE MICRODIFFUSION TECHNIQUE

To determine the efficiency of recovery of carbon tetrachloride, 1.5 μl of carbon tetrachloride and an equal volume of chloroform were added with a micro-pipette, to the tissue digestion mixture in a diffusion flask. Toluene, 1.0 ml served as the absorbing agent for the halogenated hydrocarbons. Samples were equilibrated for twenty four hours and 0.6 μl of the toluene solution was injected into an Aerograph Gas Chromatograph (Injector temperature - 80°C; column temperature - 38°C; packing material - chromatopac, chromosorb P 50-60 mesh, with 5% Ucon and 5% Chromatosol Silicon 550; hydrogen flame detector).

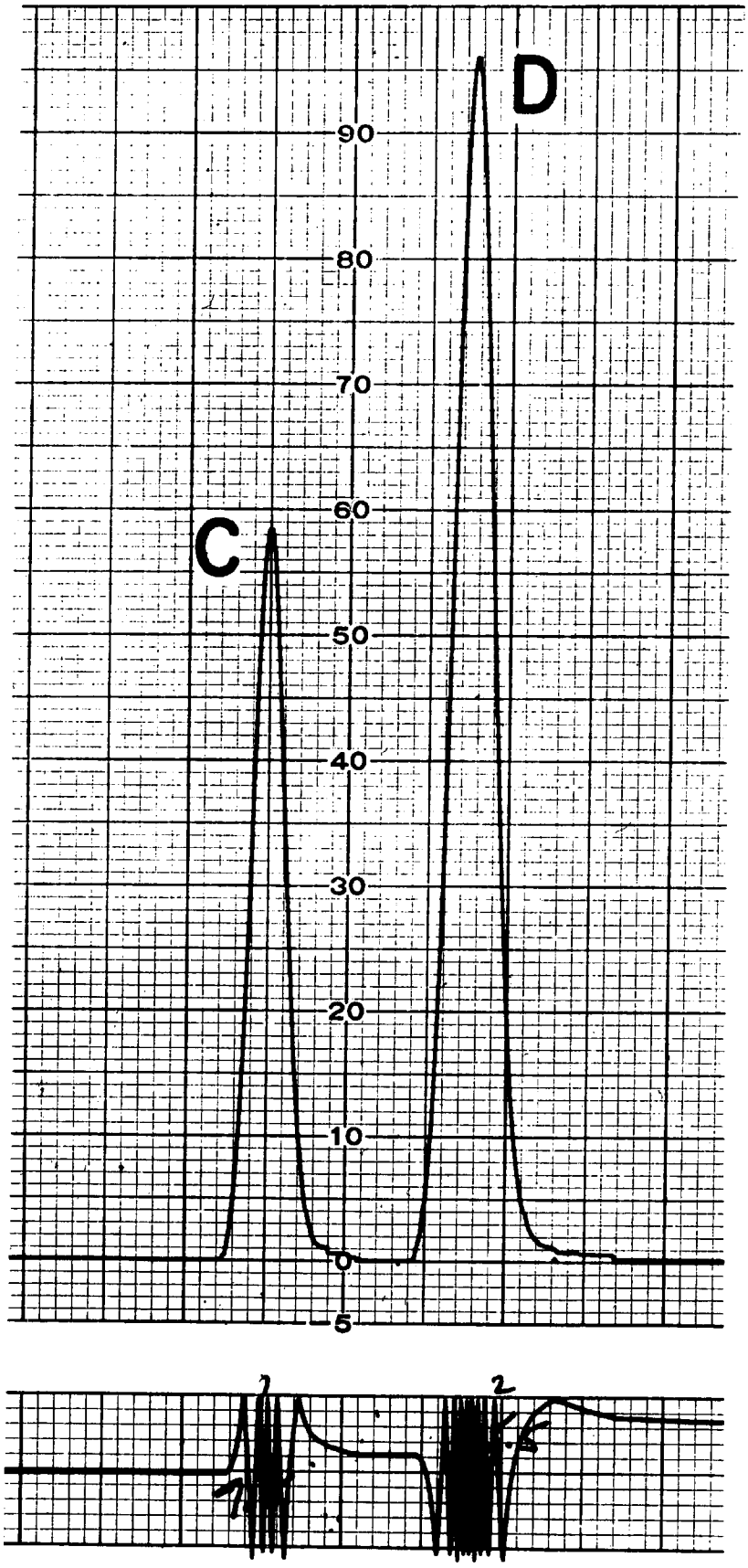
Carbon tetrachloride was eluted from the column with a retention time of 4.5 minutes, (Appendix I, peak A). Chloroform, with a retention time of 6.8 minutes, appeared next (Peak B). Toluene, with a retention time of ten minutes does not appear in the figures. These peaks were compared with those produced by 0.6 μl of a toluene solution containing 1.5 μl/ml of carbon tetrachloride and 1.5 μl/ml chloroform. Carbon tetrachloride, Appendix II, peak C, and chloroform, peak D, were eluted at their corresponding retention times of 4.5 and 6.8 minutes, respectively. Comparison of peak heights and measurement of area under the peaks showed quantitative recovery (99%) of the organic halides from the digestion mixture. Measurement of area under the elution



Appendix I

Recovery of carbon tetrachloride and chloroform using the microdiffusion technique.

peaks showed 0.185 $\mu gm/cm^2$, and 0.088 $\mu gm/cm^2$ for carbon tetrachloride and chloroform recovered from the diffusion flask. Similarly, standard solutions showed 0.190 and 0.087 $\mu gm/cm^2$ for carbon tetrachloride and chloroform, respectively. Although the gas chromatographic technique is a sensitive method for the determination of volatile chlorinated hydrocarbon in tissue and blood, only two samples can be analyzed in a day. Due to the large number of samples used in this study, the chlorinated hydrocarbons were analyzed using the colorimetric procedure described in Methods, Section II.



Appendix II

Gas chromatogram of carbon tetrachloride and chloroform.

CALIBRATION CURVE OF THE FUJIWARA CHROMOGEN

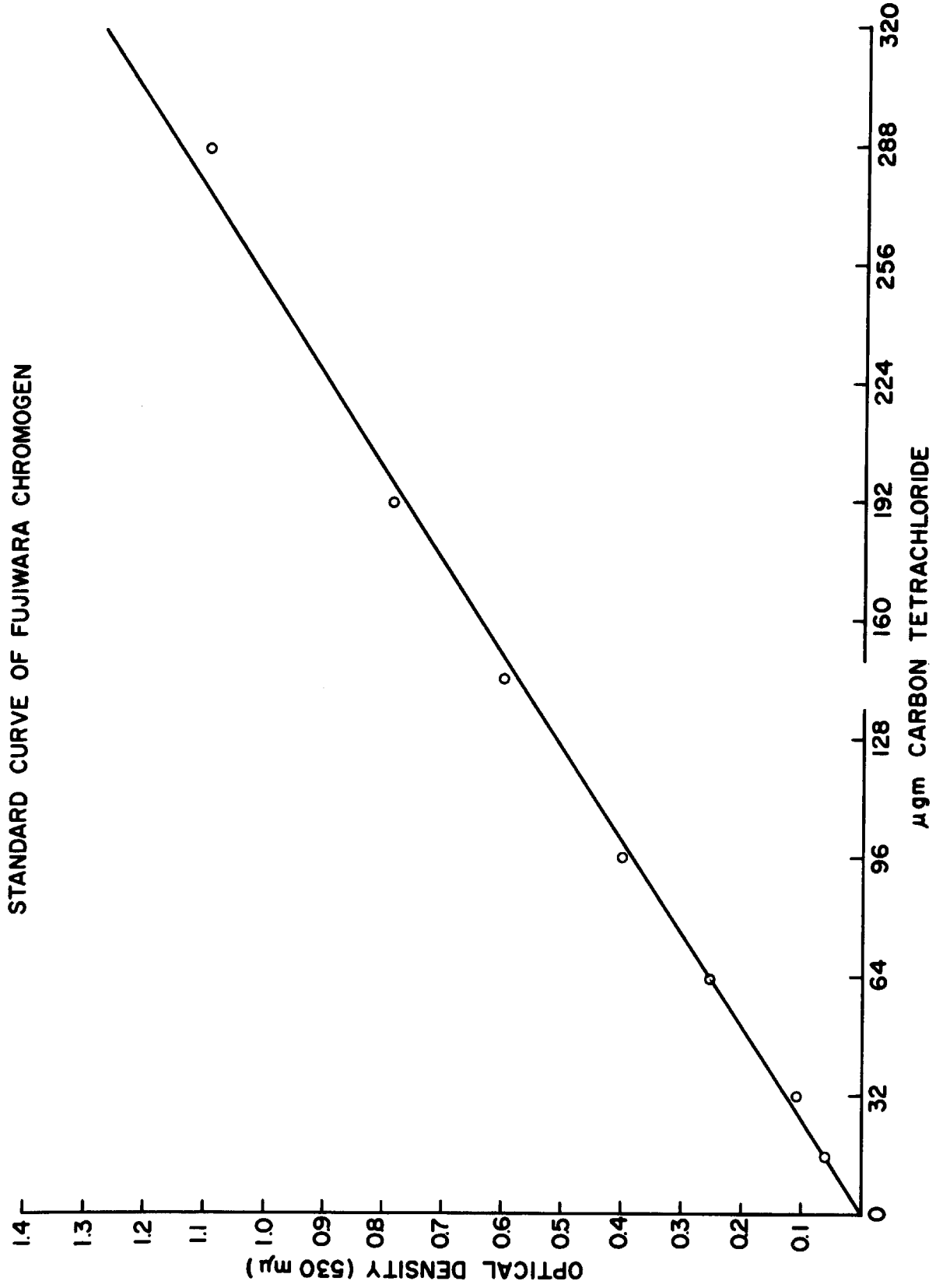
The Fujiwara chromogen reaction mixture was prepared in glass stoppered Erlenmeyer flasks by addition of reagents in the following order:

10.0 ml -- pyridine (purified)
3.0 ml -- 35 percent (w/w) potassium hydroxide
0.15 ml -- acetone (purified)

Aliquots of a standard solution of carbon tetrachloride dissolved in toluene were added to a series of tubes, each containing the above reaction mixture.

Purified toluene was added to bring the total volume of toluene to 0.5 ml in the mixture.

The Fujiwara color reaction was carried out at 70°C for 40 minutes in a water bath equipped with a reciprocating shaker. After incubation, the flasks were placed in an ice bath and the phases allowed to separate. Two ml of the upper colored phase were added to 0.5 ml of 0.01 N sodium hydroxide in a 1 cm Beckman cuvette. The contents of the cuvette were stirred vigorously and the optical density at 530 mμ was measured using a Beckman DU spectrophotometer.



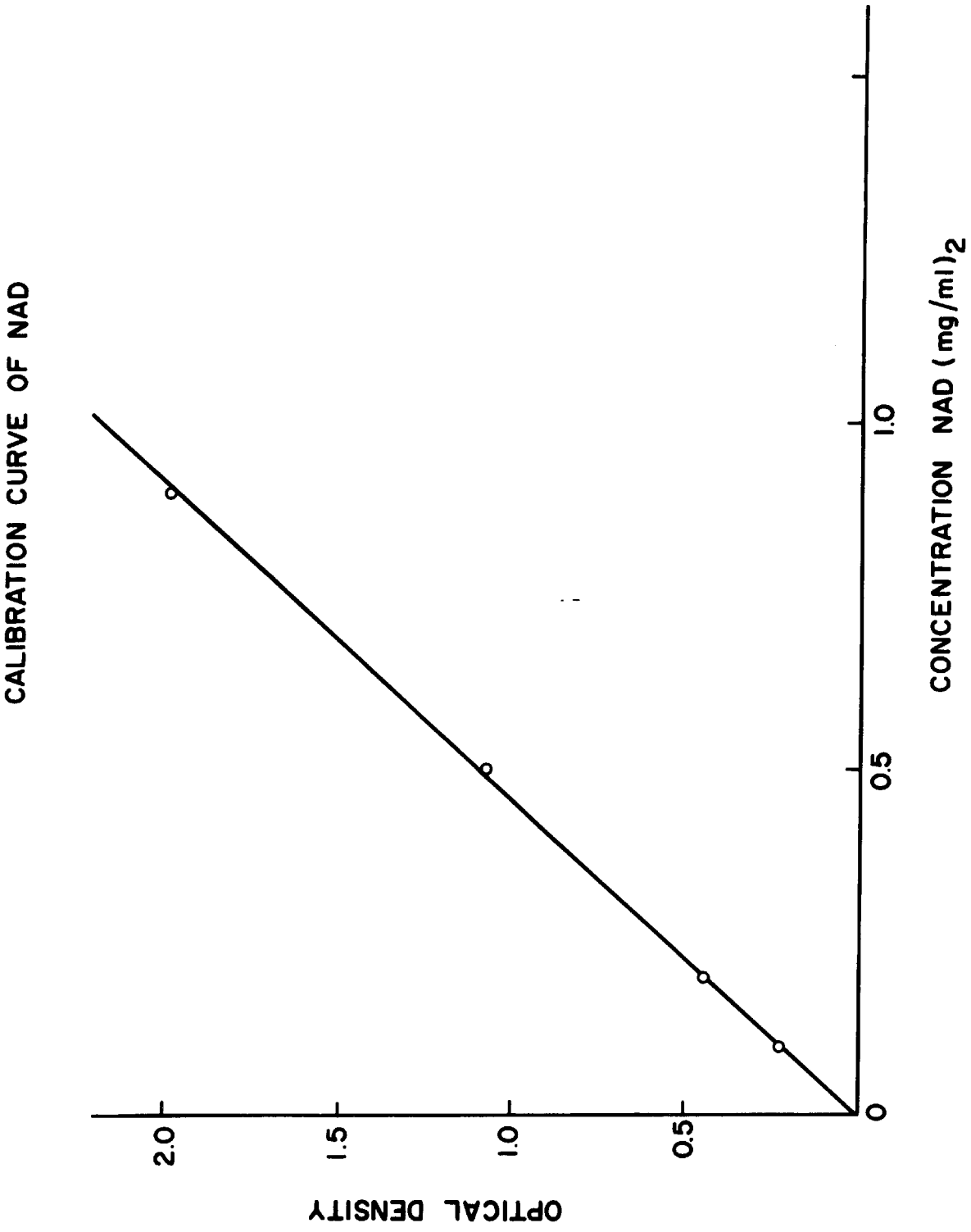
Appendix III

Calibration curve of Fujiwara chromogen.

CALIBRATION CURVES OF PYRIDINE NUCLEOTIDES

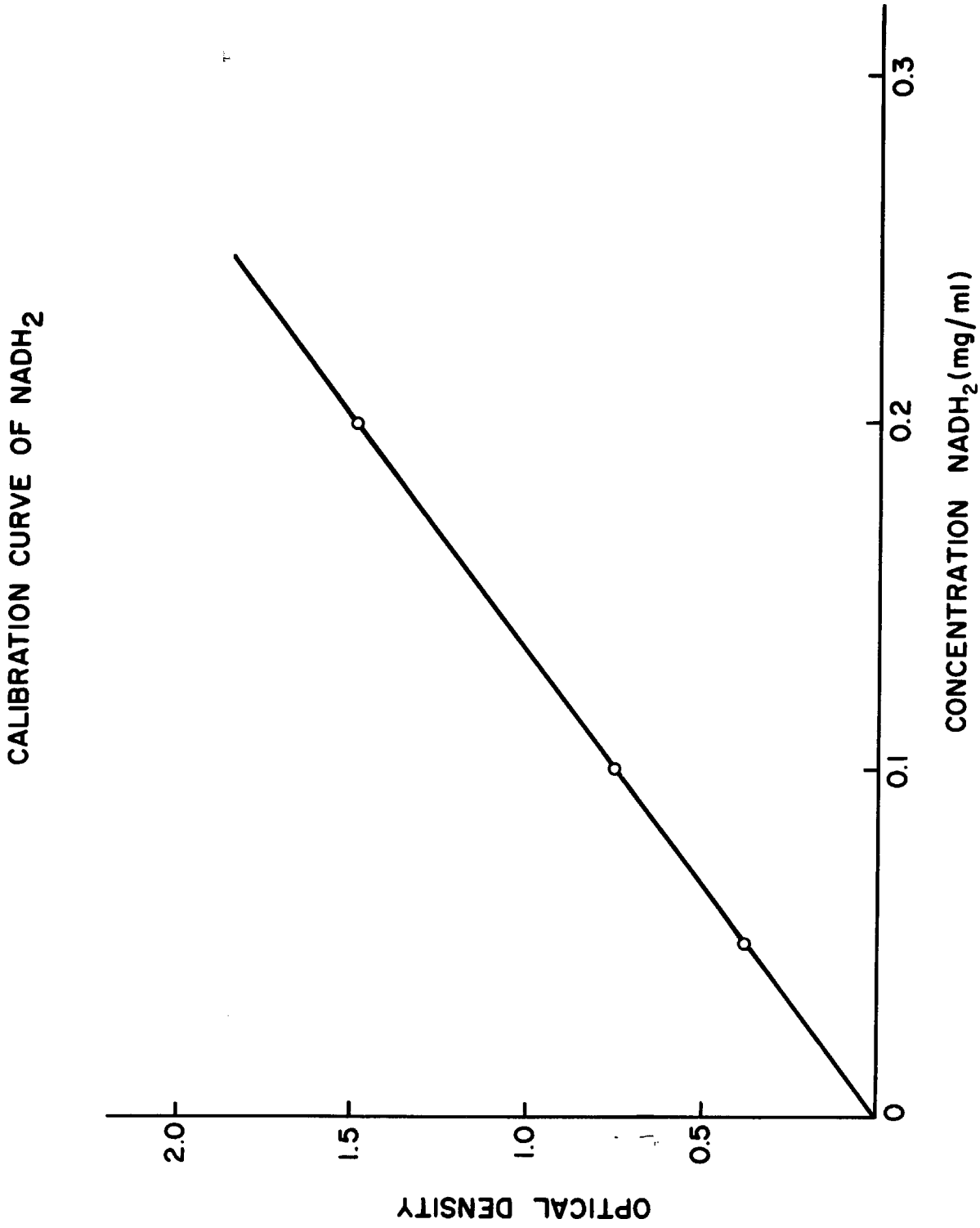
The calibration curve of β-NAD was prepared by serial dilution of the Sigma purified reagent in 5 percent trichloroacetic acid. Measurement of optical density at 260 mμ was made using the Beckman DU spectrophotometer. (Abbreviations, page xix).

Similarly, serial dilution of Sigma purified reagent β-$NADH_2$ was made with a 0.25 M sucrose solution containing 0.1 M nicotinamide and 0.1 N sodium hydroxide. Optical density was measured at 340 mμ using a Beckman DU Spectrophotometer.



Appendix IV

Calibration curve of β-NAD.



Appendix V

Calibration curve of β-$NADH_2$.

LIST OF REFERENCES

1. Abelin, I., Herren, C. and Berli, W.
   Helv. Med. Acta 25, 591 (1958).

2. Alford, W. C.
   J. Ind. Hyg. & Tox., 29, 396 (1947).

3. Artizzu, M., Baccino, F. M. and Dianzani, M. U.
   Biochim. Et. Biophys. Acta 78, 1 (1963).

4. Artom, C.
   J. Biol. Chem. 205, 101 (1953).

5. Asagoe, T.
   Naika Hokan 7, 504 (1960).
   (Chem Abstr., 55, 7624e, 1961).

6. Ashworth, C. T. and Luibel, F. J.
   Fed. Proc. 21, 305 (1962).

7. Bahor, R. and Dambrauskas, T.
   (Unpublished data).

8. Bengmark, S. and Olsson, R.
   Lab Invest., 11, 235 (1962).

9. Binet, L., Weller, G. and Goudard, H.
   C. R. Soc. Biol., 124, 1141 (1937).

10. Block, W. D. and Cornish, H. H.
    Proc. Soc. Expt'l. Biol. Med., 97, 178 (1958).

11. Bloomfield, D. K. and Bloch, K.
    J. Biol. Chem., 230, 337 (1960).

12. Boni, P., Reduzzi, F., Bile, G. and Gallero, V.
    Clin. Ter. 9, 129 (1955).
    (Chem. Abstr. 51, 3045e, 1957).

13. Bray, H.G. and James, S.P.
Biochem. J., 69, 24 (1958).

14. Bray, H.G., Thorpe, W.V. and Vallance, D.K.
Biochem. J. 51, 193 (1952).

15. Brodie, B.B. and Maikel, R.P.
Ann. N.Y. Acad. Sci., 104, 1049 (1963).

16. _____, Butler, W.M., Horning, M.G., Maikel, R.P. and Maling, H.M.
Am. J. Clin. Nutrition 9, 432 (1961).

17. _____, Maling, H.M., Horning, M.G. and Maikel, R.P.
"Drugs Affecting Lipid Metabolism."
Ed.-S. Garattini and R. Pauletti
Elsevier, Amsterdam (1961).

18. Brody, T.M.
Unpublished data. Personal communication.

19. _____.
Fed. Proc., 18, No. 4, 1017 (1959).

20. _____, Calvert, D.N., Schneider, A.F.
J. Pharm. Exp. Therap., 131, No. 3, 341 (1961).

21. Browning, E.
Ann. Occup. Hyg., 3, 231 (1964).

22. _____.
"Toxicity and Metabolism of Industrial Solvents."
Elsevier Publ. Co., N.Y., (1965).

23. Bruns, F.H., Brosswitz, E., Dannemann, H., Horn, H.D. and Noltmann, E.
Klin. Wochschr. 39, 342, (1961).

24. Butler, T.C.
J. Pharm. Exp. Therap., 134, 311 (1961).

25. Butler, W. M., Jr., Maling, H. M., Highman, B. and Brodie, B. B.
Fed. Proc., 19, 229 (1960).

26. Calvert, D. N. and Brody, T. M.
J. Pharm. & Exp. Therap., 124, No. 4, 273 (1958).

27. _____, and Brody, T. M.
J. Pharm. Exp. Therap., 134, No. 3, 304 (1961).

28. Chenoweth, M. B. and Hake, C. L.
Ann. Rev. Pharm., 2, 363 (1962).

29. Cohen, M. M.
Neurol., 7, 238 (1957).

30. Commoner, B., Townsend, J., and Pake, G. E.
Nature (London) 174, 689 (1954).

31. Cornish, H.
Toxicol. & App'l. Pharm., 4, 468 (1962).

32. _____, and Block, W. D.
Arch. Environ. Hlth., 1, 96 (1960).

33. Dambrauskas, T.
Private communication.

34. Davis, P. A.
JAMA, 103, 962 (1934).

35. Davis, P. A.
Rubber Age 25, 11 (1929).

36. Di Luzio, N. R., Poggi, M.
Fed. Proc., 22, No. 2, Pt. I, 398 (1963).

37. Dinman, B. D., Hamdi, E. A., Fox, C. F. and Frajola, W. J.
Arch. Environ. Hlth., 7, 630 (1963).

38. Docherty, J. F.
JAMA, 81, 454 (1923).

39. Eden, E. and Harrison, D. P.
Austral. J. Exp. Biol. Med. Sci., 33, 85 (1955).

40. Ershoff, B. H.
Physiol. Rev., 28, 107 (1948).

41. Fabre, R., Truhaut, R. and Laham, S.
Ann. Pharm. Franc. 9, 251 (1951).

42. Farber, E. Castillo, A. E.
Fed. Proc., 22, No. 2, Pt. I, (1963).

43. _____, Shull, K., Villa-Trevino, S. Lombardi, B. and Thomas, M.
Nature 203, No. 4940, 34 (1964).

44. Fassett, D.
Ann. Rev. of Pharm., 3, 267 (1963).

45. Feiglson P., Williams, J. N. and Elvehjem
J. Biol. Chem., 185, 741 (1950).

46. Fiore-Donati, L.
Lab. Invest., 9, 625 (1960).

47. Fleisher, G. A., Wakim, K. G., Hanson, M. L., Axelson, J. and Arns, R.
Proc. Staff Meetings Mayo Clinic, 31, 640 (1956).

48. Folch, J.
J. Biol. Chem., 191, 833 (1951).

49. Frankl, H. D., Gaertner, P. L. Kossuth, L. C. and Milch, L. J.
Texas Repts. Biol. & Med., 15, 868 (1957).

50. Gallagher, C. H.
Austral. J. Exp. Biol., 40, 241 (1962).

51. _____, Gupta, D. N., Judah, J. D., and Rees, K. R.
J. Path. Bact., 72, 193 (1956).

52. _____, Rees, K. R.
J. Path. Bact., 87, 148 (1960).

53. Gallagher, C. N.
Nature 184, Suppl. No. 18, 1407 (1959).

54. Gardner, G.H., Grove, R.C., Gustafson, R.K., Maire, E.D. Thompson, M.J., Wells, H.S. and Lamson, P.D.
J. Hopkins Hosp. Bull. 36, 107 (1925).

55. Gibb, J.W.
Doctoral thesis, University of Michigan (1965).

56. Giusti, G. and Coltori, M.
Biochim. Appl., 4, 189 (1957).
(Chem. Abstr. 52, 4859f (1958).

57. Glynn, L.E., Himsworth, H.P. and Neuberger, A.
Brit. J. Exp. Path., 26, 326 (1945).

58. Graham, E.A.
J. Exp. Med., 22, 48 (1915).

59. Griffith, H.W. and Wade, N.J.
J. Biol. Chem., 131, 567 (1939).

60. Guild, W.R., Young, J.V. and Merrill, J.P.
Ann. Int. Med., 48, 1221 (1958).

61. Habgood, S. and Powell, J.F.
Brit. J. Ind. Med., 2, 39 (1945).

62. Hall, M.C.
J. Agric. Res., 21, 157 (1921).

63. Hall, M.C.
JAMA, 77, 1641 (1921).

64. Hamilton, A.
Bull. Bureau Labor Stat., 179, 32 (1915).

65. Handschumacher, R.E., Creasey, W.A., Jaffe, J.J. Pasternak, C.A. and Hankin, L.
Proc. Nat'l. Acad. Sci., 46, 178 (1960).

66. Hardin, B.L.
Indust. Med. & Surg., 23:3, 93 (1954).

67. Hartoff, W. S.
Brit. J. Exp. Path., 29, 483 (1948).

68. Hayano, K., Tsubone, T. and Azuma, I.
J. of Biochem., 52, No. 5, 379 (1962).

69. Henley, K. S., Schmidt, E. and Schmidt, F. W.
J. Am. Med. Assoc., 173, 977 (1960).

70. Hitchcock, F. A.
Quart. J. Stud. Alcohol, 2, 641 (1942).

71. Holloway, P. W., Peluffo, R. and Wakil, S. J.
Biochem. & Biophy. Res. Comm., 12, 300 (1963).

72. Horning, M. G., Williams, E. A., Maling, H. M. and Brodie, B. B.
Biochem & Biophys. Res. Comm., 3, 635 (1960).

73. Horrocks, G. E.
Mil. Surg., 74, 246 (1934).

74. Hruzik, H. and Langer, J.
Bratislva Lekarske Listy 40, 548, (1960).
(Chem. Abstr. 55, 5781h, 1961).

75. Hutterer, F. and Leonard, V.
Fed. Proc., 22, No. 2, Pt. I, 1254 (1963).

76. Isselbacher, K. J. and Krane, S. M.
J. Biol. Chem., 236, 2394 (1962).

77. Jennings, R. B.
Arch. of Path., 59, 269 (1955).

78. Jones, D. P., Losowsky, M. S., Davidson, C. S. & Lieber, C. S.
J. Clin. Invest., 42, 945 (1963).

79. Joron, G. E., Hollenberg, C. H., Bensley, E. H.
Canad. Med. Assn. J., 76, 173 (1957).

80. Judah, J. D. and Christie, G. S.
Roy. Soc. London, Sec. B, 142, 241 (1956).

81. _____, and Rees, K. R.
Fed. Proc., 18, 1013 (1959).

82. Kalant, H., Hawkins, R. D. and Watkin, G. S.
Canad. J. Biochem. & Physiol., 41 (1963).

83. Kasbekar, D. K., Lavate, W. V., Rege, D. V. and Sreenivasan, A.
Biochem. J., 72, 384 (1959).

84. Klatskin and Krehl
J. Exp. Med., 100, 615 (1954).

85. Klausner, H. and Heimberg, M.
Fed. Proc., 23, No. 2, Part I, 251 (1964).

86. Kobayashi, M.
Med. J. Shinshu Univ. 4, 163 (1959).
(Biol. Abstr., 35, 18192 (1960).

87. Koch-Weser, D., Farber, E. and Popper, H.
Arch. of Path., 51, 498 (1951).

88. Konishi, H., Okayama Igakkai
Zasshi, 71, 1879 (1959).
(Chem. Abstr., 53, 2248e (1959).

89. Kutob, S. D. and Plaa, G. L.
J. Pharm. & Exp. Therap., 135, 245 (1962).

90. Lake, G. C.
Pub. Health Rep. 37, 1123 (1922).

91. Lambert, S. M.
JAMA, 80, 526 (1923).

92. Lamson, P. D. and Wing, R.
J. Biol. Chem., 69, 349 (1926).

93. _____, and Wing, R.
J. Pharm. & Exp. Therap., 29, 191 (1926).

94. _____, Gardner, G. H., Gustafson, R. K., Maire, E. D.
McLean, A. J. and Wells, H. S.
J. Pharm. & Exp. Therap., 22, 215 (1924).

95. Larson, R. E. and Plaa, G. L.
J. Pharm. & Exp. Therap., 147, 103 (1965).

96. Leach, C. N.
JAMA, 78, 1789 (1922).

97. Leaf, G. and Neuberger, A.
Biochem. J., 41, 20p (1947).

98. Lettow, E.
Berlin u. Munch tierarztl Wochschr.
73, 25 (1960). (Chem. Abstr. 55, 7617h, (1961).

99. Levy, A. C. and Ramey, E. R.
Proc. Soc. Expt'l. Biol. & Med., 104, 125 (1960).

100. Lewis, S. E.
Nature (London) 161, 692 (1948).

101. Lewis, C. E.
J. Occup. Med., 3, 82 (1961).

102. Lewis, L. A. and Page, I. H.
Lipid Res., 5, 216 (1964).

103. Lieber, C. S. and Schmid, R.
J. Clin. Invest., 40, 394 (1961).

104. Lindall, A. W. and Lazarow, A.
Metabolism 13, No. 3, 259 (1964).

105. Lombardi, B., Ugazio, G. and Raick, A.
Fed. Proc., 23, No. 2, Pt. I, 126 (1964).

106. Mainoli, S. and Piccinelli, O.
Arch. "E. Maragliano" Patol. E. Clin., 12, 1001 (1956).
(Chem. Abstr. 51, 9939a (1957).

107. Maling, H. M., Horning, M. G., Butler, W. M., Jr., Highman, B. and Brodie, B. B.
Fed. Proc., 19, 229 (1960).

108. _____, Wakabayashi, M. and Horning, M.G.
"Regulation of Enzyme Activity."
Ed. G. Weber, Pergamon, N.Y. (1963).

109. Mallov, S.
Am. J. Physiol., 189, 428 (1957).

110. _____,
Quart. J. Stud. Alcohol, 22, 250 (1961).

111. Mascitelli-Coriandozi, E. and Boldrini, R.
Arch. "E. Maragliano" Patol. e. Clin., 14, 45 (1958).
(Chem. Abstr., 52, 1213c (1958).

112. Matthes, K.J., Abraham, S. and Chaikoff, I.L.
Biochim et Biophys. Acta 70, 242 (1963).

113. McCollister, D.D. Beamer, N.H. and Atchison, G.J.
J. Pharm. Exp. Therap., 102, 112 (1951).

114. Miller, L.L., Ross, J.F. and Whipple, G.H.
Amer. J. Med. Sci., 200, 739 (1940).

115. Mookerjea, S. and Haines, D.S.
Fed. Proc., 22, No. 2, Pt. I, 262 (1963).

116. Moon, H.D.
Am. J. Path., 26, 1041 (1950).

117. Nelson, G.H. and Abbenhaus, J.I.
Proc. Soc. Exp. Biol. Med., 96, 5 (1957).

118. New, P.S., Lubash, G.D., Scherr, L. and Rubin, A.L.
J. Am. Med. Assoc., 181, No. 10, 903 (1962).

119. Nicholls, L. and Hampton, G.G.
Brit. Med. J., 2, 8 (1922).

120. Nicholson, J.L. and Peskoe, L.Y.
J. Kentucky State Med. Assoc., __, 1053 (1960).

121. Novikoff, A.B., Roheim, P.S. and Quintana, N.
Fed. Proc., 23, No. 2, Pt. I., 126 (1964).

122. Nunneley, T.
Brit. Med. J., 1, 685 (1867).

123. von Oettingen, W. F.
"The Halogenated Hydrocarbons Toxicity and Potential Dangers"
Pub. Hlth., Serv. Publ. No. 414 (1955).

124. Ogawa, M.
Acta Schol. Med. Univ. Kioto, 35, 229 (1958).
(Chem. Abstr. 53, 13400g (1959).

125. Olivecrona, T.
Acta Physiol. Scand., 55, 291 (1962).

126. Paul, B. B. and Rubinstein, D.
J. Pharm. & Exp. Therap., 141, No. 2, 141 (1963).

127. Perman, E. S.
Acta Physiol. Scand. 51, 68 (1961).

128. _____.
Biochem. Pharm., 8, 172 (1961).

129. _____.
Acta Physiol. Scand. 55, 189 (1962).

130. Phelps, B. M. and Hu, C. H.
JAMA, 82, 1254 (1924).

131. Plotz, W.
Biochem Zeitschr, 103, 243 (1920).

132. Reboucas, G. and Isselbacher, K. J.
J. Clin. Invest., 40, 1355 (1961).

133. Recknagel, R. O. and Anthony, D. D.
J. Biol. Chem., 234, 1052 (1959).

134. Recknagel, R. O. and Litteria, M.
Am. J. Path., 36, No. 5, 521 (1960).

135. _____, Lombardi, B. and Schotz, M. C.
Proc. Soc. Expt'l. Biol. Med., 104, 608 (1960).

136. Reichard, H.
J. Lab Clin. Med., 53, 417 (1959).

137. Reissmann, K. R., Christianson, J. F., Major, M. C., Boley, J. and Kittle, C. F.
Surg., 37, 738 (1955).

138. Reynolds, E. S.
Fed. Proc., 21, 305 (1962).
J. Cell Biol., 19, 139 (1963).

139. _____.
Fed. Proc., 22, No. 2, Pt. I, 370 (1963).

140. _____, Thiers, R. E. and Vallee, B. L.
J. Biol. Chem., 237, 3546 (1962).

141. Rikans, L. L., Arata, D. and Cederquist, D. C.
J. Nutrition 82, No. 1, 83 (1964).

142. De Ritis, F., Coltori, M. and Giusti, G.
Minerva Med. 46, 1 (1956).
Clinica Chimica Acta 2, 70 (1957).

143. Robbins, B. H.
J. Pharm. & Exp. Therap. 37, 203 (1929).

144. Robinson, D. S. and Seakins, A.
Biochem. J. 83, No. 3, 36 (1962).

145. Robinson, J. N., Levitas, F. R. and Perlzweig, W. A.
J. Biol. Chem., 170, 653 (1947).

146. Rossi, F. and Zatti, M.
Experimentia 16, 513 (1960).

147. Rubin, R. J.
Fed. Proc., 23, No. 2, Pt. I, 126 (1964).

148. Schade, A. L.
Biochim. Biophys. Acta 12, 163 (1953).

149. Schapiro, R. H., Drummey, G. D., Scheig, R., Mendelsohn, J. H. and Isselbacher, K. J.
Gastroenterology 44, 849 (1963).

150. Schapiro, R.H., Shimizu, Y., Drummey, G.D. and Isselbacher, K.J.
Clin. Res., 10, 234 (1962).

151. Schmidt, E., Schmidt, F.W., Wildhirt, E.
Klin Wochschr 38, 957 (1960).

152. Share, L. and Recknagel, R.O.
Am. J. Physiol. 197, 121 (1959).

153. Shiels, D.O.
Med. J. Austral. 2, 729 (1958).

154. Sidransky, H.
Endocrinology 72, 709 (1963).

155. _____, Verney, E., Lombardi, B.
Fed. Proc. 22, No. 2, 371 (1963).

156. Sigma Technical Bulletin
No. 410 April (1963).

157. Sigma Technical Bulletin
No. 150-UV No. 175 April (1965).

158. Slater, T.F., Strauli, U.D., Sawyer, B.C.
Biochem. J. 93, 260 (1964).

159. _____, Sawyer, B.C., Strauli, U.D.
Biochem J. 93, 267 (1964).

160. Smetana, H.
Arch. Int. Med., 63, 760 (1939).

161. Smillie, W.G. and Pessoa, S.B.
Am. J. Hyg. 3, 35 (1923).

162. Smith, M.E. and Newman, H.W.
J. Biol. Chem. 234, 1544 (1959).

163. Smuckler, E.A., Barker, E.A. and Benditt, E.P.
Fed. Proc., 22, No. 2, Pt. 1, 369 (1963).

164. _____, Iseri, O.A., Benditt, E.P.
Biochem & Biophys. Res. Comm., 5, No. 4, 270 (1961).

165. Snyder, F., Cress, E.A. and Kyker, G.C.
J. Lipid Res., 1, 125 (1959).

166. Starr, E.B.
J. Ind. Hyg., 5, 203 (1922).

167. Stein, Y. and Stein, O.
Biochim. Biophys. Acta 54, 555 (1962).

168. Stenstam, T.
Acta Med. Scand., 177, 1 (1946).

169. Sterkel, R.L., Spencer, J.A., Wolfson, S.K. Jr.
and Williams-Ashman, H.G.
J. Lab & Clin. Med. 52, 176 (1958).

170. Stern, P.H. and Brody, T.M.
J. Pharm. Exp. Therap. 141, No. 1 (1963).

171. Stewart, R.D., Torkelson, T.R., Hake, C.L. and Erley, D.S.
J. Lab Clin. Med. 56, 148 (1960).

172. Takasaka, T.
Deut. Zeitschr. ges. gerichtl Med. 6, 488 (1925).

173. Tesluk, A., Stewart, W.B.
Fed. Proc. 23, No. 2, Pt. I, 127 (1964).

174. Threshold Limit Values for 1961, 23 Annual Meeting of
the American Conference of Governmental Industrial
Hygienists, April (1961).

175. Tygstrup, N. and Windler, K.
Clin. Sci. 17, 1 (1958).

176. Vaisler, L., Lungu, A., Costiner
Acad. Rep. Populare Romine, Inst Biochim Studii Ceretari Biochem
3, 281 (1960).
Chem. Abstr. 55, 9595f (1961).
Chem. Abstr. 55, 7653h (1961).

177. Villela, G.G.
Nature 190, 807 (1961).

178. Villela, G.G. and Mitidieri, E.
Rev. Brazil Biol. 15, 285 (1955).
(Chem. Abstr. 50, 1199e (1956).

179. Wakil, S.J.
Am. J. Clin. Nutr. 8, 630 (1960).

180. Walling, C.
John Wiley & Son, Inc., N.Y. (1957).

181. Wartburb, J.P. von, Berli, W. and Aebi, H.
Helv. Med. Acta 28, 89 (1961).

182. Weber, I.
J. Lab & Clin. Med., 26, 719 (1940).

183. Williams, R.C.
"Detoxication Mechanisms."
John Wiley & Sons, Inc. N.Y. (1959).

184. Winawer, S.J., Broitman, S.A., Gottlieb, L.S. and Zamchek, N.
Fed. Proc., 23, No. 2, Pt. I, 126 (1964).

185. Wirtshafter, Z.T. and Cronyn, M.W.
Arch. Environ. Hlth., 9, 186 (1964).

186. _____, and Tsujimara, J.K.
Arch. Environ. Hlth. 2, 1c (1961).

187. Wolfson, S.K. Jr. and Williams-Ashman, H.G.
Proc. Soc. Exp. Biol. & Med. 96, 231 (1957).

188. Wool, I.G., Goldstein, M.S., Ramey, E.R., and Levine, R.
Amer. Journ. of Physiol. 178, 427 (1954).

189. Wroblewski, F. and LaDue, J.S.
Ann. Int. Med., 43, 345-360 (1955).

190. Wu, R.
Cancer Res., 19, 1217 (1959).

191. Zierler, K.L.
Ann. N.Y. Acad. Sci. 75, 227 (1958).