The Metabolism and the Effects of Chronic Carbon Tetrachloride Intoxication

THE METABOLISM AND THE EFFECTS OF CHRONIC CARBON TETRACHLORIDE INTOXICATION

by

Lewis Kanics

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Department of Biochemistry,
McGill University,
Montreal, Canada.

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Preface

A group of investigators under the direction of Dr.D. Rubinstein, in the Department of Biochemistry, McGill University, has been working on the mode of action and mechanism of toxicity of the chloromethanes for some years.

Since there was practically no biochemical data on the effects of chronic carbon tetrachloride inhalation and since the mechanism of action of the toxin was not at all understood we decided to begin an extensive examination of chronic CCl_4 inhalation. The most predominant type of exposure to CCl_4 is to small levels over a prolonged period. In view of this the cumulative effect of CCl_4 was stressed in our examination.

The second part of the thesis is an extension of the studies of Drs. B.B. Paul and D. Rubinstein on the mode of action of chloromethanes in intoxication.

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TABLE OF CONTENTS

List of Tables

List of Figures

List of Abbreviations

RER, SER	Rough-, smooth surfaced endoplasmic reticulum
ATP	Adenosine triphosphate
CoA	Coenzyme A
RNA	Ribonucleic acid
ppm	Parts per million
cpm	Counts per minute
LDLP	Low density lipoprotein
VLDLP	Very low density lipoprotein
d	Density
G	Gravity
SH	Sulfhydryl
TCA	Trichloroacetic acid
TG	Triglyceride
PL	Phospholipid
FFA	Serum free fatty acids
UFA	Liver unesterified fatty acid
SGOT	Serum glutamic-oxaloacetic transaminase
b.w.	Body weight
DPN, TPN*	di- and triphosphopyridine nucleotide

*The older nomenclature (rather than the more recent NAD, NADP) was used for the pyridine nucleotides as it was found in the entire literature reviewed for the thesis.

INTRODUCTION

Introduction

Halogenated hydrocarbons are widely used as industrial solvents for fats and oils. Therefore they present a potential health hazard when used without adequate ventilation. Acute intoxication of carbon tetrachloride is defined as the result of a single large dose. On the other hand chronic CCl_4 intoxication is the result of repeated administration of CCl_4.

I. Effects of Acute Carbon Tetrachloride Intoxication

(a) General Considerations

Many drugs and industrial solvents such as puromycin, carbon tetrachloride and ethionine produce lipid accumulation and necrosis in the liver. Choline deficiency, phosphorus and orotic acid supplemented diets cause similar changes. The extent of liver injury depends on the route of administration, the species, sex, nutritional state and other factors.

The most frequently employed experimental hepatotoxin is carbon tetrachloride. The lesion resulting from a single oral administration of CCl_4 is remarkably regular in pattern from species to species, although there is a considerable difference in susceptibility. Lehman *et al.* (1)

reported that cats are more sensitive to CCl_4 than rabbits and Lamson et al. (2) showed that rabbits succumb to CCl_4 more readily than dogs. Guinea pigs are more sensitive than rats (3). Lamson et al. (2) reported that puppies respond to CCl_4 more readily than adult dogs and in rats the opposite is true. Some communications (4, 5) indicate that females of the species are more susceptible than males.

It appears that response to CCl_4 is greatest when the toxin is administered by gastric intubation and least when it is inhaled, subcutaneous injection being intermediate (6). Prior or simultaneous alcohol intake (7) or exposure to cold (8), or a protein-free diet (9) result in augmentation of the effects of CCl_4 intoxication.

The most striking and typical hepatic alterations after administration of CCl_4 are centrilobular necrosis and fat accumulation. Using light microscopy Wahi et al. (10) showed that the earliest change noted is polymorphonuclear infiltration of the liver resulting in cloudy swelling and fatty infiltration which proceeds to necrosis when the dosage of the hepatotoxin is large. The electron microscopic studies of Stenger (11) revealed the presence of two types of morphologically altered hepatic parenchymal cells. One of these was characterized by an extensive proliferation of rough-surfaced endoplasmic reticulum (RER). The other type was a lipid-laden cell characterized by an extensive proliferation of smooth-surfaced endoplasmic reticulum (SER). Both types contained an

increased number of lysosomes indicating an accelerated intracellular autolysis, and depleted glycogen areas. Krishnan and Stenger (12) suggested that SER is responsible for detoxification since the amount of SER is reduced in starvation and CCl_4 has a greater effect in starved animals.

Rouiller et al. (13) presented evidence to the effect that swelling and degeneration of the endothelial cells of sinusoid capillaries precedes the changes in the hepatocytes. Reynolds (62) reported disruption of the cell membranes and endoplasmic reticulum together with influx of Ca^{++} ions into the cell within two hours of CCl_4 administration indicating early permeability changes. This was confirmed by Reynolds, Thiers and Vallee (63,64) who also noted a concomitant fall in K^+ and an increase in Na^+ levels. These authors suggested that the elevation of Ca^{++} concentration in the cell is a specific alteration of CCl_4-induced liver injury. These ion changes have been confirmed by many investigators (50, 65,66).

Reports on high energy phosphate levels are contradictory. Dianzani and Marinari (67) studied adenosine triphosphate (ATP) levels by four different methods in rats with CCl_4-induced fatty livers. They reported a marked decrease in adenosine triphosphate levels with a corresponding increase in adenosine diphosphate levels by all four methods. Frunder et al. (154) stated that intracellular redistribution of ATP had taken

place since they observed that in CCl_4-treated mouse liver mitochondria the ATP level was doubled while the ATP content in the supernatant was halved. However, Thiers et al. (64) measured total and acid-soluble phosphates in mitochondrial preparations of rats treated with CCl_4 and concluded that there was no change in ATP and ADP levels.

Decreases in DPN and cytochrome C concentrations in mouse liver slices after CCl_4-treatment have been noted by Nomiya (68). Decreased DPN/DPNH ratios in liver mitochondria isolated from CCl_4-treated rats have been shown by many investigators (45, 50, 66). They suggested that the depressed ratio may favor lipid synthesis rather than oxidation.

(b) CCl_4 and Lipid Metabolism

Under normal conditions the liver takes up free fatty acids and lipids from the lymph and blood streams. The lipids are hydrolyzed and the free fatty acids resynthesized into triglycerides, phospholipids and cholesterol esters, which are released back into the circulation bound to proteins.

Liver lipid accumulation can be initiated by numerous factors. Compounds and conditions which lead to fatty liver are concisely reviewed by West and Todd (14).

Wahi, Tandon and Bharadwaj (10) demonstrated by histochemical methods the presence of excess fat twenty-four hours after the subcutaneous injection of CCl_4. These results have been confirmed often in the last few years by biochemical estimations. Sreenivasan (15) found a slight increase in total liver lipids six hours after intraperitoneal injection of CCl_4 into rats, and a two-fold increase after forty-eight hours. Recknagel et al. (16, 17, 18) showed in a series of communications that the increase in liver lipids in CCl_4 intoxicated rats is almost entirely due to an increased triglyceride content. Horning, Earle and Maling (19), and Maximchuk and Rubinstein (20) demonstrated that the increase in liver triglycerides by a single dose of CCl_4 was the result of increased mobilization of fatty acids from the adipose tissue. A decrease in arachidonic acid and a twenty-fold increase in linoleic acid content of liver phospholipids were also observed (19). Maling, Frank and Horning (21) reported that after oral administration of CCl_4 injected albumin-bound palmitate-1-^{14}C disappeared from the serum within ten minutes, and thirty minutes later labelled triglycerides had reached a maximum in the serum, which was far below the values obtained in the control fasted rats. They also observed a four-fold increase in the ratio of liver triglycerides to plasma triglycerides. They concluded that in CCl_4-treated rats not only was the release of triglycerides (TG) and phospholipids (PL) from the liver impaired but also the incorporation of plasma palmitate

into TG and PL was accelerated.

Schotz (22) reported a ten minute delay in the appearance of ^{14}C labelled serum TG in rats injected with CCl_4 (0.25 ml/100 g b.w.) and four hours later with albumin-bound palmitate-1-^{14}C. Furthermore, serum and liver TG reached peak specific activities in thirty and ten minutes respectively and the secretion of TG by the CCl_4 injured livers decreased. Rubinstein and Rubenstein (23) showed that liver slices from rats intoxicated with CCl_4 (0.1 ml/100 g b.w.) administered by direct intraduodenal injection, incorporated acetate-1-^{14}C more readily into cholesterol esters and less readily into other lipid components. They also found that in the presence of CCl_4, liver slices from normal animals show increased synthesis of cholesterol esters and fatty acids from acetate-1-^{14}C.

Electron micrographs of rat livers examined three hours after CCl_4 treatment showed changes in the endoplasmic reticulum. The fact that the endoplasmic reticulum is associated with protein synthesis led to investigations on lipoprotein synthesis by CCl_4 intoxicated livers.

Seakins and Robinson (24) fasted female rats for eighteen hours and administered CCl_4 (0.5 ml/100 g b.w.) via gastric tube. Two hours later these rats were injected with d-l-leucine-1-^{14}C and the incorporation of the ^{14}C label was measured. They found a decreased incorporation into plasma and liver proteins. Furthermore, they obtained similar results with <u>in vitro</u> experiments. Using ^{32}P labelled orthophosphate they also

demonstrated decreased incorporation of ^{32}P label into liver phosphatides and high density lipoproteins.

Heimberg et al. (25,26) confirmed and extended the findings of Robinson in isolated perfused rat liver preparations. They showed that the presence of CCl_4 in the perfusion system was inhibitory towards the release of TG, PL and cholesterol into the perfusion medium and hepatic PL synthesis. They suggested that these three components were released into the $d < 1.020$ lipoprotein in discrete molar ratios to each other and that the very low density lipoproteins were released as a unit for the transport of triglycerides. Therefore, any interference with the availability or biosynthesis of the components of lipoprotein would result in fatty liver.

It was found (27) that twenty-four hours after a single injection of CCl_4 (0.1 ml/100 g b.w.) the level of ß-lipoprotein was doubled in the serum. The level declined slowly over a period of days. From this observation Ribeiro and McDonald (27) suggested that the increase was due to an effort by the liver to eliminate excess fat and cholesterol.

Ugazio and Lombardi (28) administered CCl_4 (0.25 ml in olive oil/100 g b.w.) by stomach tube to rats starved sixteen hours. On separating the various lipoprotein fractions by ultracentrifugation they found that, within four hours of the administration of CCl_4, the level of the very low density lipoproteins (VLDL) fell to 25% of that in control animals. They noted that all the components of the VLDL were reduced but pro-

portionally more lipid was bound to the protein moiety in the CCl_4-treated animals than in the controls. However, they were unable to confirm the findings of Maximchuk and Rubinstein (20) who reported an increase in the level of serum free fatty acids in CCl_4-treated rats.

Brown et al. (29) isolated amino acid-^{14}C labelled serum lipoproteins from CCl_4 poisoned rats which on injection into another animal were converted into a different class of lipoprotein. Amino acid incorporation into liver and serum proteins was also inhibited.

Schotz, Baker and Chavez (30) utilized computer analysis techniques to demonstrate the effect of orally administered CCl_4 (0.25 ml in olive oil/100 g b.w.) on liver and plasma triglyceride turnover rates. They observed that CCl_4 ingestion lowered hepatic TG secretion to one-tenth of control values. Furthermore, the rates of plasma TG turnover and hepatic uptake of plasma TG were depressed to 20% of the control rates. The rate of esterification of plasma FFA to liver TG was 50% greater in the CCl_4-treated rats.

At present there is no evidence that CCl_4 interferes with conjugation of the various lipid and protein moieties by the liver. Most of the available evidence indicates that the primary effect of CCl_4 on the liver is inhibition of the synthesis of the protein moiety of the lipoproteins.

(c) CCl_4 and Protein Metabolism

The liver occupies a central role in protein metabolism. Most of the digested protein components reach the liver through the portal vein. Some of the amino acids are used in the synthesis of blood proteins such as albumin and fibrinogen and plasma globulins. Others are broken down further: nitrogen is eliminated as urea and a large portion of the carbon skeleton is used for the synthesis of glucose and glycogen, oxidized through the tricarboxylic acid cycle to CO_2 or, through the formation of acetyl-CoA, used for fatty acid and lipid synthesis.

Increased levels of free amino acids in plasma, with the exception of arginine and glutamic acids, have been reported both two hours (10) and twenty-four hours (31) after the oral administration of CCl_4. Smuckler, Iseri and Benditt (32) observed a severe inhibition of glycine incorporation into albumin and fibrinogen in vivo, two hours after the oral administration of CCl_4 (0.5 ml/100 g b.w.) in mineral oil. This was confirmed in vivo and in vitro by Robinson and Seakins (24), who noted a decrease in leucine incorporation into lipoproteins and liver proteins. Weldon, Rubenstein and Rubinstein (33) reported a progressive inhibition of incorporation and metabolism of leucine when normal liver slices were incubated with varying amounts of CCl_4.

Hepatic ribonucleic acid depletion has been observed within two

to six hours after the administration of CCl_4 (15,34). Other investigators (35) reported increased hepatic RNA and DNA levels forty-eight hours after a small intraperitoneal dose of CCl_4. These increases were thought to be reflections of regenerative activity in the liver. A marked depression in glycine incorporation into mitochondrial protein and cytochrome C in vivo was observed as early as fifteen minutes after the oral administration of CCl_4 (36).

Smuckler and Benditt (37) showed an apparent dissociation of 79 S ribosomes into 54 S components by ultracentrifugation. Their data also showed that the depressed rate of incorporation of amino acids into microsomal protein was caused by the action of CCl_4 on the microsomes and not on the amino acid activating enzyme, the transfer enzyme, or on the sRNA. In a latter publication (38) they further linked the reduced capacity of CCl_4 intoxicated liver to synthesize proteins to changes in the ribosomes.

Seawright and McLean (39) reported a 50% drop in the incorporation of leucine into microsomal proteins by normal liver slices forty-five minutes after the addition of two µl of CCl_4. They found that the addition of SKF 525 A (diethylamino-ethyl-diphenyl-propyl acetate), a drug that inhibits microsomal hydroxylating enzymes, prevented the inhibition of protein synthesis. Icen and Huovinen (40) found a statistically significant decrease in the protein-bound sulfhydryl groups with a concomittant increase in plasma non-protein-bound sulfhydryl concentration. Kasbekar

et al. (15) observed a drop in the hepatic sulfhydryl concentration six hours after CCl_4 administration. This was confirmed by Snyder and Cornatzer (41).

It seems quite reasonable to assume that the primary effect of CCl_4 is inhibition of protein synthesis. The findings of Seakins and Robinson (24) seem to indicate that it is not CCl_4 itself which inhibits protein synthesis but a metabolite of it. This is also supported by the findings of Smuckler and Benditt (37) who showed that in contrast to their observation in vivo, they found no change of sedimentation pattern of isolated microsomes in the presence of CCl_4 in vitro.

(d) CCl_4 and Carbohydrate Metabolism

Early histological studies cited in a review by Drill (52) indicated that fatty changes due to CCl_4 intoxication were always associated with a decrease in liver glycogen. Wahi et al. (10) reported that glycogen loss occurred from the centrilobular zone within six hours of administration of the toxin. Glycogen was present only in the outer third of the lobule twenty-four hours after injection of CCl_4. Three days after the injection of CCl_4 the glycogen distribution started to follow the normal pattern. Leduc and Wilson (53) showed a simultaneous dispersal of basophilic aggregates and a loss of liver glycogen in rat liver cytoplasm as early

as fifteen minutes after subcutaneous injection of CCl_4. Maximchuk and Rubinstein (54, 55) noted an almost complete disappearance of liver glycogen within eight hours of intoxication in rats. They also noted that ergotamine was partially effective in decreasing the glycogen fall after CCl_4 poisoning. Wong et. al. (56) have shown that high liver glycogen levels produced by feeding rats p-aminosalicylic acid offered some protection against the severity of the liver lesion resulting from CCl_4 intoxication. Weldon et al. (33, 57) suggested that the sulfhydryl groups of glycogen synthetase and its microsomal-linked activation system may be attacked by CCl_4. They also showed that there was no effect on overall glucose and galactose oxidation at CCl_4 levels which drastically lowered the incorporation of these sugars into glycogen.

The effect of CCl_4 intoxication on the in vivo and in vitro metabolism of sorbitol, fructose and glucose was investigated by Hoshi et al. (58, 59). In a series of communications they showed increased glucose and fructose uptake by liver slices of CCl_4-treated rats. Furthermore, uptake of sorbitol by normal liver slices was less than that of fructose, whereas in liver slices of CCl_4 intoxicated rats the reverse was true. Also, fructose caused a marked increase in pyruvate in the medium following incubation, whereas pyruvate formation was not altered by sorbitol and glucose. Infusion of glucose, fructose and sorbitol into CCl_4-treated rabbits resulted in a decreased glucose- and fructose tolerance.

Increase in blood pyruvate levels has also been observed (60) with a marked suppression of liver glycogen synthesis from glucose and fructose but not from sorbitol.

Weldon et al. (33) incubated normal liver slices in the presence of varying amounts of CCl_4 and noted that $^{14}CO_2$ production from glucose-U-^{14}C or galactose-1-^{14}C was not significantly affected. However, they found that low CCl_4 concentrations increased glycogen synthesis from these substrates while high CCl_4 concentrations were inhibitory. On the other hand, $^{14}CO_2$ production from glucose-6-^{14}C was considerably lower while that from glucose-1-^{14}C was not affected by CCl_4. From these data and data on the inhibition of succinate, palmitate, acetate and leucine oxidation, they concluded that CCl_4 in high concentrations decreased the activity of the tricarboxylic acid cycle. A similar conclusion was reached by Shigeyoki (61) who noted an increased excretion of α-ketoglutarate by CCl_4 intoxicated rabbits.

(e) CCl_4 and Enzyme Activity

A variety of enzymes can be detected in the plasma by measuring their catalytic effects on appropriate substrates. The activity of plasma enzymes in normal animals is very low and it is unlikely that they play an important metabolic role in the plasma. Notable exceptions to this

are the enzymes concerned in blood coagulation. The enzymes present in the serum are presumably derived from the breakup of cells of various tissues; therefore measurements of various enzyme activities in the serum have proved to be of some value as diagnostic and prognostic aids for assessing tissue damage.

Glutamic-oxaloacetic- and glutamic-pyruvic transaminases are present in fairly large quantities in heart muscle and liver cells. When either of these organs is damaged, i.e. heart thrombosis or necrosis of the liver, elevated quantities of the transaminases appear in the serum. Serum GOT and GPT activity measurements are standard clinical procedures in assessing liver damage due to chloromethane intoxication. In general the enzyme activity or content is increased in the serum with a concomitant decrease in the liver.

Many workers (77,78,79,80) reported increased SGOT and SGPT levels after the administration of CCl_4. Pokrovskii et al. (47) reported that increases in transaminases, fructose-1-phosphate, aldolase and acetylesterase following CCl_4 administration could be prevented by administration of promazine. Increase in serum 5'-nucleotidase activity (48) and the appearance of isocitric dehydrogenase in serum of CCl_4 intoxicated rats have also been reported (50,51). Hanson (42) reported decreased amylase levels in the serum of CCl_4-treated rats. Increase in the activity of urea cycle enzymes for a period of up to six hours following

CCl_4 treatment followed by a marked decline in activity has been noted. These changes could be prevented by the intravenous administration of aspartic acid (44). Zöllner and Raisich (45) showed a decrease in succin-oxidase and succinic dehydrogenase activities up to ten hours after administration of the toxin. The amount of CCl_4 administered only influenced the degree of change, not the time course. Eger, King and Schroder (46) noted that lipase activity was inhibited in fatty livers induced by CCl_4 or phosphorus but was increased in fatty livers produced by ethanol. Hepatic microsome DPNH-linked cytochrome C reductase was found to be 50% greater in six hours following CCl_4 treatment (17). Grebennikova (49) reported that some changes occurred in the activity of the acetylating enzyme of pigeon liver injured by CCl_4. Furthermore, on addition of CoA-SH the activity was increased or decreased depending on the time interval which had elapsed after the administration of CCl_4.

Certain types of enzymes are bound to a special type of particle which has the same sedimentation characteristics as light mitochondria. These particles are called lysosomes. They contain practically all the acid hydrolases such as acid phosphatases, ß-glucuronidase, amylsulfatase and others. Speculations as to their physiological function have been stimulated by various morphological and biochemical studies suggesting a possible involvement of lysosomes with phagocytosis, pinocytosis, autolysis and necrosis (153).

De Duve et al. (153) reported that increased amounts of hydrolytic enzymes were released from the lysosomal particles of livers of rats treated for five days with a daily subcutaneous injection of 0.2 ml of CCl_4. They studied the release of the following enzymes: acid phosphatase, cathepsin, ß-glucuronidase, acid ribonuclease and acid deoxyribonuclease from lysosomal particles. They also observed a 50 to 70% decrease in the activities of cytochrome oxidase and glucose-6-phosphatase. Similar results on the in vivo and in vitro release of lysosomal enzymes were obtained by Dianzani et al. (97). They also showed that pharmacologically active amines do not have any effect on the lysosomal changes occurring in the livers of CCl_4-treated rats.

It is readily apparent from the above cited observations that acute carbon tetrachloride poisoning affects many biologically important reactions. Although the number of investigations into the mode of action of CCl_4, its metabolism and metabolic effects are increasing, there are still many questions waiting to be answered. Among them: what is the actual toxic agent - CCl_4 or its metabolites; what is the primary effect, is it only the inhibition of protein synthesis; why do only neutral glycerides accumulate in CCl_4 intoxicated livers?

II. The Effects of Inhalation and Chronic Carbon Tetrachloride Poisoning

The high volatility of the chloromethanes presents a great hazard for their users. Statistics cited by Von Oettingen(69) show that the hazards of accidental inhalation of CCl_4 are not widely enough suspected and appreciated, particularly in places where ventilation may be inadequate. A characteristic of chronic CCl_4 exposure is that the effect of CCl_4 is additive regardless of the dose employed and the route of administration. This presents the greatest danger to its users. Post et al. (75) observed that repeated CCl_4 injury decreased the power of restoration of normal liver function.

A relatively small number of studies have been carried out on chronic CCl_4 intoxication when compared to the number of studies on acute intoxication. Furthermore, most of the chronic intoxication studies were restricted to the measurement of various enzyme activities.

Lehmann (1,70) and Lamson et al. (2) described the CCl_4 concentrations necessary to produce different stages of intoxication including anaesthesia. Both groups described mainly the behaviour of chronically intoxicated dogs. The various stages were:

(a) almost immediate restlessness at 40 mg/liter of air;

(b) increased salivation and stiffening of all muscles at 51 mg/liter within two minutes;

(c) coarse intermittent head tremors at 57 mg/liter within six minutes;

(d) loss of coordination and balance at 73 mg/liter within ten minutes;

(e) complete anaesthesia and relaxation at 133 mg/liter within fifteen minutes of placing the dogs in the exposure chamber.

After the oral administration of CCl_4 (0.5 ml/kg) to dogs three times a week for nine months and studying the following parameters

1. bromesulfatein clearance;
2. plasma cholesterol and cholesterol esters;
3. plasma protein levels;
4. serum bilirubin levels;
5. galactose clearance;
6. prothrombin concentration;

Hoffbauer (71) wrote "the results of these tests when compared with the histological appearance of the liver are disappointing." His only consistent finding was a decrease in protein concentration. Bollman (72) observed extremely low levels of prothrombin along with blood accumulation in the intestinal tract in rats chronically exposed to CCl_4 (1 ml of CCl_4/ 10 liter air/minute). The exposure time was thirty minutes three times per week until the animal died. He found that vitamin K is ineffective in raising the prothrombin levels in the presence of CCl_4.

An increase in all serum lipoprotein levels was observed (73) in rabbits injected chronically with CCl_4 (1 ml/kg twice a week for a total

of eleven injections). On cessation of CCl_4 administration the serum lipoprotein levels returned to normal. Pierce et al. (73) suggested that the increase was due to impaired function, possibly in the liver, of the degradation and synthetic systems involved.

Rats exposed to CCl_4 vapours at 1000 parts per million (ppm), 500 ppm or 100 ppm for fifty days, every day for six hours, exhibited a marked decrease in serum esterase activity. The level returned to normal after the exposure was discontinued (74).

Increased anaerobic glycolysis was observed in rats receiving three to five subcutaneous injections daily of 0.2 ml of 20% CCl_4 in olive oil. This was considered as a compensation for the decrease in oxidative glycolysis (76).

The most widely used clinical tests in assessing liver damage are the measurements of various serum enzyme activities since blood is fairly easily obtained without major or minor surgical intervention.

Block and Cornish (77,78) compared the effect of CCl_4 inhalation on various rat serum enzymes. They measured serum glutamic-oxaloacetic transaminase (SGOT), xanthine oxidase and serum esterase activities with the following results. After exposure to 250 ppm of CCl_4 for four hours only the SGOT activity was increased; at 1000 ppm or 1500 ppm both the SGOT and xanthine oxidase activities were increased and in addition, at the latter concentration, serum esterase activity was decreased. Dinman and

co-workers (79,80) measured a series of enzyme activities in rabbits exposed for six hours to 200 ppm or 500 ppm of CCl_4 vapours. They determined the following enzyme activities; glutamic-oxalacetic and pyruvic transaminase, isocitric-, malic-, glutamic-, and lactic dehydrogenase, aldolase and phosphohexose isomerase. Their conclusion was that as CCl_4-induced hepatotoxicity progressed and necrosis appeared serum enzyme activity increased. With the onset of mitochondrial alterations such as disruption of the mitochondrial membrane and loss of pyridine nucleotides, glutamic dehydrogenase appeared in the serum. In a different series of experiments they demonstrated that administration of phenergan reduces elevation of the activities of the above described serum enzymes (81).

It has been observed that the activity of ornithine transcarbamylase in the blood rises sharply in rabbits receiving 1.0 ml of CCl_4/kg (82). Rossi et al. (43,83) reported that treatment with CCl_4 for four days led to a marked decrease in the activities of all the enzymes of the urea cycle with the exception of argininosuccinase. However, Salvatore et al. (84) demonstrated the protective effect on rats of ornithine and aspartic acid in chronic CCl_4 poisoning. These compounds were regarded as catalysts for urea biosynthesis from ammonia and other nitrogen containing catabolic substances which are constantly produced by the injured liver.

A molecular complex of ATP-methionine-glutarate was effective in raising FAD and CoA-SH concentrations to normal levels in both whole liver and mitochondria of rats receiving 0.2 ml of CCl_4 per day for five days (85).

III. Mechanism of Action

The exact mechanism by which carbon tetrachloride produces its characteristic biochemical changes has not as yet been fully elucidated. The main theories regarding the mechanism of toxicity of CCl_4 in liver are discussed below.

(a) Anoxia Theory

It was observed (86) that oxygen had a protective effect on the liver during chloroform anaesthesia and a similar protection of carbon tetrachloride induced liver injury (87) has been demonstrated. It was also known that restriction of blood flow to the liver resulted in anoxia with centrilobular necrosis (88). Glynn and Himsworth (89) and Drill (52) proposed the theory that CCl_4 caused anoxia, necrosis and fatty changes of the liver. They based their theory on the similarity of the histological appearance of the liver in anoxia and in CCl_4 induced liver injury. The investigations of Glynn and Himsworth (89), Cameron (52), Loeffler (52) and McMichael (52) showed that CCl_4 and $CHCl_3$ produced parenchymal swelling which constricted the sinusoids of the liver. This sinusoidal constriction in turn resulted in ischemia followed by centrilobular necrosis.

Support for the anoxia theory came from Moore and Brody (155). They showed that mitochondrial damage caused by ligation of the blood

vessels to the liver and mitochondrial damage caused by the in vivo administration of CCl_4 are similar. Signs of mitochondrial damage such as increase in Mg^{++} - stimulated ATPase, decreased P/O ratio and decreased ability to carry out DPN-linked oxidations were common following both. Further support to this theory was given by Wakim and Mann (88) and Seneviratne (156) who showed a decrease in blood flow due to CCl_4 intoxication.

The anoxia theory was opposed by Stoner (157) who reported a fall in the temperature of the liver but no decrease in blood flow.

(b) Physical Disruption of Mitochondrial Function

Christie and Judah (90) put forward the theory that CCl_4 physically attacks the liver cell causing a change in the permeability of the cell membrane and dissolution of the mitochondrial lipid membranes thus disorganizing the respiratory enzymes located there. In a later publication by Judah (91) it was suggested that alteration of cell membrane structures allows increased amounts of Na^+ and Ca^{++} to enter. ATP which is held to be responsible for the maintenance of normal internal ion concentrations is used to a greater extent in an effort to restore normal Na^+ and Ca^{++} concentrations. This increased ATP requirement in the presence of CCl_4 may bring down the ATP to fatally low levels, or allow Ca^{++} and Na^+

ions into vulnerable sites leading to fatal cell injury. Supporting Christie and Judah's theory is the evidence supplied by Dianzani and co-workers (92,93,94,95). They presented evidence for the uncoupling of oxidative phosphorylation, decreased ATP levels and loss of mitochondrial pyridine nucleotides. Dianzani et al. (96,97) also presented evidence for increased activities of lysosomal enzymes such as arylsulfatase A and B, ribonuclease, acid phosphatase, ß-glucuronidase, cathepsin and uricase in vivo and in vitro.

Criticism of the theory of Christie and Judah was put forward by Recknagel et al. (158, 159) who pointed out that accumulation of fat in CCl_4-injured liver can be demonstrated long before the changes in mitochondrial enzymes are demonstrable. They showed that mitochondrial enzyme defects appear ten to twelve hours after administration of CCl_4 while lipid was already accumulating after two to three hours. As further support of their criticism, they showed that octanoate oxidation was unaffected by liver mitochondria from rats ten and fifteen hours post-CCl_4 feeding, whereas by this time the livers were fatty. Moreover, the concentration of CCl_4 in the liver reached its peak level one to three hours after administration which again suggested a primary site of action other than the mitochondria.

(c) The Role of the Sympathetic Nervous System in CCl_4 Poisoning

Calvert and Brody (66) observed that CCl_4 stimulates the release of catecholamines by the peripheral sympathetic nervous system. The increased catecholamine levels would lead to anoxia by constricting the blood vessels of the liver. This would result in centrilobular necrosis. Furthermore, the increase in catecholamines resulted in an increased release of fatty acids from the peripheral fat depots. Brody (98) also showed that the effects of CCl_4 could be markedly reduced by prior administration of adrenergic blocking agents and high spinal transection (99, 100). Rubinstein (55) noted a decrease in adrenal epinephrine levels, with a concomitant increase in serum epinephrine levels, two hours after the intraduodenal injection of CCl_4 in rats. A simultaneous increase in liver phosphorylase activity and blood glucose concentration was observed.

Since by four hours the serum catecholamine level has returned to normal (55), whereas Calvert and Brody (66) found increased catecholamine levels after sixteen hours or more, it is concluded that adrenalin has little to do with intoxication.

(d) Failure of Triglyceride Release by the CCl_4 Injured Liver

Recknagel, Lombardi and Schotz (18) suggested that fatty infiltration of the liver in carbon tetrachloride intoxication is due to blockage of

the triglyceride secretory mechanism. They proposed that the liver synthesizes triglycerides normally in CCl_4 poisoning but cannot release them. This view is based on the following observations. A rapid increase in liver triglycerides after oral administration of CCl_4 resulting in doubling of hepatic levels by three hours post-CCl_4, even though the mitochondrial respiratory control was still unaffected. The serum triglyceride level was greater in normal animals than in the CCl_4-treated rats after Triton injection. Triton is a surface active agent which prevents the uptake of triglyceride from the serum.

The theory of Recknagel et al. (18) was considerably extended when Seakins and Robinson (108, 109, 110, 24) demonstrated an in vivo and in vitro inhibition of protein synthesis in the liver of ethionine or CCl_4-treated rats. They suggested that the accumulation of liver lipid is due to a failure of lipoprotein synthesis. Smuckler et al. (32, 36) demonstrated decreased amino acid incorporation into the liver-produced proteins, albumin and fibrinogen. Furthermore, they showed, by electron microscopy, dislocation of ribosomes from the endoplasmic reticulum. They found no impairment of uptake of the amino acid into the liver and no loss of ability on the part of the liver to release formed protein. Smuckler and Benditt (37, 38) have shown by analytical ultracentrifugation an apparent dissociation of the 80 S ribosomes into 54 S subunits in CCl_4 intoxicated liver preparations. They concluded that the reduction in polysome

size and numbers, aegradation of ribosomes into 54 S subunits, and the decreased rate of amino acid incorporation into proteins are the immediate cause of the decreased rate of lipoprotein synthesis induced by CCl_4. Smuckler (160) did not observe any functional or structural alterations in the polysomes or in ribosomes on addition of CCl_4 and concluded that CCl_4 acts indirectly in producing the changes in polysomes in vivo.

Heimberg et al. (25, 112, 113, 114) showed that the presence of CCl_4 in isolated and perfused rat liver preparations was inhibitory towards the release of TG, PL and cholesterol into the perfusion medium. They suggested that a decrease in hepatic PL synthesis and inhibition of protein synthesis are the causes of CCl_4 produced fatty liver.

Further expansion of Recknagel's theory was brought about by the observations of Gallagher (101, 102) and Diluzio et al. (103). They have shown that treatment of rats with antioxidant substances, such as diphenyl-para-phenylene-diamine (DPPD), vitamin E, α-tocopherol and sodium selenite has a very strong preventive action on all changes induced by CCl_4. Dianzani et al. (104) suggested that an oxidation-reduction reaction takes place during the first stages of CCl_4 metabolism and such a reaction might then be concerned with peroxidation mechanisms. In fact, he demonstrated an in vitro and in vivo increased subcellular lipid peroxidation following CCl_4 intoxication.

In the presence of CCl_4, free radical attack on the double bonds

of poly-unsaturated fatty acids are characterized by diene absorption in rat liver microsomal lipids (105). Recknagel and Ghoshal (105) showed that decrease in glucose-6-phosphatase activity, which has been known to occur (17, 106), parallels the peroxidative degeneration of the lipid components of the endoplasmic reticulum. This peroxidative degeneration of the endoplasmic reticulum would lead to decreased protein synthesis resulting in a decrease in the release of lipoproteins and fatty liver.

Furthermore, Roubal and Tappel (107) demonstrated the destructive effects of transient free radicals, produced by peroxidative reactions, on a variety of proteins, resulting in denaturation and loss of enzyme activity. Desai and Tappel (161) demonstrated in vitro lipid peroxidative destruction of cytochrome C. They have incubated cytochrome C and linolenic acid in the presence of appropriate buffer solution and oxygen. Molecular oxygen is paramagnetic and bears two impaired electrons. Thus, molecular oxygen is itself a di-free radical and its reaction with organic free radicals to form corresponding organic peroxide free radicals occurs extremely rapidly (162). The following mechanism has been suggested (161) for the inactivation of proteins:

$$L \cdot + O_2 \longrightarrow LOO \cdot$$

$$LOO \cdot + PSH \longrightarrow \cdot LOOSP + \cdot H$$

$$\cdot LOOP + O_2 \longrightarrow \cdot OOLOOSP$$

$$\cdot OOLOOP + P \longrightarrow POOLOOSP$$

where L. is a linolenic free radical, LOO . a linolenate peroxy radical, PSH cytochrome C or other proteins, and . OOLOOP a multi-functional linolenate-protein peroxy radical.

Butler (127) and Wirtschafter and Cronyn (132) suggested that any of the above free radicals could react with CCl_4 to produce a trichloromethyl (.CCl_3) free radical and mono atomic chlorine (.Cl). Thus the breakdown products of CCl_4 will also propagate a peroxidative breakdown of the lipid components (arachidonic acid, linolenic acid) of the endoplasmic reticulum.

The anoxia theory for the mechanism of CCl_4 toxicity can be disregarded since it was based on histological observations only. The adrenalin theory is really an extension of the anoxia theory with some biochemical observations. These observations are contradictory though since Rubinstein showed an increased serum epinephrine level two hours after the administration of CCl_4 which returned to normal by four hours. The theory of physical disruption of mitochondrial function has been disproven by the investigations of Recknagel (158, 159).

The best working hypothesis for the mode of action of CCl_4 appears to be the expanded theory of Recknagel et al. (18). That is, the inhibition of lipoprotein release due to a failure in the synthesis of the protein moiety of the lipoprotein. The failure of protein synthesis in turn is the result of malfunctioning ribosomal particles caused by destructive lipid peroxidation reaction.

IV. Metabolism of the Chloromethanes

It has been shown that the chloromethanes are metabolized *in vivo* and *in vitro*. Since one of the metabolites could be the active toxin, information concerning their metabolism would be useful.

It was believed by Kast (117) that methylene chloride and chloroform, used as anaesthetics, were quite stable *in vivo* and were eliminated unchanged from the body. Robbins (118) found that orally administered CCl_4 was eliminated by the lungs - none being excreted by the kidneys of recipient dogs.

One of the major detoxification mechanism of the liver is conjugation of foreign molecules with glucuronic acid. Deichmann and Thomas (119) were unable to show any chloromethane-glucuronides while Gross (120) and Sperling *et al*. (121) reported that CH_2Cl_2 was converted to formate which was found in the urine of the experimental animals.

The possibility that fatty degeneration of the liver after chloroform anaesthesia is due to intracellular decomposition of the chloroform into phosgene ($COCl_2$) was first raised by Meyer and Gottlieb (122). Phosgene is a very highly reactive chemical capable of attacking many biologically active groups such as the sulfhydryl-, amino- and hydroxyl groups of proteins and enzymes.

It was observed (123) that rabbit and dog liver preparations are

able to convert chloroform to inorganic chloride. Heppel and Porterfield (124) extended this observation to methylene chloride. They also demonstrated increased chloride and formaldehyde production from added $CHCl_3$ and CH_2Cl_2 by homogenates prepared from livers of previously exposed rats. This observation indicated an inducible enzyme for the following postulated conversion:

$$CH_2Cl_2 + H_2O \xrightarrow{\text{Enzyme}} 2H^+ + 2Cl^- + HCHO$$

This enzyme lost its activity upon dialysis and ammonium sulfate fractions were inactive without the addition of cysteine or reduced glutathione. This indicates that the enzyme has one or more functional sulfhydryl groups. A five-fold increase in activity of this enzyme was observed in the 45 - 55% ammonium sulfate fraction.

Bray et al. (125) suggested that chloride ions are liberated from organic chloro-compounds as a result of non-enzymatic reaction between the chloro-compound and sulfhydryl groups of proteins, according to the general scheme:

$$R - SH + Cl - R' \longrightarrow R - S - R' + HCl$$

(where R and R' are aliphatic radicals).

They demonstrated a five-fold increase in chloride liberation from chloral hydrate in the presence of cysteine and rabbit liver extract. The native liver extract gave only 20% greater activity than boiled preparations. They assumed that $CHCl_3$ behaves similarly to chloral hydrate in the

presence of cysteine and the liver extract. However, they did not offer any experimental evidence for their assumption.

Evidence on the fate of the carbon atom of the chloromethanes began to be accumulated with the availability of ^{14}C labelled chloromethanes. McCollister et al. (126) exposed monkeys to a mixture of air and $^{14}CCl_4$ (50 ppm) obtaining the following results: 10-20% of $^{14}CCl_4$ was present as $^{14}CO_2$ in the expired air. After sacrificing the animals the highest concentration of radioactive material was associated with lipids and in decreasing amounts with bone marrow, blood, brain, kidney, heart, spleen, lung and bone. Some ^{14}C label appeared in urea, urinary carbonates and in a non-volatile urinary product.

Butler (127) using the gas-liquid chromatographic technique identified $CHCl_3$ in the expired air of dogs following the administration of CCl_4 by inhalation. He also showed the presence of $CHCl_3$ after incubating CCl_4 with mouse liver homogenates and reduced glutathione. Upon incubation of $CHCl_3$ with mouse liver homogenate a well defined peak of CH_2Cl_2 appeared on the gas chromatograms. However, Butler maintained that the dehalogenation of carbon tetrachloride is a non-enzymatic reaction, since he obtained some dehalogenation in the presence of cysteine, GSH and vitamin C in one week.

Paul and Rubinstein (128) using a similar gas-liquid chromatographic technique showed the presence of $^{14}CHCl_3$ and a very small amount of

$^{14}CH_2Cl_2$ after incubation of rat liver slices with $^{14}CCl_4$. They also reported that the expired air analyzed following the intraduodenal injection of $^{14}CHCl_3$ or $^{14}CCl_4$ in rats contained more $^{14}CO_2$ from $^{14}CHCl_3$ than from $^{14}CCl_4$. Among the tissues tested, liver slices converted $CHCl_3$ and CCl_4 to CO_2 most rapidly, kidney, adipose tissue, blood and muscle tissue showed progressively less ability to convert chloromethanes to CO_2. However, in each tissue the rate of CO_2 production from $CHCl_3$ was greater than from CCl_4. Slight stimulation of the conversion of chloromethanes to CO_2 was observed upon the addition of citric acid cycle intermediates, while inhibition occurred on treatment with iodoacetate, cyanide or arsenate. Heat denaturation (100°C for ten minutes), acids, or alkali destroyed the ability of the liver slices to convert CCl_4 and $CHCl_3$ to CO_2. They also observed that homogenization of the liver tissue resulted in a reduced ability of the tissue to convert CCl_4 and $CHCl_3$ to CO_2. In effect they demonstrated that enzymes are involved in the dehalogenation reactions.

Kanics and Rubinstein (129) reported that the addition of pyridine nucleotides to liver homogenates raised the rate of conversion of $^{14}CCl_4$ and $^{14}CHCl_3$ to $^{14}CO_2$ to that obtained with liver slices. They showed that there are at least two enzymes involved in the conversion of chloromethanes to CO_2; one in the post-microsomal supernatant, another in the microsomes.

Cessi et al. (130) incubated polylysine, albumin and liver proteins with phosgene-^{14}C and $^{14}CCl_4$. The ^{14}C labelled products were then isolated and subjected to acid hydrolysis. The liberated $^{14}CO_2$ was measured and from the similarity in $^{14}CO_2$ liberation from the phosgene-^{14}C and $^{14}CCl_4$ labelled proteins he concluded that $COCl_2$ is a metabolite of CCl_4. Phosgene produced from CCl_4 would then react with the α-amino groups of proteins and possibly with other groups, leading to cross-linked carbonyl derivatives with impaired biological activities. It was reported (131) that non-volatile products appear in the bile of sheep within fifteen minutes of oral administration of $^{14}CCl_4$. Acyl-chlorides produced from CCl_4 had also been suggested (62) as metabolites. Commoner et al. (133) presented evidence for the presence of free radicals in biological material at a level of approximately 10^{-8} mol/g. Wirtschafter and Cronyn (132) proposed that CCl_4 and $CHCl_3$ may undergo free radical initiated reactions. They postulated that the following reaction takes place

$$CCl_4 + RO\,. \longrightarrow RO : Cl + .\,CCl_3$$

where RO . may be a ready source of free radicals such as from the breaking of the very labile peroxide bond through homolytic fission.

$$R - O : O - R \longrightarrow 2\,RO\,.$$

Slater (134) proposed that CCl_4 is activated to a free radical ($.\,CCl_3$) form by interaction with normally occurring homolytic processes under

metabolic control. He based his theory on the fact that protection against CCl_4 poisoning is obtained by blocking the endogenous radical system either with antioxidants such as vitamin C or with drugs such as Phenergan which is known to have powerful free radical quenching properties (135).

Since carbon tetrachloride is metabolized by mitochondria-free preparations of rat liver homogenates and lipoproteins are synthesized on the ribosomal particles it is possible that one or more of the CCl_4 metabolites are directly involved in the inhibition of lipoprotein synthesis thus causing fatty liver.

EXPERIMENTAL

MATERIALS

Acetate-1-^{14}C, uracil-2-^{14}C, L-leucine-1-^{14}C, palmitic acid-1-^{14}C, palmitic acid-9-10-^{3}H, methylene dichloride-^{14}C and chloroform-^{14}C were obtained from New England Nuclear Corporation, Boston, Massachusetts. $^{14}CCl_4$ was supplied by Merck, Sharpe and Dohme of Canada Ltd., Montreal. Diphosphopyridine nucleotide, TPN and serum-GOT kits were obtained from Sigma Chemical Company, St. Louis, Mo. Purified phospholipids were purchased from Nutritional Biochemicals Corp., Cleveland, Ohio. All other chemicals used were of reagent grade or purer. Male hooded rats, weighing 180-200 g were obtained from the McIntyre Animal Centre, McGill University.

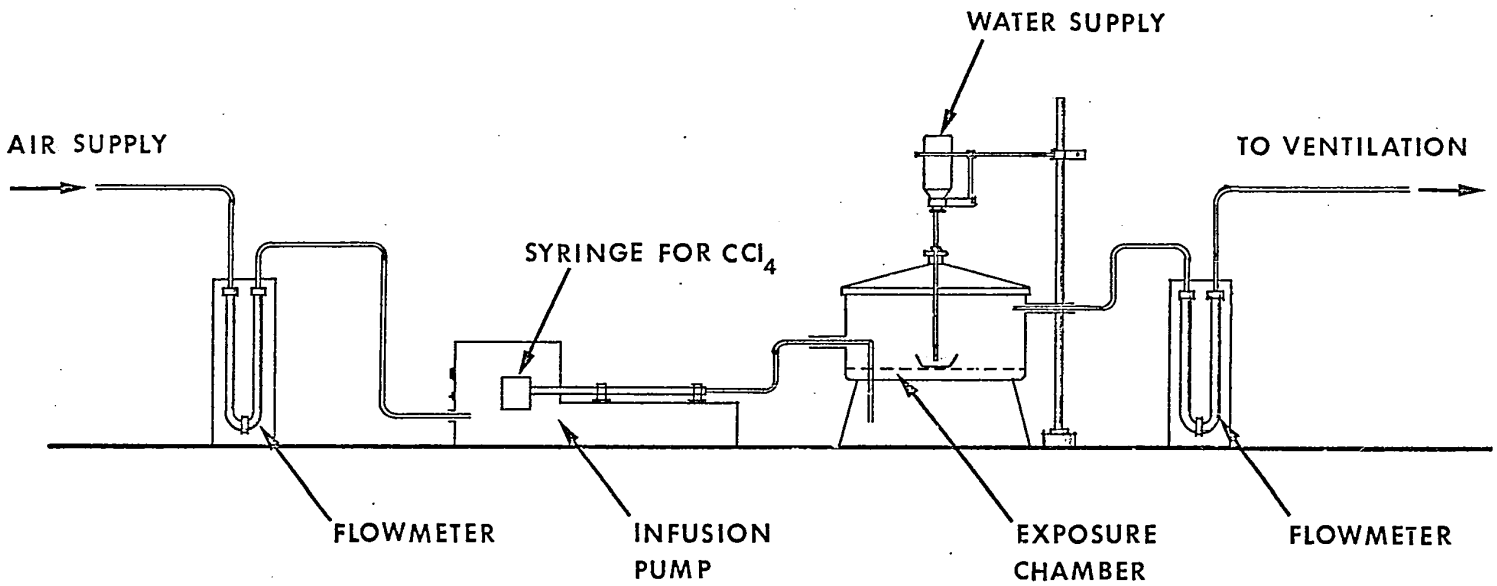
METHODS

1. Chronic Exposure

To investigate the effects of chronic CCl_4 intoxication the animals were exposed to mixtures of air and CCl_4 vapour (Fig. 1), eight hours a day for a maximum period of twelve consecutive days. The exposure chamber consisted of two dessicators equipped with a central air supply and two rubber stoppers, one of which was used as an air-CCl_4 mixture entrance and the other for exit and water supply. CCl_4 was injected into

Figure 1

Schematic Representation of the Exposure Chamber


WATER SUPPLY
AIR SUPPLY
TO VENTILATION
SYRINGE FOR CCl_4
FLOWMETER
INFUSION PUMP
EXPOSURE CHAMBER
FLOWMETER

the air supply line which was constricted in order to increase the rate of air flow insuring complete evaporation of the haloalkane. The flow of air was measured by flow meters placed before and after the exposure chamber and regulated with a needle valve. Carbon tetrachloride was injected into the air stream at a steady rate by the use of a dual infusion-withdrawal pump (Harvard Apparatus Co. Inc., Dover, Mass., Model No. 600-910, Set No. 1062). Changes in CCl_4 concentration (ppm) were achieved by either increasing or decreasing the pump speed (0.78 µl - 6.8 µl/min) and the amount of air supplied (100-120 L/hour).

It was found that the CCl_4 exposed animals lost appetite during exposure; therefore during the exposure periods both the control and the CCl_4 exposed rats were deprived of food but allowed water ad libitum.

(a) In vivo experiments: blood samples were collected from the tail, allowed to clot and the serum quickly removed. Serum-GOT activity was determined according to the method of Reitman and Frankel (136). Glycogen was isolated by precipitation with alcohol following digestion of the liver tissues in hot 30% KOH, dissolved and reprecipitated several times. Glycogen was estimated colorimetrically using the anthrone reagent (137). Lipids were extracted with chloroform-methanol (2:1) according to the method of Folch et al. (138) and free fatty acids were removed by the Borgstrom procedure (139). The neutral glycerides and phospholipids were separated on silicic acid columns (140) and estimated using the ferric

perchlorate reagent (141).

In another series of experiments the rats were exposed for a certain number of days and at the end of the exposure period the animals were anaesthetized with Nembutal. Albumin-bound palmitate-1-^{14}C was administered via the jugular vein, and blood samples were taken from the tail at the time intervals noted. Albumin-bound palmitate was prepared according to the procedure of Milstein and Driscoll (145). Serum triglycerides were isolated, counted and measured colorimetrically by the method of Van Handel and Zilversmit (146) following the removal of free fatty acids by the Borgstrom technique (139). Free fatty acids were determined by the procedure of Dole (147).

Liver lipids were isolated, measured as previously described and an aliquot counted in the liquid scintillation counter.

For the investigations on the effect of CCl_4 on serum low density lipoproteins (LDLP), animals were anaesthetized with Nembutal at the end of the exposure period. L-leucine-1-^{14}C and, thirty minutes later, albumin-bound palmitate-9-10-^{3}H were then injected into the jugular vein. A series of blood samples were taken from the tail, the first thirty minutes after the injection of palmitate. Low density lipoproteins were isolated by two different procedures.

1. Ultracentrifugal preparation of LDLP; aliquots of the separated serum samples were placed in plastic centrifuge tubes and their density

adjusted to 1.21 by the addition of concentrated NaBr solution. These samples were subjected to an average centrifugal force of 360,000 x G (60,000 rpm in SB 405 head) for sixteen hours in a preparative ultracentrifuge (International Preparative Ultracentrifuge, Model B-60). The top 1 cm of the D = 1.21 fraction was collected after puncturing the tube and its density measured and adjusted to D = 1.064 by the addition of distilled water. The samples were made up to a final volume of 3.8 ml with NaBr solution (D = 1.064) and centrifuged again at 360,000 x G for twelve hours. The top 1 cm was collected as before and its protein content measured by the Lowry procedure (142). Aliquots of the D < 1.064 fractions were dried on filter paper strips and the radioactivity measured in the liquid scintillation counter at a setting for combined counting of ^{14}C and ^{3}H with an efficiency of 64% for ^{14}C and 10% for ^{3}H.

For the isolation of very low density lipoproteins the serum densities were adjusted to D = 1.006 with distilled water and made up to volume with NaBr solution (D = 1.006). After centrifugation at 360,000 x G for sixteen hours the top 1 cm was separated by slicing the tube. The density of the remaining solution (D > 1.006) was adjusted to 1.064 with NaBr solution and centrifuged again at 360,000 x G for sixteen hours. The top 1 cm was again separated by slicing.

2. Heparin sulfate precipitation method. In some experiments the ß-lipoproteins were precipitated as a heparin-complex by the method of

Faulkner and Jordan (148) as modified in our laboratory by Buckley (unpublished). The radioactivity and protein content of the precipitate were measured as described above.

(b) In vitro experiments: liver slices (of 250-300 mg, three slices total weight) obtained from chronically exposed rats, were incubated in the presence of various substrates for two hours in Krebs-Ringer Tris buffer (pH 7.4) after gassing with pure oxygen for five minutes. $^{14}CO_2$ produced from the various substrates was absorbed on filter paper saturated with 30% KOH, dried and counted in the Packard Tricarb Liquid Scintillation Counter (Model 3003) using 0.03% of 1,4-bis-2-(5-phenyl oxazolyl)-benzene and 1.2% of diphenyloxazole dissolved in toluene as the scintillation medium at a counting efficiency of 85% for ^{14}C.

Liver proteins were obtained by homogenizing the slices in 5% trichloroacetic acid (TCA). The precipitated protein was washed several times with TCA, ethanol and ether, dissolved in formic acid, put on filter paper strips, dried, and counted in the liquid scintillation counter. Hepatic RNA was prepared according to the method of Smith and Thannhauser (143). Its ribose content was determined using the orcinol reagent (144).

II. Metabolism of the Chloromethanes

Other experiments were carried out in order to gain some insight into the fate of the chloromethanes in biological systems.

To ascertain the purity of the ^{14}C labelled chloromethanes we proceeded as follows: cold carrier carbon tetrachloride, chloroform and methylene chloride were mixed in a ratio of 1:1:1. This mixture of chloromethanes was divided into three parts, to the first part $^{14}CCl_4$, to the second $^{14}CHCl_3$ and to the third part $^{14}CH_2Cl_2$ was added to give a final concentration of 10,000 cpm/20 µl. Twenty microliters of the $^{14}CCl_4$ labelled chloromethane mixture was injected into a four foot long gas-liquid chromatographic column (30% hexadecane on chromosorb W). The fractions were eluted from the column (F & M Scientific Corporation, Model 500 Programmed Temperature Gas Chromatograph) at room temperature, with the following flow rates: carrier He 40 ml/min, air 550 ml/min, and H_2 60 ml/min. The apparatus was equipped with a hydrogen flame detector (F & M Scientific Corporation, Model 1609 Flame Ionization Attachment) which contained a sample splitter (F & M Scientific Corporation, Model 609 FD, Fraction Delivery) so that approximately 25-30% of the sample passed through the detector. The remainder of the column effluents were collected in counting vials containing toluene-phosphor and cooled in an alcohol dry ice bath by the method of Paul and Rubinstein (128). A typical

gas-liquid chromatograph is shown in Figure 2. Recoveries of the chloromethanes are given in Table I.

Investigations on the incorporation of ^{14}C labelled chloromethanes into various cell constituents were carried out as follows: male hooded rats, weighing between 180 and 220 g were decapitated and their livers quickly removed, chilled and washed with ice cold saline. Liver slices were prepared with a Stadie-Riggs microtome or a 25% liver homogenate was made in 0.33M sucrose solution using a Potter-Elvehjem homogenizer (three passes at 5000 rpm). The homogenates, containing 250 mg liver or 2 - 3 slices (total weight 250-300 mg) were incubated under the conditions indicated in the section of results. The produced $^{14}CO_2$ and liver proteins were measured as previously described.

To verify the fate of the ^{14}C label of carbon tetrachloride among the various lipid fractions a thorough separation of liver lipids was necessary. Neutral glycerides and phospholipids were separated on silicic acid columns by the procedure of Getz et al. (150). The purity of the various phospholipids was checked by thin-layer chromatography (151). Hydrolysis of the phospholipids by lecithinase C was carried out according to the method of MacFarlane and Knight (152). The hydrolyzed phospholipid components were then extracted with heptane. The water layer containing the glycerophosphatide bases was put on an ion exchange column and eluted with 2N NH_4OH. The counting of radioactivity was carried out as previously described.

Figure 2

A Typical Gas-Liquid Chromatogram of the Chloromethanes

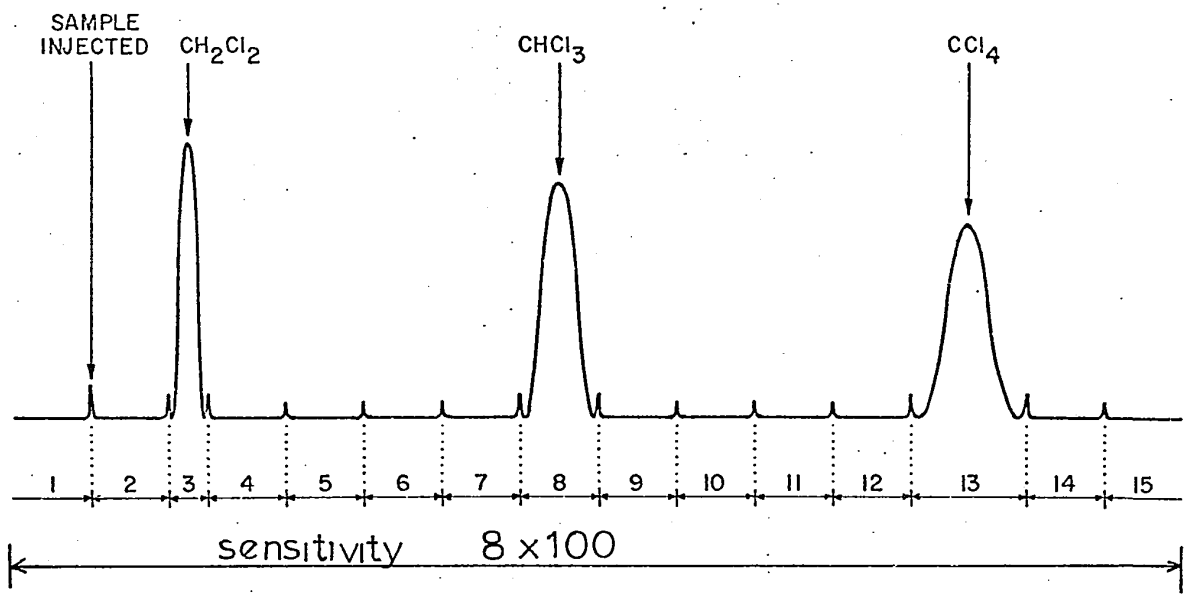

SAMPLE
INJECTED
CH_2Cl_2
$CHCl_3$
CCl_4
1
2
3
4
5
6
7
8
9
10
11
12
13
14
15
sensitivity 8 x100

TABLE I

Recovery of Chloromethanes after Gas-Liquid Chromatography

Fraction	1	2	3	4	5	6	7	8	9	10	11	12	13	14	15
Chloromethane			CH_2Cl_2					$CHCl_3$					CCl_4		
$^{14}CH_2Cl_2$	-	100	6800	75	-	-	-	-	-	-	-	-	-	-	-
$^{14}CHCl_3$	-	-	-	-	-	5	87	6575	125	-	-	-	-	-	-
$^{14}CCl_4$	-	-	-	-	-	-	-	-	-	-	20	370	6320	59	-

Each labelled chloromethane containing 10,000 cpm in 20 µl was injected into the column in the presence of all three unlabelled chloromethanes.

The optimal DPN and TPN concentrations in the presence of the chloromethanes were determined by the procedure reported earlier by the author (149).

RESULTS

I. Chronic Carbon Tetrachloride Intoxication

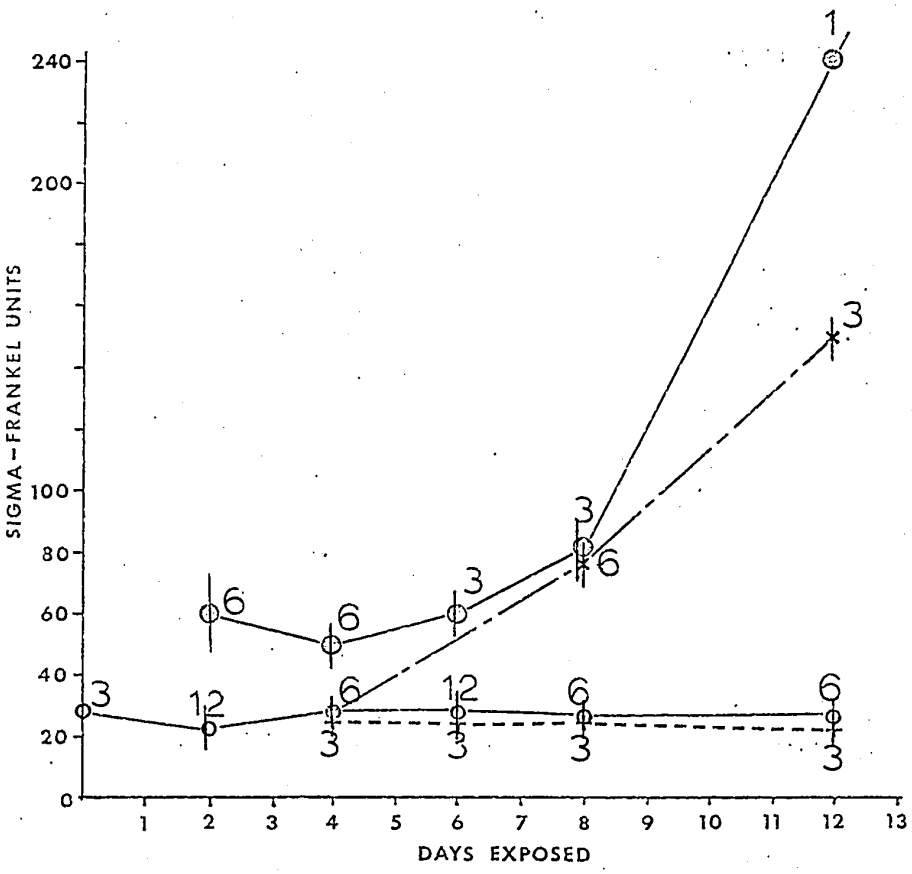
(a) Preliminary experiments

Experiments were carried out to determine the inhalation toxicity of various concentrations of CCl_4. The parameters studied were serum glutamic-oxaloacetic transaminase (SGOT) activity and liver lipid and glycogen levels. As seen in Figure 3, daily eight hour exposure to very low concentrations of CCl_4 (16 ppm) did not alter SGOT activity. As the CCl_4 concentration was increased to 68 ppm SGOT activity was elevated only after four days (eight hours per day) of exposure and continued rising with time. At the end of the eighth day of daily eight hour exposure the SGOT activity had increased three-fold and at the end of the twelfth day of exposure it had increased seven-fold over the experimental controls. When the CCl_4 concentration was further increased (680 ppm) the SGOT activity was increased by 150% after two days of exposure and reached 200% after eight days and 1000% by the end of the twelfth day of daily eight hour exposure. Thus, these results indicate that the measurement of SGOT activity cannot be used as a criterion for impaired liver function in cases where low doses of CCl_4 (0-40 ppm) are administered chronically.

The corresponding liver glycogen levels are presented in Figure 4. The liver glycogen content of the experimental controls decreased to about 20 mg/g liver by four days and remained at this level. When the

Figure 3

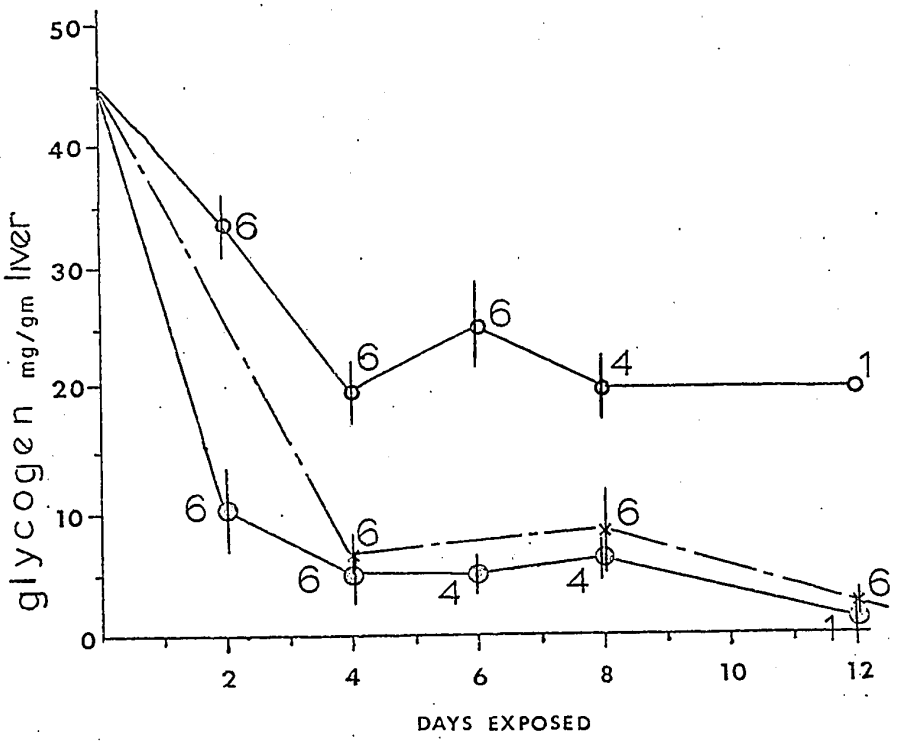
Serum GOT Levels after Exposure to Various CCl_4 Concentrations



Numbers refer to number of determination, the vertical bars represent 1 Standard Error.

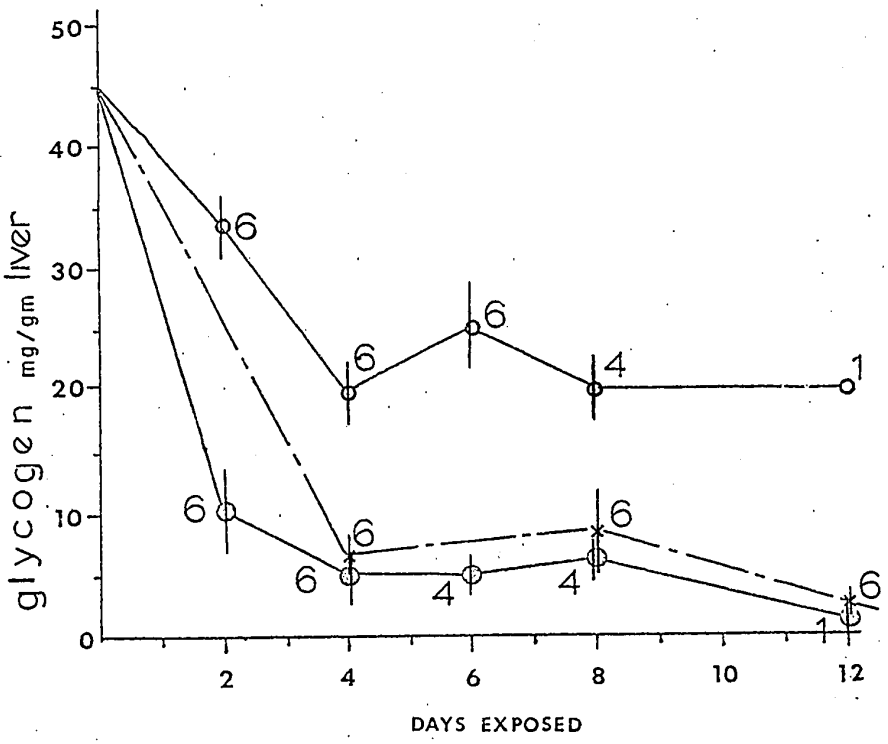
Figure 4

Liver Glycogen Levels of Control and CCl_4 Exposed Animals



o — CONTROL
× — 68 ppm
⊙ — 680 ppm

Numbers refer to number of determinations, the vertical bars represent 1 Standard Error.



o — CONTROL
x — 68 ppm
⊘ — 680 ppm

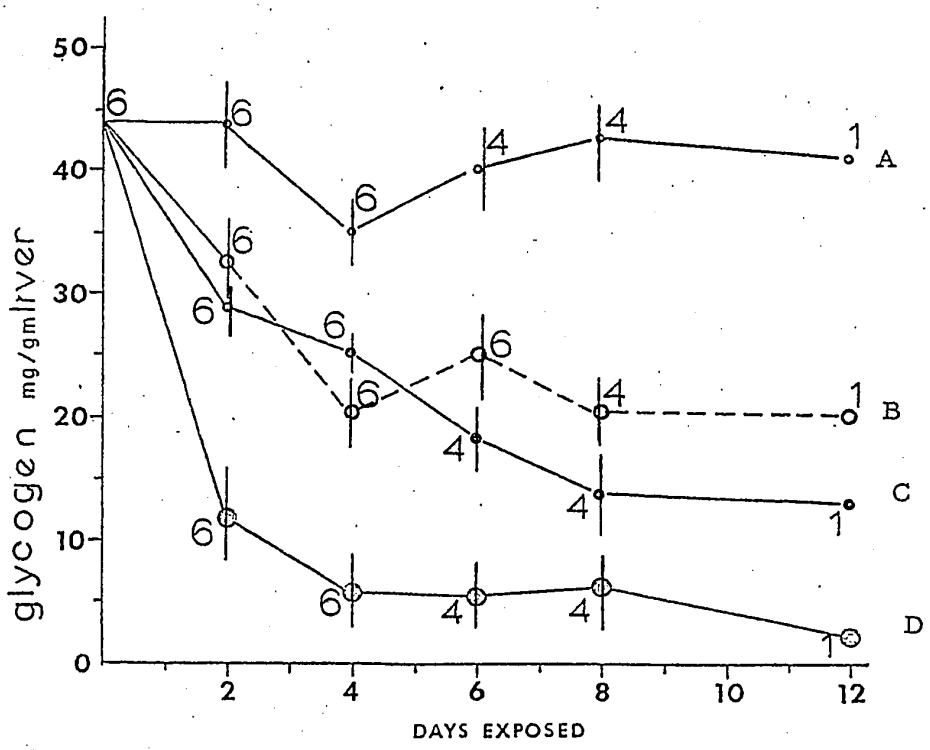
Numbers refer to number of determinations, the vertical bars represent 1 Standard Error.

CCl_4 concentration was very low (16 ppm - not indicated in Figure 4) the liver glycogen content followed very closely that of the controls. However, as the concentration of CCl_4 was increased (68 and 680 ppm) the liver glycogen content was reduced to about one half that of the controls. Since the liver glycogen level of normal fed animals is about 45 mg/g and our experimental controls showed an average liver glycogen level of about 20 mg/g after four days of eight hours per day starvation, the possibility existed that the decrease in the liver glycogen content of the CCl_4 exposed animals might also be due to starvation. To investigate this possibility we let the exposed and control animals rest, re-fed for eighteen hours, and then observed the glycogen levels. The results obtained are graphically presented in Figure 5 along with those obtained immediately after the exposure. The glycogen levels of the control animals returned to the levels of normal fed animals (57). The exposed animals tested eighteen hours after the exposure also increased their liver glycogen contents as compared to values seen immediately after exposure but never approached those of the normals. Moreover, it seemed that as the number of days of exposure increased, the ability of the liver to restore its glycogen content decreased. Thus, after two days of exposure the recovery was almost twice as efficient as that after 12 days of eight hours per day exposure.

Hepatic neutral glyceride levels of control and CCl_4-exposed

Figure 5

Liver Glycogen Levels of Control and CCl_4 Exposed Animals with 0 and 18 Hour Recovery Periods



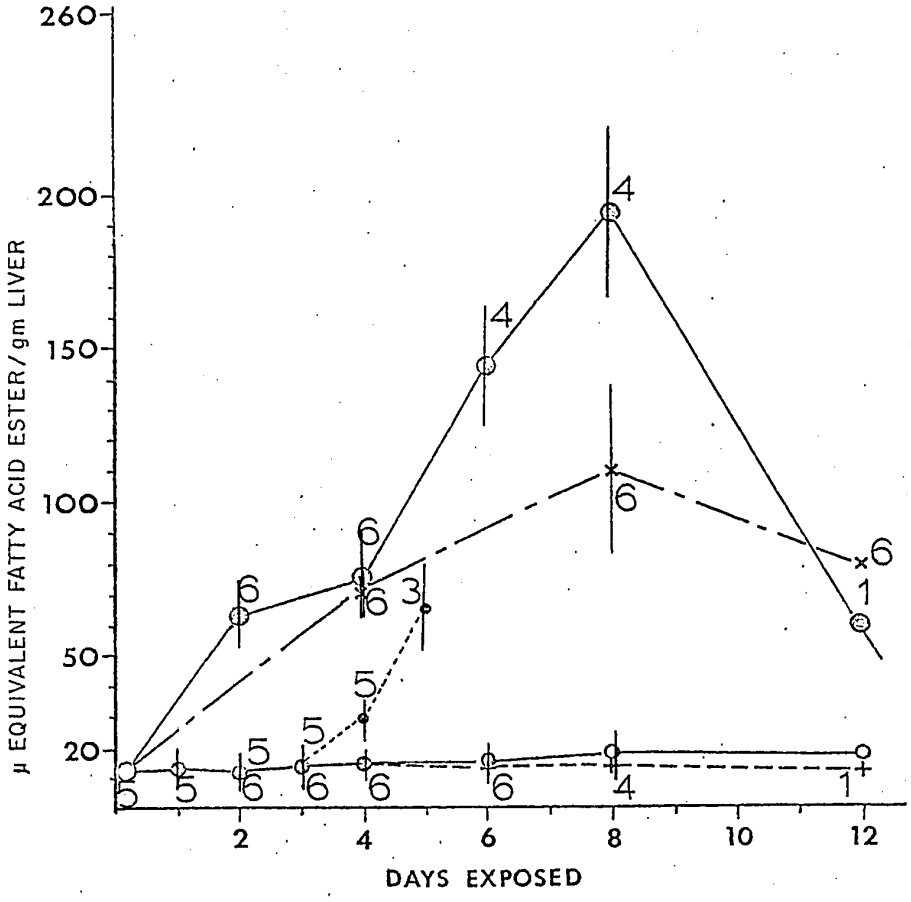
D Immediately after the exposure (680 ppm)
C 18 hours after the exposure (680 ppm)
B Control, immediately after exposure
A Control 18 hours after exposure

Numbers refer to number of determinations, the vertical bars represent 1 Standard Error.

animals are reported in Figure 6. The neutral glyceride levels at very low CCl_4 (16 ppm) concentrations were found to be normal. When the CCl_4 concentration was increased to 43 ppm there was no change in neutral glyceride levels during the first three days of daily eight hour exposure. Subsequently, the neutral glyceride content started to increase and by the fifth day of exposure it had increased about five-fold. At a somewhat higher CCl_4 (68 ppm) concentration after thirty-two hours (four days) of exposure the quantity of neutral glycerides was increased five-fold and continued to rise as the exposure period was increased. The liver neutral glyceride level reached a maximum after eight days of daily eight hours exposure. It then declined rapidly and after twelve days it was only 75% of the value obtained after eight days of exposure. A similar observation was made when the CCl_4 concentration was increased to 680 ppm; the neutral glyceride level began to rise and by two days it was six times the normal value. After eight days it had reached a maximum and further exposure again resulted in a sharp decline. The results obtained when the animals receiving 680 ppm CCl_4 by inhalation were allowed to rest and eat are presented in Figure 7. As seen these results are somewhat higher than those obtained immediately after the exposure. The peak neutral glyceride concentration occurred earlier, after six days of exposure plus eighteen hours rest, or seven days after the start of the exposure. This was followed again by a sharp decline.

Figure 6

Hepatic Neutral Glyceride Levels of Control and CCl_4 Exposed Animals

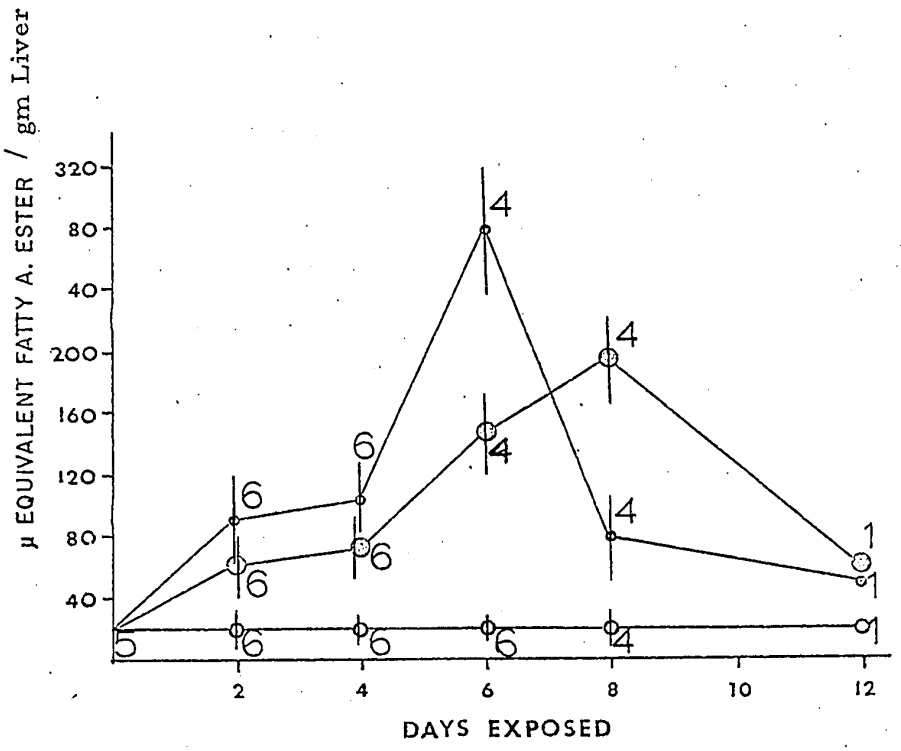


⊙ — 680 ppm
× — 68 ppm
∘ — 43 ppm
O — CONTROL
\+ — 16 ppm

Numbers refer to number of determinations, the vertical bars represent 1 Standard Error.

Figure 7

Hepatic Neutral Glyceride Levels of Control and Exposed Animals with 0 and 18 Hour Recovery Periods



⊙ — 680 ppm
⊙ — 680 ppm RESTED
○ — CONTROL

Numbers refer to number of determinations, the vertical bars represent 1 Standard Error.

Comparison of the two curves of Figure 7 suggests that fasting offers some protection against the effects of CCl_4 inhalation.

(b) In vitro experiments

Horning et al. (19) and Maximchuk and Rubinstein (20) demonstrated that the increase in liver triglycerides by a single dose of CCl_4 was the result of increased mobilization of fatty acids from the adipose tissue. Therefore, the possibility that the increase in liver neutral glyceride content of CCl_4 exposed (68 ppm for five days eight hours per day) animals was due to an increase in the ability of the liver to esterify fatty acids was investigated in vitro. Results presented in Table II indicate a significantly ($p < 0.01$) increased lipogenesis from acetate-1-^{14}C by liver slices of chronically exposed rats. However, neither the rate of acetate-1-^{14}C oxidation nor its incorporation into phospholipids was affected. When palmitate-1-^{14}C was used as the substrate no significant differences were observed either in its metabolism or in its rate of esterification, although the evidence suggested that palmitate metabolism was slightly increased in liver slices of the CCl_4 exposed animals.

Another possible mechanism by which fat could accumulate in the liver may lie in the lipid secretory mechanism. It has been shown by Seakins and Robinson (24, 163) that repeated injections of puromycin

TABLE II

Effect of Chronic CCl_4 Exposure on in vitro Incorporation of Acetate-1-^{14}C and Palmitate-1-^{14}C into CO_2 and Hepatic Lipids

Substrate	Acetate-1-^{14}C cpm/g liver		p**	Palmitate-1-^{14}C cpm/g liver		p
	Normal (3)	CCl_4 Exposed*(3)		Normal (6)	CCl_4 Exposed*(6)	
Neutral glycerides	9172 ± 1094	22255 ± 2558	0.01	76767 ± 9623	92166 ± 9704	N.S.
Phospholipids	8541 ± 178	9276 ± 917	N.S.	34383 ± 8000	34008 ± 4855	N.S.
CO_2	253329 ± 15436	242831 ± 23793	N.S.	3344 ± 349	4853 ± 804	N.S.

Each flask contained liver slices (250-300 mg) in 3.0 ml of Krebs-Ringer solution with 125 µmoles of Tris buffer (pH 7.4). Concentration of acetate and palmitate were 1 µM containing 620,000 cpm or 720,000 cpm respectively. Incubation time: two hours. Results are presented as the Arithmetic Mean ± Standard Error. The numbers in brackets indicate the number of experiments.

*Animals exposed to 68 ppm of CCl_4 for eight hours per day for five days.

**Significance: CCl_4 vs control calculated by student's "t" test.

into rats resulted in fatty livers similar to those seen in CCl_4 intoxication. That is, protein synthesis is necessary for the release of lipid from the liver. This possibility was investigated by studying the incorporation of L-leucine-1-^{14}C and uracil-2-^{14}C into liver proteins and RNA respectively, by liver slices of chronically exposed rats. These results are shown in Table III. Liver slices were prepared using a Stadie-Riggs microtome (0.5 mm) and were incubated in the presence of all amino acids as suggested by Heimberg et al. (25) for two hours. It appeared that liver slices from chronically exposed animals incorporated more leucine than the controls. Even though the difference is not statistically significant it is probably a valid finding and suggestive of a regenerating liver. When the animals were acutely intoxicated (0.2 ml/100 g b.w.) the rate of incorporation of leucine showed a marked decline ($p < 0.01$).

The incorporation of uracil-2-^{14}C into liver RNA was not affected by either chronic or acute poisoning after two hours of incubation.

(c) In vivo experiments

A third possibility for the accumulation of liver lipids in chronic carbon tetrachloride poisoning could be a failure in the release of the lipid-protein complexes. To investigate this aspect the chronically intoxicated (five days, eight hours daily) animals were injected immediately

TABLE III

Effects of CCl_4 on *in vitro* Incorporation of L-Leucine-1-^{14}C and Uracil-2-^{14}C into Liver Proteins and RNA Respectively

	Protein			RNA		
Mode of CCl_4 administration	No. Exp.	Specific activity (cpm/µg protein)	p***	No. Exp.	Specific activity (cpm/100µg ribose)	p
Control	9	57.1 ± 4.1	-	4*	13.7 ± 0.6	-
Chronic*	10	80.6 ± 11.8	N.S.	5	14.7 ± 0.8	N.S.
Acute**	5	14.3 ± 2.3	0.01	5	11.8 ± 2.1	N.S.

Results are presented as the Arithmetic Mean ± Standard Error. Each flask contained 1.3 µmoles L-leucine-1-^{14}C containing 400,000 cpm or 0.1 µmoles uracil-2-^{14}C containing 3.7×10^{-6} cpm and all amino acids as suggested by Heimberg et al. (25). For incubation conditions see Table II.
*Animals were exposed to 68 ppm of CCl_4 for eight hours per day for five days.
**0.2 ml CCl_4 per 100 g body weight injected into the duodenum, three hours previously to the removal of the liver.
***Significance: CCl_4 vs. control.

after the exposure with albumin-bound palmitate-1-^{14}C via the jugular vein. The results shown in Table IV indicate that at fifteen and thirty minutes both the quantity and radioactivity of serum triglycerides were significantly lower ($p < 0.01$) in the serum of the exposed animals. However, the specific activities at these time intervals were not significantly different. All the noted differences had disappeared after sixty minutes.

As further support of the above observation the liver neutral glyceride levels sixty minutes after the injection of the palmitate-1-^{14}C are presented in Table V. These results showed that the liver neutral glyceride levels were much higher in the CCl_4 exposed animals, although the specific activities were the same. The liver phospholipid levels indicated that the exposed animals incorporated more palmitate than the controls into this fraction. This was indicated by the significant difference in their specific activities.

To obtain a more complete view of the fate of the injected palmitate-1-^{14}C, a more intensive time-activity study was carried out. Figure 8 shows that the CCl_4 injured liver released labelled triglycerides at a slower rate than the controls. This difference in the rate of release was statistically significant at fifteen minutes after the injection of the palmitate in animals exposed to 68 ppm of CCl_4, while at this time the rate of release was not significantly decreased in animals exposed to 55 ppm. The maximum rate of release of labelled serum triglycerides occurred thirty

TABLE IV

Effect of CCl_4 Exposure on Serum Triglyceride Levels after the Injection of Palmitate-1-^{14}C

Time	μequivalent FAE ml serum			cpm/ml serum			specific activity cpm/μequiv.FAE		
	Control (6)	Exptl.*(5)	p**	Control	Exptl.	p	Control	Exptl.	p
15	1.22 ± 0.13	0.53 ± 0.14	0.01	11202 ± 1104	6120 ± 973	0.01	9794 ± 1490	6607 ± 728	N.S.
30	1.01 ± 0.06	0.51 ± 0.08	0.01	10143 ± 3322	4989 ± 995	N.S.	8777 ± 1409	7875 ± 976	N.S.
60	0.70 ± 0.07	0.69 ± 0.06	N.S.	1538 ± 486	2208 ± 437	N.S.	2761 ± 1076	3099 ± 215	N.S.

Results are presented as the Arithmetic Mean ± Standard Error. The numbers in brackets indicate the number of experiments.

*Animals exposed to 68ppm of CCl_4 for eight hours per day for five days, then injected via the jugular vein 2.1 μmoles per 100 g body weight of albumin-bound palmitate-1-^{14}C containing 2×10^6 cpm.
**Significance: CCl_4 vs. control.

TABLE V

Hepatic Lipid Levels of Control and CCl_4 Exposed Animals Sixty Minutes Post Intravenous Injection of Palmitate-1-^{14}C

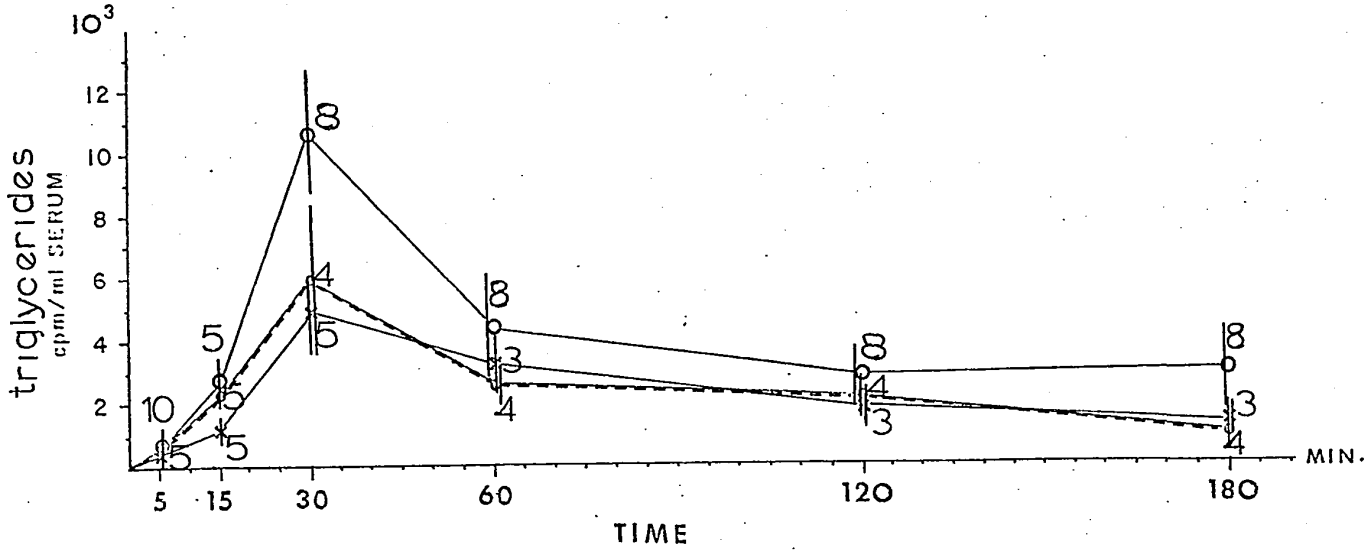
Lipid fraction	cpm/g liver			μequiv. FAE g liver			specific activity cpm/μequiv. FAE		
	Control (6)	Exptl.*(6)	p**	Control	Exptl.	p	Control	Exptl.	p
Neutral glycerides	26233 ± 2269	180072 ± 23560	0.01	7.5 ± 0.4	42.7 ± 6.5	0.01	3454 ± 285	4781 ± 1142	N.S.
Phospho-lipids	33020 ± 2872	128756 ± 24610	0.01	44.2 ± 7.0	62.8 ± 3.9	N.S. (0.06)	766 ± 101	1989 ± 257	0.01

Results are presented as the Arithmetic Mean ± Standard Error. The numbers in brackets indicate the number of experiments.

*Exposure conditions and amount of palmitate-1-^{14}C injected were same as in Table IV.
**Significance: control vs. CCl_4.

Figure 8

In Vivo Incorporation of Palmitate-1-^{14}C into Serum Triglycerides



o — CONTROL
o — 55 ppm
x — 68 ppm

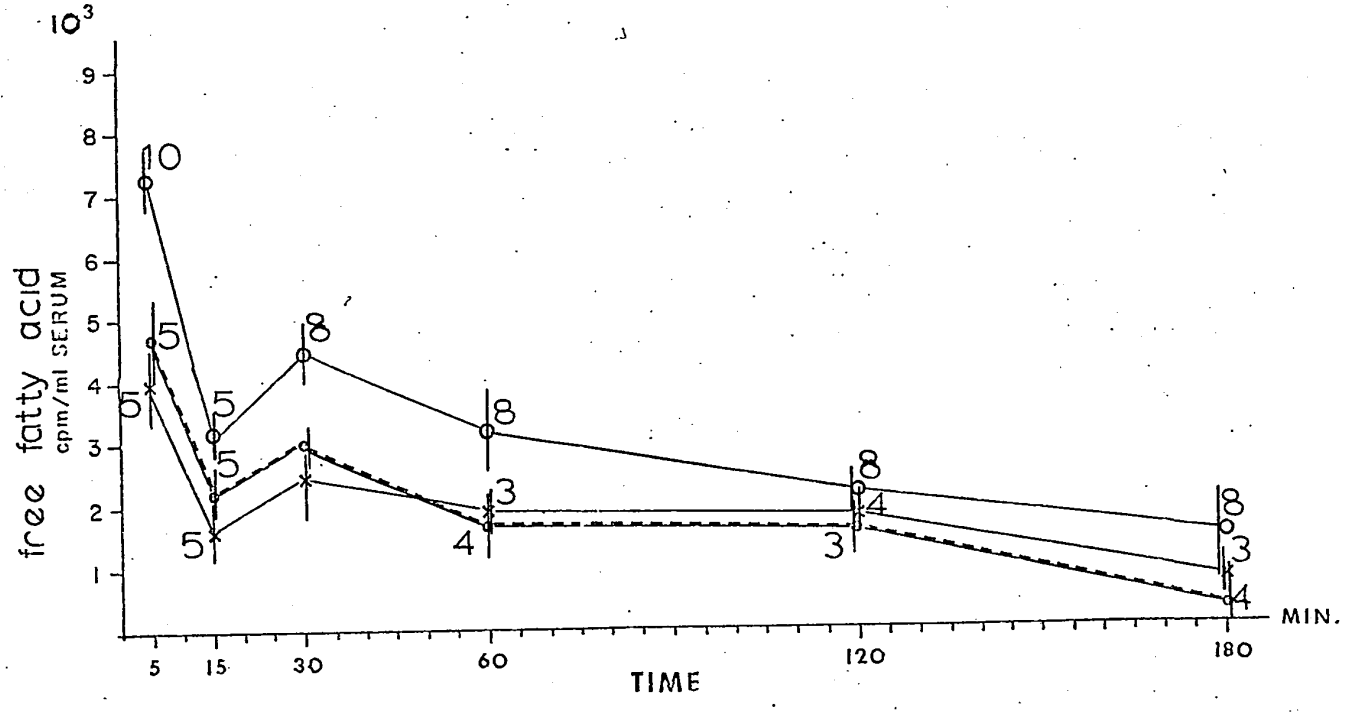
Numbers refer to number of determinations, the vertical bars represent 1 Standard Error.

minutes after the injection of the palmitate in both the control and the exposed (55 - 68 ppm) animals. The rate of release by the latter groups was significantly lower ($p < 0.01$) than the controls. One hour after the injection of the palmitate the above differences were equalized.

The differences in the release of triglycerides from the livers may not be the only way by which fat can accumulate in the CCl_4 poisoned liver. It is possible that aside from inhibition of the release of triglycerides from the liver the uptake of free fatty acids by the poisoned liver might be increased. Therefore, the level of unesterified fatty acids remaining in the serum was also measured. These results are shown in Figure 9. As seen in Figure 9, the CCl_4 exposed (55 ppm) liver took up free fatty acids faster ($p < 0.01$) at any point than the corresponding control animals. This difference was further increased with increased CCl_4 concentration (68 ppm). The secondary increase that occurred at thirty minutes after the injection of the palmitate in all samples is possibly due to the fact that the maximal rate of release of lipoproteins labelled with ^{14}C-palmitate occurred at this time. Furthermore, evidence obtained in our laboratory suggests that the free fatty acids can be released by the liver attached to lipoproteins. Increased uptake of serum free fatty acids could result in increased neutral glyceride formation and therefore the liver neutral glyceride levels were also measured three hours after the injection of palmitate. These results are

Figure 9

Serum Free Fatty Acid Levels after I.V. Injection of Palmitate-1-^{14}C



o — CONTROL
o — 55 ppm
× — 68 ppm

Numbers refer to number of determinations, the vertical bars represent 1 Standard Error.

presented in Table VI. The liver neutral glyceride levels showed similar results to those in Table V, that is, increased incorporation of labelled palmitate into neutral lipid by the exposed animals. Furthermore, the specific activity of the neutral lipid was much lower in the experimental than in the controls. There was no difference in palmitate incorporation into phospholipids.

It has been shown in our laboratory that both the rate of appearance and level of labelled lipoproteins are fairly constant for an individual animal (Figure 10A). It is well known that the maximal appearance of labelled lipid in newly synthesized lipoproteins is approximately thirty minutes after the introduction of labelled albumin-bound palmitate (22, 30).

To obtain a direct correlation for the effects of chronic CCl_4 exposure on individual animals, we decided to carry out further experiments as follows. Normal animals deprived of food eight hours per day for five days were used as controls. At the end of the five day period the animals were injected with L-leucine-1-^{14}C followed thirty minutes later by the administration of palmitate-9-10-^{3}H, both via the jugular vein. Samples were then taken at the specified intervals. A few days after this procedure blood samples were taken and radioactivity measured. We found no measurable radioactivity present either in the protein or in the lipid moieties of the lipoprotein. The animal was then exposed for five days,

TABLE VI

Hepatic Lipid Levels of Control and CCl_4 Exposed Animals

Three Hours after the Intravenous Injection of Palmitate-1-^{14}C

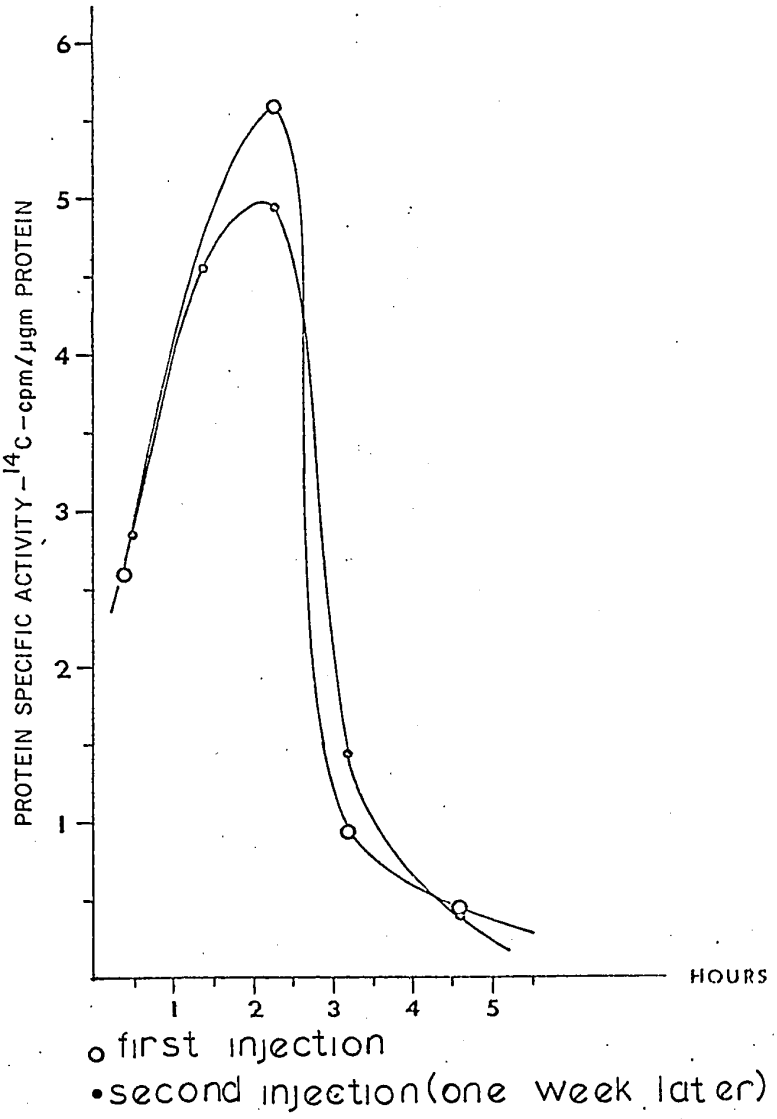
Lipid fraction	cpm/g liver			μequiv.FAE/g liver			specific activity cpm/g liver		
	Control (4)	Exptl.*(4)	p**	Control	Exptl.	p	Control	Exptl.	p
Neutral glycerides	32812 ± 5617	100749 ± 11248	0.01	11.7 ± 1.6	70.9 ± 12.8	0.01	2796 ± 224	1574 ±296	0.01
Phospho-lipids	139098 ± 8984	111390 ± 13292	N.S.	114.4 ± 8.0	100.0 ± 5.1	N.S.	1218 ± 32	1130 ±161	N.S.

Results are presented as the Arithmetic Mean ± Standard Error. The numbers in brackets indicate the number of experiments.

*Exposure conditions and amount of palmitate-1-^{14}C injected were same as in Table IV.
**Significance: control vs. CCl_4.

Figure 10A

Rate of Appearance of Low Density Lipoproteins Following Intravenous Injection of L-Leucine-1-^{14}C


PROTEIN SPECIFIC ACTIVITY – ^{14}C – cpm/µgm PROTEIN
6
5
4
3
2
1
HOURS
1
2
3
4
5
o first injection
• second injection (one week later)

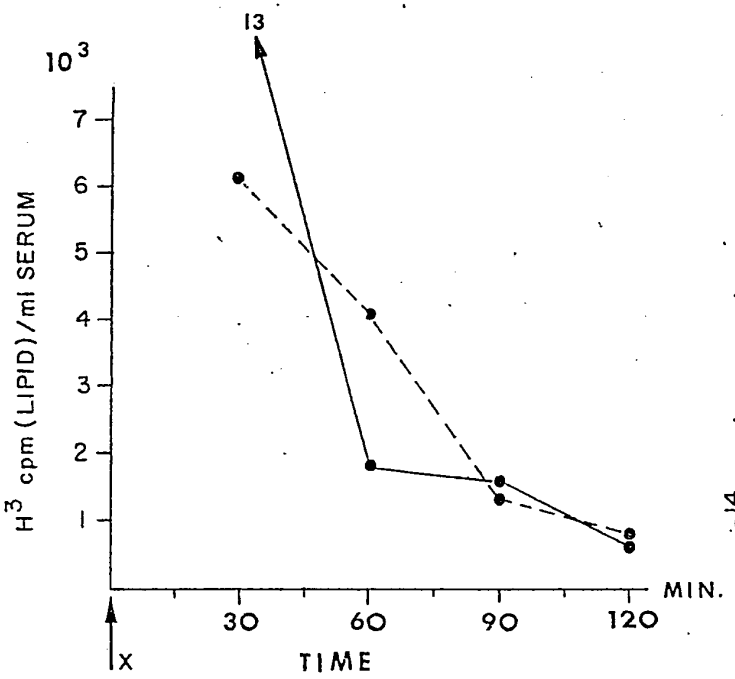
eight hours per day this time to 68 ppm of CCl_4. At the end of the exposure period the animals were injected with L-leucine-1-^{14}C followed thirty minutes later by albumin-bound palmitate-9-10-^{3}H, as before. Thus each animal served as its own control. A typical pattern obtained under these conditions is shown in Figure 10B. Similar results were observed in four out of five experiments although the actual counts per minute per milliliter of serum varied greatly. As seen in Figure 10B the lipid component of the low density serum lipoprotein was lower only at the thirty minute point after chronic CCl_4 exposure. From thirty to ninety minutes the lipid component decreased very slowly and two hours after the injection of albumin-bound palmitate the amount of labelled palmitate incorporated into low density lipoproteins was identical to the control.

The protein component of the LDLP complex showed that sixty minutes after the injection of L-leucine-1-^{14}C the rate of incorporation of leucine was slower after the animal had been exposed to CCl_4. Furthermore, the peak which occurred sixty minutes after the injection of leucine in the control animal was delayed by thirty minutes in the CCl_4 exposed animal. Moreover, the second peak which occurred two hours after the injection of leucine in the control was absent in CCl_4 exposed animals.

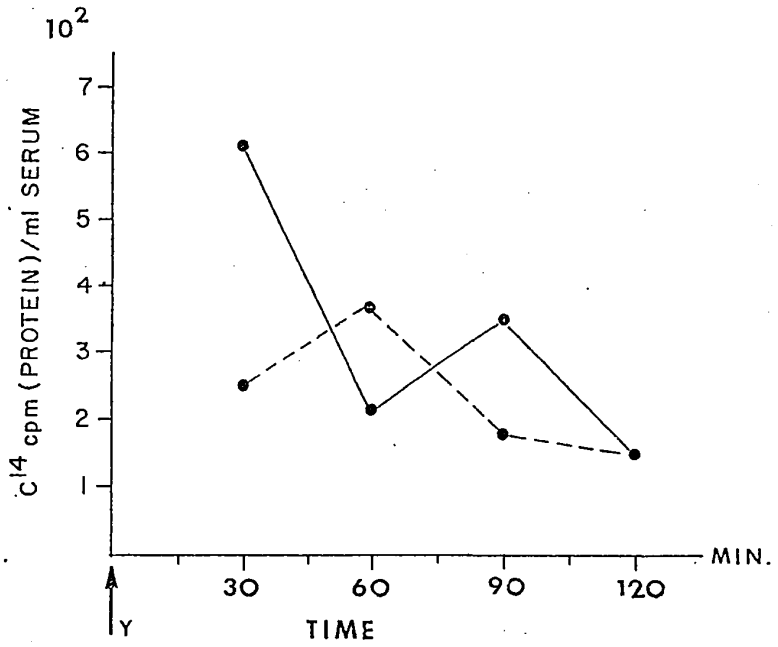
Measurement of the rate of lipoprotein secretion was approached from two additional directions. Firstly, we changed the isolation procedure for LDLP from ultracentrifugation to precipitation by complexing the ß-lipo-

Figure 10B

Typical Serum Low Density (D < 1.064) Lipoprotein Levels Before and After Chronic CCl_4 Exposure



—— BEFORE
– – – AFTER
X = PALMITATE INJECTION.



—— BEFORE
– – – AFTER
Y = PALMITATE INJECTION 30 min. AFTER LEUCINE INJECTION.

proteins with heparin, thus reducing the possibility of contamination of the low density lipoprotein fraction ($D < 1.064$) by α-lipoprotein or by albumin. The second approach involved a ten-fold increase in CCl_4 concentration. That is, the animals were exposed to 680 ppm of CCl_4 for five days, eight hours per day, instead of exposing them to 68 ppm. We felt that by increasing the CCl_4 concentration ten-fold the effects of chronic exposure would approach those of acute intoxication.

The animals were exposed to 68 ppm and 680 ppm of CCl_4 for daily eight hour periods over five days. At the end of the exposure period all three groups of animals received 2.1 μmoles of albumin-bound palmitate-1-^{14}C containing 2×10^6 cpm/100 g b.w. via the jugular vein, thirty minutes prior to taking blood samples. The results are shown in Table VII. As seen, the lipid specific activities of the heparin-precipitated ß-lipoprotein after a total of forty hours of exposure to 68 ppm of CCl_4 were not statistically different from the controls ($p = 0.06$). However, when the CCl_4 concentration was increased ten-fold, the difference between the lipid specific activities of the normal and exposed animals was highly significant ($p < 0.01$). As indicated, the serum free fatty acid levels were equally reduced by exposure to 68 ppm or 680 ppm of CCl_4. Although the differences in the specific activities of the serum free fatty acids were statistically insignificant, the differences between the serum free fatty acid levels of control and exposed animals (68 ppm or 680 ppm) were highly

TABLE VII

Specific Activities of Serum Free Fatty Acids and Heparin-Precipitated ß-Lipoproteins Following I.V. Injection of Palmitate-1-^{14}C

	Normal (6)	Exposed			
		68 ppm (3)	p*	680 ppm (4)	p
LDLP-lipid cpm/µg TG	95 ± 13	55 ± 15	N.S. (0.06)	30 ± 13	0.01
Serum FFA µeq/ml serum	0.80 ± 0.05	0.53 ± 0.08	0.02	0.53 ± 0.02	0.01
(cpm/µefa)	2698 ± 176	3172 ± 660	N.S.	2793 ± 259	N.S.

Results are presented as the Arithmetic Mean ± Standard Error. The numbers in brackets indicate the number of experiments. Animals were exposed to 68 and 680 ppm of CCl_4 eight hours per day for five days. 2.1 µmoles of albumin-bound palmitate-1-^{14}C containing 2×10^6 cpm/100 g body weight was injected via jugular vein thirty minutes prior to taking blood samples.

*Significance: control vs. CCl_4.

significant. Corresponding lipid levels are presented in Table VIII. Thirty minutes after the injection of albumin-bound palmitate the liver neutral glyceride specific activities were significantly lower regardless of the CCl_4 concentration employed. This decrease in specific activity is the result of higher amounts of non-labelled lipid present in the exposed livers since both the uptake of palmitate and its incorporation into neutral glycerides by the exposed animals were actually increased.

Specific activities of the liver phospholipids were the same for the control and animals exposed to 68 ppm of CCl_4 while those of animals exposed to 680 ppm of CCl_4 were significantly lower.

It was noted earlier (Table VII and Figure 9) that when animals were chronically exposed to CCl4 vapour (eight hours per day for five days), serum free fatty acids were taken up at a faster rate by the injured livers. This increased uptake of serum free fatty acids could result in either an increase in the liver unesterified fatty acid (UFA) pool or an increase in the turnover rate of UFA in the livers of exposed animals. This possibility was investigated and the results obtained are also presented in Table VIII. As seen, the unesterified fatty acid contents of the CCl_4 intoxicated livers were the same as those of controls. That is, CCl_4 intoxication did not alter the UFA pool size in the liver. However, the specific activities of UFA in the exposed livers were significantly lower than the controls. This is possible if the turnover rates of UFA in the

TABLE VIII

Liver Lipid Levels of Control and CCl_4 Exposed Animals 30 Minutes Post Intravenous Injection of Palmitate-1-^{14}C

Fraction	Units	Control (6)	Exposed			
			68 ppm (3)	p*	680 ppm (4)	p
Neutral glycerides	cpm/g liver	163000 ± 11500	240000 ± 10200	0.01	256000 ± 10700	0.01
Specific activity	cpm/µg TG	58.0 ± 3.1	8.1 ± 2.1	0.01	8.1 ± 1.1	0.01
Phospholipids specific activity	cpm/µg TG	39.5 ± 3.1	32.8 ± 9.6	N.S.	23.3 ± 2.9	0.01
Unesterified fatty acids	µeqFA/g liver	0.55 ± 0.07	0.55 ± 0.06	N.S.	0.57 ± 0.03	N.S.
Specific activity	cpm/meFA	27.6 ± 1.9	15.5 ± 4.5	0.05	15.1 ± 2.0	0.01

Conditions: See Table VI. Results are presented as the Arithmetic Mean ± Standard Error.
*Significance: control vs. CCl_4, calculated by Student's "t" test.

exposed animals were increased.

Experiments were conducted to measure protein in the low density lipoproteins precipitated by heparin. Animals were exposed to CCl_4 eight hours daily for five days. Immediately after the last exposure, blood samples were taken followed by the intravenous administration of 1.2 µmoles of L-leucine-1-^{14}C containing 2.6×10^6 cpm/100 g b.w. One hour after the injection of leucine, blood samples were taken and the serum ß-lipoproteins were precipitated with heparin sulfate. These results are presented in Table IX. There was no chemically measurable difference in the protein levels of ß-lipoproteins due to the injection of leucine. The ß-lipoprotein-protein levels and the amount of ^{14}C label incorporated into this fraction by the exposed animals (680 ppm) was lower. This is suggestive of either a decrease in protein synthesis or an inhibition of the release of ß-lipoprotein.

It has been reported that acute CCl_4 poisoning mostly affects the very low density ($D < 1.006$) lipoproteins (25, 26). Therefore, it was decided to investigate this in chronically intoxicated rats.

Animals were exposed to 68 and 680 ppm of CCl_4 for five days, eight hours a day. The rats were injected, immediately after the last exposure, with 1.2 µmoles of L-leucine-1-^{14}C and thirty minutes later with 2.1 µmoles of albumin-bound palmitate-9-10-^{3}H per 100 g b.w. containing 2.6×10^6 cpm and 2×10^6 cpm respectively. One hour after

TABLE IX

Protein Levels and Specific Activities of Heparin-Precipitated ß-Lipoproteins Following I.V. Injection of L-Leucine-1-^{14}C

Protein	Normal (4)	Exposed	
		68 ppm (5)	680 ppm (3)
Before injection µg/ml serum	152 ± 24	170 ± 17	91.1 ± 22
After injection µg/ml serum	161 ± 21	175 ± 15	127 ± 22
cpm/ml serum	1813 ± 267	1596 ± 221	1281 ± 158
Specific activity cpm/µg	11.1	9.1	10.5

Results are presented as the Arithmetic Mean ± Standard Error. Numbers in brackets indicate the number of experiments. Animals were exposed to 68 and 680 ppm of CCl_4 for eight hours per day for five days. 1.2 µmoles of L-leucine-1-^{14}C containing 2.6×10^6 cpm per 100 g body weight was injected via the jugular vein sixty minutes prior to taking blood samples.

the injection of leucine blood samples were taken and separated. The serum density was adjusted with NaBr to D = 1.006 and the very low density lipoproteins (VLDLP) were separated by ultracentrifugation. Results obtained are presented in Table X. Although the values obtained at low CCl_4 concentration (68 ppm) are lower than the controls there was no significant difference in either the lipid or the protein moieties of VLDLP.

At the higher CCl_4 concentration (680 ppm) the lipid moiety of the VLDLP was approximately 60% less than the controls. Lipid specific activities showed a similar decrease. The protein component of VLDLP in these animals (680 ppm) was approximately 35% of the controls; however, the protein specific activity of the exposed animals was twice that of the controls.

After removal of the very low density lipoproteins, the density of the samples was adjusted to D = 1.064 with NaBr and the low density lipoproteins (LDLP) separated by ultracentrifugation. These results are presented in Table XI. As seen, there is no quantitative difference in the lipid moieties of the LDLP regardless of CCl_4 concentration employed. The lipid specific activity of the LDLP after exposure to 680 ppm of CCl_4 was similar to that of the controls. After 68 ppm of CCl_4 intoxication the lipid specific activity of the LDLP fraction was twice that of the controls.

The protein moiety of the LDLP (μg/ml serum) was decreased

TABLE X

Effect of Chronic Carbon Tetrachloride Exposure on Serum Very Low Density (D < 1.006) Lipoprotein Levels

Fraction	Very Low Density Lipoproteins (D < 1.006)				
	Control (3)	68 ppm (4)	p	680 ppm (3)	p
Lipid					
μgTG/ml serum	497 ± 18	425 ± 55	N.S.	179 ± 40	0.01
cpm/ml serum	56523 ± 6205	40334 ± 3191	N.S.	8731 ± 345	0.01
specific activity (cpm/μgTG)	108.9 ± 9	96.9 ± 7.1	N.S.	40.8 ± 11	0.01
Protein					
μg/ml serum	266 ± 13	212 ± 26	N.S.	102 ± 25	0.01
cpm/ml serum	1594 ± 82	1219 ± 179	N.S.	1074 ± 118	0.02
specific activity (cpm/μg protein)	6.0 ± 0.31	5.81 ± 0.80	N.S.	11.59 ± 1.30	0.01

Results are presented as the Arithmetic Mean ± Standard Error. Numbers in brackets indicate the number of experiments.

Significance: control vs. CCl_4 calculated by Student's "t" test.

TABLE XI

Effect of Chronic Carbon Tetrachloride Exposure on Serum Low Density (D< 1.064) Lipoprotein Levels

Fraction	Low Density Lipoproteins (D< 1.064)				
	Control (3)	68 ppm (4)	p	680 ppm (3)	p
Lipid					
µgTG/ml serum	115 ± 8	107 ± 5	N.S.	119 ± 4	N.S.
cpm/ml serum	848 ± 38	1456 ±296	N.S.	852 ± 47	N.S.
specific activity (cpm/µgTG)	7.33± 0.90	16.0 ± 1.8	0.01	7.02± 0.40	N.S.
Protein					
µg/ml serum	255 ± 18	201 ± 2	0.02	161 ± 3	0.01
cpm/ml serum	925 ± 147	680 ± 39		941 ± 57	N.S.
specific activity (cpm/µg protein)	4.04± 0.30	3.40± 0.1	N.S.	5.96 ± 0.31	0.01

Results are presented as the Arithmetic Mean ± Standard Error. Numbers in brackets indicate the number of experiments.
Significance: control vs. CCl_4 calculated by Student's "t" test.

approximately 25% in animals exposed to 68 ppm and it was decreased approximately 50% after exposure to 680 ppm of CCl_4. The protein specific activity at low CCl_4 concentration (68 ppm) was not significantly different from that of the controls. Exposure to 680 ppm of CCl_4 resulted in an approximately 50% increase in protein specific activity.

The results obtained in these experiments (VLDLP and LDLP) clearly demonstrate that chronic CCl_4 intoxication inhibits either the release or formation or both, of these VLDLP and LDLP fractions.

II. Metabolism of the Chloromethanes

(a) Incorporation of chloromethanes into liver components

It has been shown that liver slices and homogenates are able to convert CCl_4 and $CHCl_3$ to CO_2. Although the ability of liver homogenates to convert these two chloromethanes to CO_2 was greatly diminished this was due to a dilution of the intracellular coenzymes(129).

To investigate the possibility that the chloromethanes may be attached to various lipid and protein components liver slices and homogenates were incubated in the presence of ^{14}C labelled haloalkanes. The results obtained are shown in Table XII. As seen, $^{14}CHCl_3$ and $^{14}CH_2Cl_2$ were converted to $^{14}CO_2$ by liver slices and homogenates at about the same rate and more rapidly than $^{14}CCl_4$.

The three labelled chloromethanes were incorporated into liver proteins to the same extent. However, the amount of ^{14}C label incorporated into homogenate proteins was twice as high as that incorporated by slices. Carbon tetrachloride-^{14}C was incorporated into liver lipids more readily than were $^{14}CH_2Cl_2$ and $^{14}CHCl_3$. The total lipid fraction was separated into its components by column chromatography. The results of a typical experiment obtained are shown in Table XIII. About 80% of the total radioactivity from $^{14}CCl_4$ was incorporated into phospholipids. Among the phospholipids, phosphatidyl serine, inositol and lecithin contained most of

TABLE XII

Incorporation of Chloromethanes into Lipid, Protein and CO_2 by Rat Liver Slices and Homogenates

Chloromethane added	$C^{14}O_2$ cpm/g liver		Lipid cpm/g liver		Protein cpm/g liver	
	slice	homogenate	slice	homogenate	slice	homogenate
$C^{14}Cl_4$	2558 ± 208	3483 ± 347	3742 ± 803	2343 ± 440	1274 ± 24	3143 ± 274
$C^{14}HCl_3$	6326 ± 324	6114 ± 362	1492 ± 348	385 ± 32	1094 ± 198	2472 ± 430
$C^{14}H_2Cl_2$	7488 ± 453	6669 ± 714	1593 ± 239	594 ± 39	1694 ± 406	2746 ± 106

Results are presented as the average of four experiments ± Standard Error. Each flask contained liver slices or an equivalent weight (250 mg) of 25% homogenate in 0.33M sucrose, made up to 3.0 ml with Krebs-Ringer solution containing 0.2M Tris buffer (pH 7.4), 10 µmoles of DPN and 0.2 ml 30% KOH in the centre well. After gassing for five minutes with pure oxygen 10 µl (1.0×10^6 cpm) of the chloromethane was injected into the side arm of the flask and allowed to volatilize. Incubation time: two hours at 37°C.

TABLE XIII

The Incorporation of $C^{14}Cl_4$ into various Lipid Fractions by Liver Slices

Fraction	cpm/g liver
Total lipids	6,600
Neutral lipids	1,290
Phospholipids	4,150
Serine phosphatides	910
Ethanolamine phosphatides	360
Inositol and lecithin	1,340
Sphingomyelin	550

Incubation conditions: as in Table XII.

the radioactivity.

When the phospholipids were subjected to the action of lecithinase C (Table XIV) practically all the incorporated ^{14}C label was recovered in the fraction containing the phosphate esters of the bases.

Since the conversion of CCl_4 to CO_2 is an enzymatic process (128, 129) the question arose whether or not the incorporation of CCl_4 into phospholipids was also enzymatic. Purified phospholipids were incubated in the presence of Krebs-Ringer medium and $^{14}CCl_4$. The results, presented in Table XV, show that $^{14}CCl_4$ was incorporated into phospholipids to an approximately equal extent indicating a non-enzymatic reaction. Since CCl_4 is a very stable molecule it was rather surprising to find that it was, non-enzymatically, incorporated into phospholipids.

Another possibility was that the purified phospholipids may have contained some lipid-bound enzyme as an impurity. To clarify this point it was decided to incubate the purified phospholipids in hexane:$CHCl_3$ (1:1) medium and later in CCl_4. These results are also shown in Table XV. In both incubation mediums, hexane:$CHCl_3$ (1:1) and in CCl_4, the amounts of labelled $^{14}CCl_4$ incorporated into the phospholipids are practically identical.

The fact that an isotope dilution effect was not observed (during incubation with CCl_4) suggested that the $^{14}CCl_4$ used in the experiment

TABLE XIV

Distribution of Radioactivity from $C^{14}Cl_4$ Following Hydrolysis of Phospholipids by Lecithinase C

Fraction	Experiment		
	(1)	(2)	(3)
Total phospholipids	6740	5300	3900
Unhydrolyzed phospholipids	0	200	0
Fatty acids	0	0	0
Phosphate ester of base	6000	4700	3410

Total phospholipids obtained after elution from silicic acid columns were digested with phospholipase C (Cl welchii). Liberated lipids extracted with heptane. Bases were eluted with 2N NH_4OH from a Dowex 50 (H^+) column.

TABLE XV

A Typical Experiment on the Incorporation of $C^{14}Cl_4$ into Purified Phospholipids

Purified Lipid	Incubation medium		
	Krebs-Ringer Tris cpm	Hexane:$CHCl_3$ cpm	CCl_4 cpm
Dipalmityl lecithin	720	750	-
Distearoyl lecithin	1035	615	-
Phosphatidyl ethanolamine	1150	1040	1490
L-α-lecithin	1060	1000	970
Lysolecithin	740	850	-
Phosphatidyl serine	1130	1200	850
Sphingomyelin	820	850	-

Each flask contained 10 mg phospholipid in 3 ml of medium indicated, and 10 µl of CCl_4 containing 1.5×10^6 cpm. Incubation time: two hours at 37°C.

contained some impurity. However, infra-red and nuclear magnetic resonance studies, carried out in the Department of Chemistry, McGill University, shed no light on whether or not any impurity was present in the ^{14}C-labelled carbon tetrachloride. As a further test of purity the peak observed upon gas-liquid chromatography after injecting $^{14}CCl_4$ into the column was collected in two fractions. Fraction one (before the peak was reached) was the ascending side of the peak and fraction two was the descending side including the peak itself. These two fractions were incubated with liver slices and the results obtained are shown in Table XVI. As seen, both fractions were incorporated into lipids, protein and CO_2 to an approximately equal extent. From these results it appears that there was some impurity in the $^{14}CCl_4$ preparation, otherwise fraction two should have been incorporated to a much greater extent since its specific activity is five times that of fraction one. Attempts to further isolate this impurity have thus far not been carried out.

(b) Reduction of DPN in the presence of chloromethanes

It was reported that diphosphopyridine nucleotide (DPN) is a necessary cofactor for the metabolism of the chloromethanes (129). Therefore, experiments were carried out in a Beckman DU spectrophotometer at 340 mμ in order to measure the formation and re-oxidation of DPNH by liver homogenates in the presence of chloroform.

TABLE XVI

Incorporation of Various CCl_4 Fractions into CO_2, Lipid and Protein by Liver Slices

	Fraction one cpm/g liver	Fraction two cpm/g liver
CO_2	143 ± 7	258 ± 11
Lipid	184 ± 29	135 ± 32
Protein	146 ± 21	138 ± 17

Flasks were allowed to equilibrate at 37°C for five minutes. Other incubation conditions same as in Table XII. Each flask contained from Fraction one 12,000 cpm in 10 µl of CCi_4, from Fraction two 60,000 cpm in 10 µl of CCl_4. Results presented are the average of five experiments ± Standard Error. Numbers in brackets indicate the number of experiments.

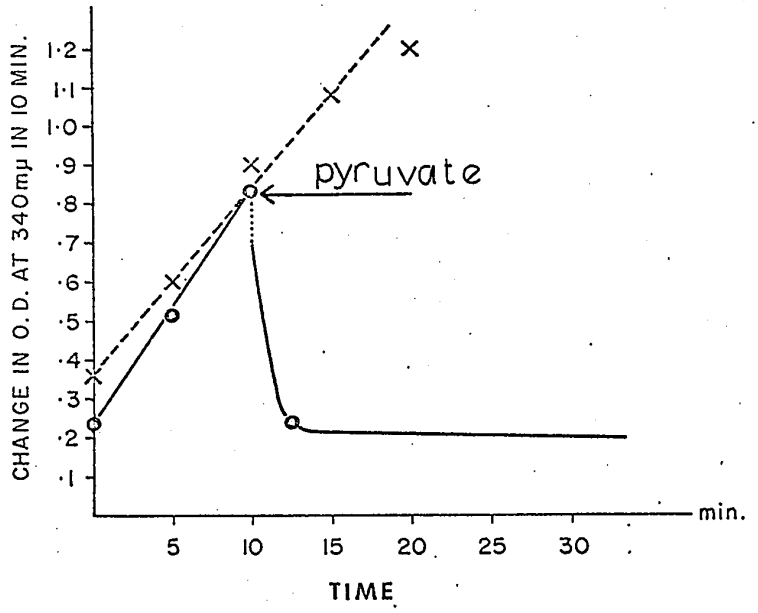
The results obtained are graphically presented in Figure 11. As seen, DPN is reduced proportionally to time. Furthermore, the addition of pyruvate and lactic dehydrogenase resulted in an almost immediate decrease in optical density. This indicates that DPN is reduced in the presence of chloroform and liver homogenate.

Table XVII contains evidence that the enzyme responsible for the reduction of DPN in the presence of $CHCl_3$ is present only in the final supernatant (homogenate free of microsomes). It also indicates that the enzyme is specific for DPN since addition of triphosphopyridine nucleotide did not result in an increase in optical density at 340 mμ.

Enzyme-substrate specificity studies and experiments on optimal chloromethane concentrations for the reduction of DPN by liver homogenates were also carried out. These results are presented in Figure 12. As seen, the enzyme appears to have a much higher specificity toward $CHCl_3$ than toward CCl_4 or CH_2Cl_2, since more DPN was reduced in the presence of $CHCl_3$ than in the presence of CCl_4 or CH_2Cl_2. Optimal chloromethane concentrations were found to be 2 mμmoles for $CHCl_3$ and CCl_4 and 20 mμmoles for CH_2Cl_2.

Figure 11

Formation of DPNH by Liver Extracts and in the Presence of $CHCl_3$



× DPN

°DPN, Lactic Dehydrogenase, Pyruvate

TABLE XVII

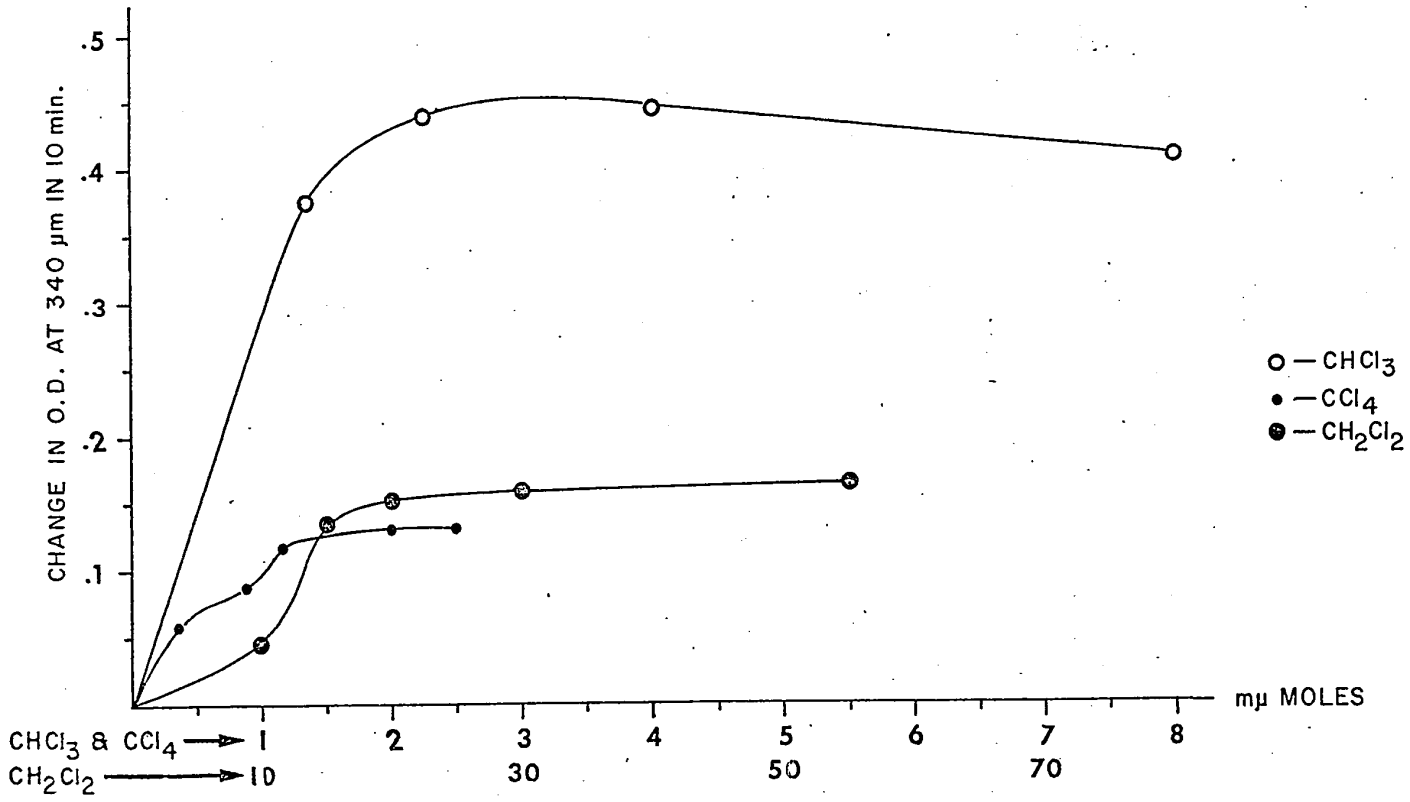
Reduction of Pyridine Nucleotides by the Subcellular Components of Rat Liver Homogenates in the Presence of $CHCl_3$

Fraction	Change in optical density at 340 mμ	
	DPN	TPN
Whole homogenate	0.200	0.0
Supernatant (free of nuclei)	0.190	0.0
Mitochondria	0.0	0.0
Microsomes	0.0	0.0
Supernatant (mitochondria-free)	0.210	0.0
Supernatant (free of microsomes)	0.205	0.0

Each cuvette contained: 0.05 ml of subcellular fraction, 5 μmoles of DPN, 0.03 M Tris buffer, pH 8.25 saturated with chloroform and Krebs-Ringer solution to 3.0 ml. Temperature: 37°C.

Figure 12

Optimal Chloromethane Concentrations for the Reduction of DPN by Rat Liver Homogenates


CHANGE IN O.D. AT 340 μm IN 10 min.
.5
.4
.3
.2
.1
O — $CHCl_3$
• — CCl_4
⊛ — CH_2Cl_2
mμ MOLES
$CHCl_3$ & CCl_4 → 1
CH_2Cl_2 → 10
1
2
3
30
4
5
50
6
7
70
8

DISCUSSION

I. Chronic CCl_4 Intoxication

As pointed out earlier acute CCl_4 poisoning is very rare. On the other hand, the effects of CCl_4 inhalation are not widely enough recognized. In the past investigations on chronic CCl_4 inhalation were mostly concerned with measurements of various serum enzyme levels and activities and some histochemical estimations of liver glycogen control. Direct comparison of the effects of chronic inhalation and acute intoxication is extremely difficult due to the fact that different responses were observed when the same dose of CCl_4 was administered by different routes (6). However, as will be seen in the following pages, the effects of chronic CCl_4 inhalation are in general in good agreement with those of acute intoxication.

Chronic inhalation of low concentrations of CCl_4 did not affect the serum GOT levels. At medium CCl_4 concentration the SGOT levels were affected only after four days of exposure while chronic inhalation of very high concentrations of CCl_4 resulted in an almost immediate release of GOT into the serum (Fig. 1). These results show that the liver is able to handle only small amounts of inhaled CCl_4; furthermore, they indicate that as the dose of CCl_4 is increased the number of damaged cells increases (as indicated by the rapidly rising SGOT levels).

These results are similar to those obtained by Rubenstein (55) seven hours after a single intraduodenal injection of CCl_4.

Liver glycogen fell almost immediately when rats were exposed to medium or high concentrations of CCl_4. The initial drop was followed by a low but relatively stable glycogen level which after the eighth consecutive day of exposure further declined (Fig. 2).

Some loss of the liver glycogen, in these chronically exposed rats, was due to the particular conditions used during exposure. However, comparison of curves C and D of Figure 3 indicates a partial reversibility especially in the early periods of exposure. These observations support the findings of Weldon et al. (33) who incubated normal liver slices in the presence of CCl_4, and those of Maximchuk and Rubinstein (20) in acutely intoxicated rats. Both groups suggested that the physical presence of CCl_4 decreases the glycogen storage capacity of the liver and that liver amylase is activated by Cl^- ions which accompany the influx of Na^+ and Ca^{++} ions into the injured liver. However, the decrease in liver glycogen level after the eighth day of in vivo exposure is probably due to a loss of structural carbohydrate components following cell destruction.

Comparing Figures 3, 4 and 6 a fine separation of lipid accumulation from necrosis can be observed. Lipid accumulation occurred up to the eighth day of exposure with a low but stable glycogen level while the SGOT level was steady although slightly increased. After this point

necrosis occurred, characterized by rapid destruction of liver cells with a very rapid increase in the SGOT activity and a loss of liver glycogen and neutral glycerides. Exposure to medium and high doses of CCl_4 produced an almost immediate rise in liver neutral glycerides. The level reached a maximum around the eighth day of daily eight hour exposure and then declined rapidly.

Lipid accumulation in the livers of acutely intoxicated rats, as pointed out in the introduction, is due to an inhibition of lipoprotein synthesis. The causes of pre-necrotic lipid accumulation in the livers of chronically exposed rats will be discussed in the following pages.

The amount of FFA in the serum of rats exposed to 68 ppm of CCl_4 eight hours per day over a period of five days was found to be lower than the controls (Table V). This decrease in the level of serum FFA could be the result of increased uptake of FFA by the liver or a drop in the synthesis of carrier albumin by the intoxicated liver. A combination of the two effects seems most likely.

Maximchuk and Rubinstein (20) showed an increase in serum free fatty acids eight hours after acute intoxication; however, Lombardi and Ugazio (28), also working with acutely intoxicated animals, were unable to confirm this finding. The results presented in this thesis, showing a decrease in serum FFA, can be partially accounted for by a difference in the mode of administration of the toxin.

In support of the arguments for a decreased level of FFA, Smuckler et al. (32) have shown a decrease in the FFA carrier, albumin, in rats acutely poisoned with CCl_4. It has also been reported (20) that acute CCl_4-treatment results in an increased discharge of catecholamines which increase the transport of free fatty acids from the peripheral fat depots to the liver. The decreased carrier level and increased mobilization should result in an increase in the turnover of the FFA which should be reflected in the specific activities of the serum free fatty acids of the chronically exposed rats. After injection of albumin-bound palmitate-1-^{14}C the specific activity of the serum free fatty acids of the exposed animals should be much greater than the controls, since there is less FFA present in the exposed animals. However, there was no significant difference between the serum FFA specific activities of the chronically exposed and control animals at thirty minutes after the injection. This is possible only if FFA from the serum of exposed rats is taken up faster. Further supporting evidence for the increased uptake and turnover of serum FFA in chronically exposed animals is indicated by the increased disappearance of injected labelled palmitate (Fig. 7). Hence, we have shown that there is an increase in the turnover rates of serum free fatty acids and this could lead to increased esterification in the livers of chronically intoxicated rats.

Experiments with liver slices from chronically exposed rats showed increased lipogenesis from acetate. The rate of palmitate esterification or

CO_2 production from both acetate and palmitate was unchanged. The increased lipogenesis may be due to an inhibition of acetyl-CoA oxidation through the tricarboxylic acid cycle or, more likely, to a loss of reduced pyridine nucleotides from the mitochondria as suggested by Dianzani (52).

The above noted increased lipogenesis from acetate and the unchanged rate of palmitate esterification are similar to the findings of Weldon et al. (33) on incubating normal liver slices in the presence of CCl_4. However, we found the amount of CO_2 produced by liver slices of chronically intoxicated rats was the same as the amount produced by the controls while Weldon et al. (33) obtained a decrease in CO_2 production by normal liver slices in the presence of CCl_4. This difference is probably due to the amount of CCl_4 present in the liver slices since Weldon et al. (33) observed a progressive decrease in CO_2 production as the CCl_4 concentration was increased.

Evidence from Table VIII suggests that the pool sizes of unesterified fatty acids in the livers of exposed and control animals are not different. The amount of labelled free fatty acid released back into the circulation by the liver is likely negligible. Therefore it appears that the labelled palmitate is incorporated into neutral glycerides more rapidly by chronically exposed rats, since the liver unesterified fatty acid specific activities of the exposed animals were decreased thirty minutes after the injection of

palmitate.indicating faster turnover of unesterified fatty acids in the livers of exposed rats. These in vivo observations are in agreement with those of Schotz et al. (30) who reported a 50% increase in the rate of free fatty acid esterification in rats acutely intoxicated with CCl_4.

The increase in the rate of esterification of palmitate by the livers of chronically exposed animals should also be reflected in the amount of labelled triglycerides present in the liver. As seen in the in vivo results presented in Tables V, VI, VIII, the amount of ^{14}C-palmitate incorporated into liver neutral glycerides is in fact much greater in the exposed animals. This observation is similar to those obtained in acutely intoxicated rats by other investigators (30, 20, 21).

The increased fatty acid turnover, both in the liver and in the serum of the exposed animals therefore resulted in an increase in the synthesis and deposition of neutral glycerides. This increase should be accompanied by an increase in the serum triglyceride levels. Results presented in Table IV and Figure 6 show that the rate of appearance of newly synthesized triglycerides in the serum is similar in both the control and in the exposed animals. The appearance of labelled triglyceride reaches a maximum thirty minutes after the injection of palmitate in both; however, the total radioactivity appearing in the serum of exposed rats was lower than the controls. These results suggest a decrease in lipoprotein secretion although it is possible that the slower rate of lipid release as

measured by the radioactivity of serum triglyceride is only due to the lower specific activity of the liver lipids in the exposed animals. This seems unlikely in view of the fact that protein synthesis is lowered in exposed animals as will be shown. Further supporting evidence for the decreased release of TG into the serum is the fact that the amount of TG present in the serum of the exposed animals was found to be lower than that in the controls. The above findings are similar to those obtained in vivo by Schotz et al. (30) in acutely intoxicated rats and to those of Heimberg et al. (25) in isolated rat livers perfused in the presence of CCl_4.

L-leucine-1-^{14}C and uracyl-2-^{14}C were incorporated into total liver proteins and RNA respectively to a greater extent by liver slices of chronically exposed rats. This is probably the result of regenerating processes, which are known to occur (62,63,64). This, however, was a rather surprising finding since it was reported by Smuckler et al. in vitro (36,37,38) and in vivo by Seakins and Robinson (24) that acutely intoxicated livers incorporated less L-leucine into hepatic protein. We have confirmed the findings of Smuckler et al. in acute intoxication. Furthermore, we have also observed a slight decrease in the in vitro incorporation of uracyl-2-^{14}C into hepatic RNA with acute poisoning. The inhibition of protein synthesis in acutely intoxicated animals was attributed (37) to a direct effect of CCl_4 on the microsomal protein synthesizing system. The slight

decrease in the incorporation of uracyl into hepatic RNA may be explained by the activation of lysosomal RNAase. This activation of lysosomal RNAase in the livers of animals acutely intoxicated with CCl_4 has been established in vivo and in vitro by several laboratories (17, 67).

Since essentially all the TG in the serum are present as lipoproteins it was safe to assume that the decrease in serum TG levels, in the chronically exposed animals, represented a decrease in lipoprotein levels.

Studies on the release of newly synthesized low density ($D < 1.064$) lipoprotein (LDLP) levels indicated that the newly synthesized lipid moiety of the LDLP appeared at a slower rate than the controls in the serum of rats chronically exposed to low levels of CCl_4 (68 ppm). This is suggestive of an interference either in the release of preformed LDLP or in the rate at which the protein moiety of the LDLP is synthesized. Interference with the synthesis of the protein moiety of the LDLP is supported by the decreased incorporation of L-leucine into LDLP. Moreover, it has been shown in our laboratory by Mr. T. J. Delahunty that when L-leucine-1-^{14}C is repeatedly injected into normal animals the rate of appearance of labelled low density lipoprotein in the serum is constant for an individual animal. The absolute values of radioactivity and the concentration of the protein moiety may be somewhat different without influencing the shape of the curve (Fig. 10A). L-leucine-1-^{14}C incorporation into LDLP was diminished on chronically exposing the animals and the time of maximum appearance of label in this

fraction was delayed by thirty minutes (Fig. 10B).

The delay is possibly due to a larger pool of unlabelled leucine in the livers of chronically exposed rats, while the lower incorporation of ^{14}C label indicates an interference with the synthesis of the protein moiety of the LDLP.

Serum ß-lipoprotein levels in chronically exposed rats one hour after leucine injection were not significantly different when measured by the heparin sulfate precipitation method, although the values obtained at high CCl_4 concentration suggest a decrease in the ß-lipoprotein levels. To draw the correct conclusions on lipoprotein levels in chronically exposed animals is extremely difficult. Measurements on the synthesis and release of lipoproteins are done very shortly, within two hours after the injection of the precursors, and a slight decrease in the synthesis of these complexes may not be clearly seen. Furthermore, it seems from the available literature that there is a contradiction in the results obtained in acute intoxication experiments. Heimberg et al. (25, 112, 113, 114), working with isolated perfused rat livers and Brown et al. (29), Aiyar et al. (111) and others (115, 116), working with in vivo systems have reported a decrease in the synthesis and release of lipoproteins when rats were treated with 0.25 ml/100 g b.w. or more of CCl_4. On the other hand, Ribeiro and McDonald (27) and Ugazio and Lombardi (28) reported an increase in lipoprotein levels when they treated rats with less than 0.25 ml/100 g b.w. of CCl_4.

Since all ß-lipoproteins are precipitated by the heparin sulfate method, it is conceivable that one class of the ß-lipoproteins might have been significantly affected by exposure to CCl_4. This effect could have been masked by the other ß-lipoproteins. As seen in Table X the very low density lipoproteins(VLDL; $D < 1.006$) levels at low CCl_4 concentrations were not significantly affected, although these results suggest a decrease in this lipoprotein class. On the other hand a significant decrease occurred in both lipid and protein moieties of VLDL at high CCl_4 concentrations. The increase in the specific activity of the VLDL protein moiety after exposure to high levels of CCl_4 implies an even greater decrease in protein synthesis.

Since we have found an increase in serum free fatty acid turnover along with an increase in the rate of esterification of unesterified fatty acids in the livers of chronically exposed animals in vivo the decrease in the very low density lipoproteins is possibly due to an interference with the protein synthesizing mechanism.

There was no significant difference in the level of low density lipoproteins; however, the lipid specific activity of this fraction is significantly increased which is possibly due to the increased rate of esterification and a very rapid coupling of the newly synthesized TG to protein of the LDLP. The protein levels of the low density lipoprotein fraction indicate a progressive inhibition of protein synthesis. Again the

specific activity of the protein moiety after exposure to CCl_4 was higher than that of the controls, suggesting that the protein synthetic mechanism and the leucine pool were interfered with.

II. Metabolism of the Chloromethanes

Butler (127) suggested that the dehalogenation of CCl_4 is a non-enzymatic process and results from a reaction between a sulfhydryl group and a dehalogenated free radical, leading to oxidation of the - SH compound and reduction of the free radical. Rubinstein and Kanics (129) proposed that only the first step in the dehalogenation process is non-enzymatic. After the formation of $CHCl_3$ from CCl_4 the conversion of $CHCl_3$ to CO_2 is enzymatic as has been shown by Rubinstein and Paul (128). Heppel and Porterfield (124) reported the presence of an enzyme system which is able to oxidize CH_2Cl_2 to formaldehyde. Rubinstein and Kanics (129) reported that addition of pyridine nucleotides to liver homogenates restored the ability of the homogenate to convert CCl_4 and $CHCl_3$ to CO_2. These observations have been confirmed and extended to $^{14}CH_2Cl_2$ in the present thesis. The ^{14}C label of each of the chloromethanes was incorporated to an approximately equal extent into liver proteins. However, homogenates incorporated more of the ^{14}C label than liver slices. This probably was due to a larger contact area. The ^{14}C label of the chloromethanes might have been attached to the proteins as phosgene ($COCl_2$). Phosgene, a very reactive chemical, would attack amino groups and other functional groups, resulting in cross-linked carbonyl derivatives with impaired biological activities. However, we were unable to detect the presence of phosgene.

On the other hand Cessi et al. (130) concluded that phosgene is a metabolite of CCl_4.

Table XII also shows that the ^{14}C label of the chloromethanes was also associated with liver lipids. The mechanism of this reaction is probably an interaction between a peroxidizing lipid and a free radical derivative of the chloromethanes. Free radical formation from chloromethanes may be initiated by naturally occurring free radicals in biological systems. Commoner et al. (133) have presented evidence for the presence of free radicals in biological systems. Wirtschafter and Cronyn (132) and Slater (134) proposed that the chloromethanes may undergo free radical initiated reactions by interaction with normally occurring homolytic processes under metabolic control, such as lipid peroxidation.

Fractionation of the lipids revealed that over 80% of the ^{14}C label incorporated from $^{14}CCl_4$ into lipids was present in the phospholipid fraction. Moreover, the ^{14}C label was entirely associated with the phosphate esters of the various bases (i.e. serine, choline, ethanolamine). Commercially available phospholipids on incubation in Krebs-Ringer or hexane-chloroform or in carbon tetrachloride solution also incorporated ^{14}C label from $^{14}CCl_4$. The mechanism of this reaction of phospholipids with CCl_4 (or derivatives of CCl_4) is not known. It may occur through either a free radical type intermediate or an acylchloride intermediate (i.e. phosgene type reaction). Heimberg et al. (25) observed that serum lipoproteins contain phospholipids,

cholesterol esters and triglycerides in a definite ratio to each other. The fact that ^{14}C label from $^{14}CCl_4$ was found associated with phospholipids may affect their incorporation into lipoproteins thus inhibiting lipoprotein formation and release, which could result in fatty liver.

However, an isotope dilution effect for CCl_4 in these experiments was not observed. This is taken as an indication of some impurity in the $^{14}CCl_4$ preparation. Infrared (minimum detectable impurity 0.1%) and nuclear magnetic resonance (minimum detectable impurity 1.0%) studies did not show the presence of any impurity. The batch of $^{14}CCl_4$ used in these experiments was obtained from a different supplier than that used in earlier experiments. Furthermore, $^{14}CCl_4$ was prepared according to the following scheme:

$$^{14}CH_3OH \xrightarrow{PCl_5} {}^{14}CH_3Cl \xrightarrow[Cl_2]{U.V.} {}^{14}CCl_4$$

Radioactive purity according to the manufacturer was 100% on gas-liquid chromatography. However, on our instrument we were able to show (Table VI) that the $^{14}CCl_4$ had an impurity associated with it. The material collected under the ascending side of the GLC peak of CCl_4 was incorporated into CO_2, lipid and protein to a greater extent than the ^{14}C label incorporated from the fraction collected under the peak and on the descending side. The impurity in the $^{14}CCl_4$ preparation presents great difficulty in discussing these results, however, it is felt that these results

(CO_2, protein and lipid) are valid but exaggerated by the presence of the impurity.

It has been shown (129) that there are at least two enzymes involved in the dehalogenation of the chloromethanes to CO_2, since both the microsomal and the supernatant fractions are required for activity. In addition we have found that one of these enzymes, located in the supernatant fraction, is also responsible for the reduction of DPN in the presence of chloromethanes. Addition of lactic dehydrogenase and pyruvate reversed the increase in optical density at 340 mμ indicating that DPNH was formed, not merely a complex between DPN and some other compound. Furthermore, this reaction appears to be DPN specific since no additional reduction of TPN occurs upon addition of the chloromethanes. Thus the sequence of events in the conversion of chloromethanes to CO_2 appears to be:

1. Non-enzymatic reduction of CCl_4 to $CHCl_3$. This reduction has been shown to occur by Butler (127), Rubinstein and Paul (128) and Kanics (149).

2. Conversion of $CHCl_3$ to CO_2 mediated through the reduction of DPN, i.e. an enzymatic reaction. Chloroform appears to be the best substrate for the reduction of DPN in the presence of liver extracts. DPN is reduced approximately two and a half to three times as fast in the presence of $CHCl_3$ as in the presence of CCl_4. This increased reduction of DPN compares favorably with the $^{14}CO_2$ results obtained with these two chloromethanes.

Methylene chloride may be an intermediate in the metabolism of chloroform. Methylene chloride formation has been demonstrated in vitro by Rubinstein and Paul (128). Methylene chloride could then be oxidized by the enzymatic mechanism suggested by Heppel and Porterfield (124), i.e. $CH_2Cl_2 \xrightarrow{\text{enzyme}} HCHO$. However, recent evidence suggests that another mechanism may be involved which proceeds through a hydroxylation process followed by oxidation of the hydroxylated chloromethane to phosgene. Seawright and McLean (39) have demonstrated in vitro that SKF 525 A (diethylamino-ethyl-diphenylpropyl acetate), a drug that inhibits microsomal hydroxylating enzymes, prevents the effect of CCl_4 on protein synthesis. In addition Cessi et al. (130), from the similarities in the effects of CCl_4 and phosgene, concluded that phosgene is a metabolite of CCl_4.

It may be suggested here that investigations on the following aspects of chloromethane metabolism would be valuable for further elucidation of the mechanism of chloromethane poisoning: the role of DPN in chloromethane metabolism; the presence of chlorinated intermediates as well as ^{14}C intermediates; the structure of the derivatives associated with lipids and proteins; the nature of chloromethane inhibition of lipoprotein secretion and synthesis.

Summary

Investigations were carried out to determine the toxic effects of chronic carbon tetrachloride inhalation. It was found that:

1. The degree of liver injury, due to chronic CCl_4 exposure, as determined by serum GOT, liver glycogen, liver triglyceride, and serum lipoprotein measurements, is dose dependent.
2. Serum glutamic-oxaloacetic transaminase activity measurement is not a dependable index of liver injury.
3. There is a rapid initial loss of liver glycogen followed by a low but relatively stable glycogen level up to the eighth day of daily eight hour exposure. This is followed by a further decline.
4. Liver triglyceride levels show a six-fold increase by four days of exposure to 68 and 680 ppm of CCl_4. After eight days of exposure the liver triglyceride levels are increased ten- to twenty-fold in animals exposed to 68 and 680 ppm of CCl_4. The accumulation of triglycerides in the livers of rats chronically exposed to CCl_4 vapours is due to: (a) increased uptake and turnover of serum free fatty acids by the CCl_4 injured liver;
   (b) higher turnover of unesterified fatty acids in the exposed liver;
   (c) increased lipogenesis from acetate;

(d) increased esterification of intravenously injected albumin-bound palmitate;

(e) decreased release of very low density lipoproteins which is probably due to:

(i) inhibition of the synthesis of the protein moiety of the very low density lipoproteins as evidenced by a decreased incorporation of i.v. injected leucine;

(ii) possible interaction of chloromethanes with the phospholipid moiety of the very low density lipoproteins.

Further investigations on the metabolism of the chloromethanes were also carried out. It was found that:

(a) methylene chloride is converted to CO_2;

(b) more of the ^{14}C label of the chloromethanes is incorporated into phospholipids than into proteins;

(c) DPN is reduced in the presence of liver extract and chloromethanes while TPN is not;

(d) the enzyme responsible for the reduction of DPN in the presence of chloromethanes is located in the microsome-free supernatant of rat liver homogenates.

Contribution to Knowledge

1. The effects of CCl_4 exposure are cumulative. This is based on measurements of serum glutamic-oxaloacetic transaminase activity, liver glycogen and triglyceride levels.

2. The increase in liver triglycerides in the livers of chronically exposed rats is due to:
   (a) increased turnover and uptake of free fatty acids along with an in vivo increase in esterification;
   (b) decreased synthesis of the protein moiety of the very low density lipoproteins.

3. Methylene chloride-^{14}C is converted to $^{14}CO_2$ by liver slices and homogenates.

4. DPN is reduced during the metabolism of the chloromethanes by a microsome-free supernatant of rat liver homogenate.

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