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The Effect of Modifiers of Microsomal Electron Transport On Carbon Tetrachloride Hepatoxicity

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THE EFFECT OF MODIFIERS OF MICROSOMAL ELECTRON TRANSPORT ON CARBON TETRACHLORIDE HEPATOTOXICITY

BY

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ABSTRACT

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Phenobarbital pretreatment significantly enhanced the rise in SGOT and SGPT immediately following a three-hour exposure of rats to carbon tetrachloride by inhalation. However, these parameters of hepatotoxicity were significantly lower in rats pretreated with 3-methylcholanthrene when compared to rats pretreated with vehicle and exposed to carbon tetrachloride vapor. Levels of hepatic microsomal NADPH cytochrome c reductase and CO-binding pigment were elevated by phenobarbital pretreatment, but 3-methylcholanthrene had no effect on hepatic microsomal NADPH cytochrome c reductase. Although carbon tetrachloride exposure reduced CO-binding pigment content by 61 per cent in phenobarbital pretreated and by 39 per cent in 3-methylcholanthrene pretreated rats, microsomal NADPH cytochrome c reductase was reduced by only 6 per cent and 20 per cent, respectively. In phenobarbital pretreated rats, exposure to carbon tetrachloride produced a greater decrease in aminopyrine demethylase activity than in saline treated carbon tetrachloride exposed controls. However, in 3-methylcholanthrene pretreated rats, exposure to carbon tetrachloride produced a lesser decrease in p-nitroanisole demethylase activity than in corn oil treated controls. Twenty-one hours after exposure, the difference in SGOT and SGPT values of the phenobarbital and 3-methylcholanthrene pretreated rats was more divergent. Histological

evidence at this time period revealed extensive damage in the phenobarbital pretreated animals and a sparing effect in the 3-methylcholanthrene pretreated animals.

While phenobarbital pretreatment enhanced the microsomal diene conjugation absorption indicative of lipid peroxidation following carbon tetrachloride exposure, 3-methylcholanthrene pretreatment had the opposite effect. Carbon monoxide, but not hypoxia, enhanced the increase in SGOT, SGPT, and microsomal diene conjugation absorption following exposure to carbon tetrachloride. These data suggest that the differential effects of 3-methylcholanthrene and phenobarbital pretreatment on NADPH cytochrome c reductase and CO-binding pigment may be responsible for the observed protective effect of 3-methylcholanthrene in carbon tetrachloride exposed rats.

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INTRODUCTION

The study of carbon tetrachloride hepatotoxicity has led to many hypotheses concerning the initial biochemical lesions responsible for the toxicity of this agent. Recently, Recknagel (1967) authored a comprehensive review emphasizing the relationship between the underlying mechanisms for these lesions and the study of hepatocellular injury.

The lipid peroxidation hypothesis described by Recknagel (1967) is currently accepted as the most tenable mechanism for carbon tetrachloride induced liver damage. According to the hypothesis, the interaction of carbon tetrachloride with liver microsomal electron transport components produces free radicals that attack unsaturated fatty acids yielding unstable addition products that undergo peroxidative decomposition. The disruption of cytoplasmic membrane lipid components is followed by alterations in cellular metabolism and tissue necrosis.

Slater (1966) has suggested that flavin linked enzymes in the microsomal chain may be sites of free radical production following carbon tetrachloride exposure. Pretreatment of rats with phenobarbital produces an increase in the flavoprotein NADPH cytochrome c reductase and CO-binding pigment. A consequence of phenobarbital pretreatment is an increased sensitivity to carbon tetrachloride hepatotoxicity (Gaines and McLean, 1969).

Polycyclic hydrocarbons such as 3-methylcholanthrene are also inducers of drug metabolizing enzymes but differ from phenobarbital in the spectrum of enzymes induced and the mechanism of induction (Sladek and

Mannering, 1969). Specifically, 3-methylcholanthrene treatment induces CO-binding pigment without producing any changes in NADPH cytochrome c reductase.

If NADPH cytochrome c reductase is the site responsible for activation of carbon tetrachloride to toxic free radicals, then increased levels of CO-binding pigment may enhance electron flow through the terminal electron acceptor decreasing the availability of reducing equivalents for the activation of carbon tetrachloride at the flavoprotein site. The net result would be a decrease in carbon tetrachloride toxicity in 3-methylcholanthrene treated animals.

This hypothesis will be tested by comparing the effects of modifiers of microsomal electron transport on carbon tetrachloride induced lipid peroxidation and parameters of carbon tetrachloride hepatotoxicity.

LITERATURE SURVEY

The toxicity of carbon tetrachloride was recognized shortly after its introduction as an anesthetic by Simpson, the discoverer of chloroform anesthesia (Robinson, 1946). The inhalation of chloroform or carbon tetrachloride frequently produced an acute yellow atrophy of the liver that often proved fatal. While both agents produce similar pathological changes, carbon tetrachloride is considerably more toxic (Drill, 1952). Chloroform has been used for anesthesia but is almost never used for that purpose today. Although carbon tetrachloride was used for some time as an anthelminthic (Hall, 1921), it has now been replaced by more effective and less toxic compounds. The primary interest in carbon tetrachloride and related halogenated hydrocarbons is toxicological as these agents are often responsible for accidental poisoning. Carbon tetrachloride and chloroform have both been used as tools for the production and investigation of cirrhotic liver disease (Drill, 1958).

Carbon tetrachloride produces hepatic lesions characterized at first by necrotic degeneration in the midzonal region, followed by ballooning of the cells, and finally by acute yellow atrophy of the liver (see Recknagel, 1967). Biochemically, the lesions are characterized by abnormalities in calcium metabolism, glycogen depletion, depression of microsomal enzyme activity, uncoupling of mitochondrial oxidative phosphorylation, accumulation of triglycerides and the release of intracellular enzymes into the plasma (see Recknagel, 1967).

The literature on carbon tetrachloride hepatotoxicity is substan-

tial and contains many hypotheses attempting to explain the initial events leading to the fatty infiltration, mitochondrial degeneration and tissue necrosis produced by this compound (see reviews by Moon, 1934; Drill, 1952; and Recknagel, 1967).

The Phospholipid Hypothesis

One of the earliest thorough investigations of the pathological changes induced by carbon tetrachloride was reported by Meyer and Pessoa (1923). They observed that carbon tetrachloride produced severe macroscopic lesions in the kidney and the liver of the dog. The severity of the lesions was proportional to the administered dose and was preceded by fatty infiltration. Lesions appeared at 12 hours and were maximal at about 48 hours. Cameron and Karunaratne (1936) also studied carbon tetrachloride toxicity using the rat as a model for cirrhosis in relation to liver regeneration. Subcutaneous injections of 0.3 to 1.00 ml of carbon tetrachloride produced congestion of the liver followed by hydropic degeneration, fatty infiltration and eventual necrosis. Again lesions induced were dose dependent and often reversible if damage was not excessive.

The consistent finding of early fatty infiltration in the liver following carbon tetrachloride exposure suggested to many investigators that altered lipid metabolism was a critical initial event responsible for hepatotoxicity.

During the 1930's and 40's most investigators in the field of lipid metabolism considered plasma phospholipids to act as carriers of fatty acids from organ to organ. Peters and Van Slyke (1946) believed that only fatty acids incorporated into phospholipids were available for oxidation, and these phospholipids were presumably synthesized in the

liver. A disruption of phospholipid synthesis would then lead to an accumulation of lipid in the liver. The increase in neutral fat and the relative decrease in phospholipid in livers from rats maintained on choline deficient diets was, therefore, believed to be due to failure of phospholipid synthesis. If alterations in phospholipid synthesis could account for dietary fatty liver, then it seemed a reasonable conclusion that carbon tetrachloride induced fatty liver might also be due to failure in the synthesis of phospholipids.

Advances in lipid biochemistry have shown that fatty acids can be mobilized from peripheral tissue by the action of epinephrine on triglyceride lipase (Sutherland et al., 1968). Failure of phospholipid synthesis in the liver is, therefore, not essential for accumulation of lipid in the liver following carbon tetrachloride; mobilization of lipid from peripheral stores can explain that phenomenon. Based on these findings, the phospholipid hypothesis as originally proposed no longer seems tenable. However, failure of hepatic lipid transport to the plasma may be responsible for the pathological lipid accumulation in the liver (Recknagel, 1967).

The Mitochondrial Hypothesis

As evidence accumulated to negate the importance of alterations in hepatic phospholipid metabolism as an initial lesion responsible for carbon tetrachloride hepatic necrosis, investigators focused attention on the mitochondria and its relation to the maintenance of cell function.

Christie and Judah (1954) were among the first to emphasize the importance of alterations in mitochondrial structure and function in the series of events leading to hepatic necrosis after carbon tetrachloride. They observed a loss of diphosphopyridine nucleotide dependent dehydrog-

enase activity as early as 15 hours after carbon tetrachloride poisoning. Partial restoration of activity by addition of diphosphopyridine nucleotide suggested that an alteration in mitochondrial permeability induced by carbon tetrachloride was responsible for the loss in enzyme activity.

Carbon tetrachloride produces mitochondrial swelling under *in vivo* (Dianzani and Fahr, 1954) and *in vitro* (Malamed *et al.*, 1957) conditions. Recknagel and Malamed (1958) have shown this mitochondrial swelling to be due to an increase in the permeability of the mitochondrial membrane. A consequence of the increased permeability is a leakage *in vitro* of cytochrome *c* (Dianzani and Viti, 1955), citrate (Recknagel and Lombardi, 1961) and vitamin B_{12} (Kasbekar *et al.*, 1959) into the medium.

Observations on the disruption of mitochondrial function suggested that the uncoupling of mitochondrial oxidative phosphorylation following carbon tetrachloride poisoning was responsible for the accumulation of lipid in the liver (Dianzani, 1954). A deficient supply of adenosine triphosphate for the activation of fatty acids prior to oxidation could be responsible for accumulation in the liver. However, electron microscopic and biochemical studies have shown little impairment of mitochondrial structure (Oberling and Rouiller, 1956) or function (Reynolds *et al.*, 1962) at a time when there is a marked alteration in the appearance of the granular endoplasmic reticulum (Oberling and Rouiller, 1956). Arbiasu and Dianzani (1962) reported no changes in the P/O ratio in mitochondria isolated from carbon tetrachloride poisoned rats as late as one hour after poisoning but noted a significant decrease at two hours. Increased liver lipids as early as one hour after poisoning strongly suggests that mitochondrial changes are not responsible for the accumulation of fat following carbon tetrachloride. Alterations in mitochondrial structure and function may still be important in the necrogenic action of

carbon tetrachloride but are not likely to be involved as an initiating event (Recknagel, 1967).

The Catecholamine Hypothesis

An interesting feature of carbon tetrachloride hepatotoxicity is that the damage is primarily centrolobular. The peripheral cells are the first exposed to carbon tetrachloride and yet are not usually involved in the overall pathology (Drill, 1952). As carbon tetrachloride has been shown to reduce hepatic blood flow (Wakim and Mann, 1942), Drill (1952) felt that hepatic anoxia due to restricted blood flow might be responsible for the necrosis produced by this agent. Indeed, McMichael (1937) had found that impaired hepatic blood flow produced lesions similar to those found after carbon tetrachloride. It seemed reasonable to assume that the ischemia produced by carbon tetrachloride was responsible for the centrolobular necrosis as a result of tissue anoxia.

Calvert and Brody (1960) proposed that carbon tetrachloride produced a massive sympathetic discharge that restricted hepatic blood flow producing ischemia and hepatotoxic anoxia leading to necrosis. According to the "catecholamine hypothesis," anoxia due to ischemia produced tissue necrosis while the mobilization of fats from peripheral stores was responsible for the fatty infiltration of the liver. Much of the hypothesis was based on indirect evidence obtained by experimental manipulation of the sympathetic nervous system. Pharmacological methods such as reserpine pretreatment or use of adrenergic blockers to decrease responses following sympathetic stimulation afforded protection against carbon tetrachloride induced necrosis and fatty infiltration. The physiological interruption of sympathetic activity by transection of the spinal cord offered even greater protection against these changes.

The hypothesis presented by Calvert and Brody (1960) was later supported by Larson and Plaa (1963a) who confirmed the protection afforded by cord transection of carbon tetrachloride hepatotoxicity. However, animals receiving large doses of carbon tetrachloride were maximally protected whereas animals receiving small doses had more severe damage than intact controls. After determining that cord transection had no effect on the absorption of carbon tetrachloride following oral administration, Larson and Plaa (1963b) performed experiments to determine whether the loss of ability to regulate temperature following cord transection might alter the response to carbon tetrachloride. These experiments showed that cord transected rats maintained normothermic in an incubator were no longer protected against carbon tetrachloride induced centrolobular necrosis. In addition, hypothermia produced by immersion of animals in water was found to protect against carbon tetrachloride toxicity in intact rats (Larson and Plaa, 1965). They also observed a 50 per cent reduction in whole body oxygen consumption and postulated that a decrease in metabolism was in some way responsible for the protection afforded by cord transection induced hypothermia.

While necrosis produced by catecholamine induced tissue anoxia no longer seemed tenable, the fatty infiltration of the liver due to mobilization of peripheral stores of lipids seemed a reasonable explanation for the fatty degeneration of the liver produced by carbon tetrachloride. However, Rubinstein (1962) reported only transient increases in plasma levels of epinephrine following intradermal administration of carbon tetrachloride. This finding suggests that the sympathetic activation following carbon tetrachloride poisoning is not of sufficient intensity or duration to elevate plasma fatty acid levels to produce fatty infiltration of the liver due to hyperlipidemia alone.

The Lipid Peroxidation Hypothesis

An early approach to the study of carbon tetrachloride hepatotoxicity involved the use of agents effective in preventing or decreasing the severity of the lesions in an effort to elucidate the mechanism of toxicity. Much of the evidence accumulated from such investigations suggested that antioxidants were particularly effective in reducing the pathological consequences of carbon tetrachloride poisoning (Recknagel, 1967).

Hove (1948) reported that rats on vitamin E deficient diets were more susceptible to carbon tetrachloride poisoning than rats fed diets supplemented with alpha-tocopherol. Hove (1953) emphasized that alpha-tocopherol was an antioxidant *in vitro* and that carbon tetrachloride could act as a pro-oxidant in a nonbiological system. DiNunio (1964) reported that a commercial antioxidant containing butylated hydroxytoluene, butylated hydroxyanisole and propyl gallate offered protection against the fatty infiltration of the liver produced by carbon tetrachloride or ethanol. He suggested a relationship between the toxicity of carbon tetrachloride and the formation of lipid peroxides. According to this hypothesis, the lipid peroxides were responsible for the loss of mitochondrial function and cellular degeneration. Alternatively, BHT and other antioxidants may protect by competing with microsomal enzymes responsible for activation of carbon tetrachloride to a toxic metabolite (Carthorne *et al.*, 1970).

Recknagel and Ghoshal (1966) presented a hypothesis linking the hepatotoxicity of carbon tetrachloride to the peroxidation of microsomal lipids. The hypothesis was based on the following considerations: 1) the speculations of Butler (1961) and of Wirtschafter and Cronyn (1964) that carbon tetrachloride is metabolized to the trichloromethyl free

radical in the liver; 2) the finding of Rubinstein and Kanics (1964) that carbon tetrachloride and chloroform are metabolized to carbon dioxide by rat liver homogenates; 3) the protective effect of antioxidants on carbon tetrachloride hepatotoxicity (DiLuzio, 1964); and 4) the organic chemical theory of rancidity which postulates the formation of lipid peroxides during autoxidation of polyunsaturated fatty acids (Dahle et al., 1962). According to the lipid peroxidation hypothesis, free radicals produced by the homolytic cleavage of carbon tetrachloride abstract a hydrogen atom from the methylene bridges of unsaturated fatty acids of microsomal lipids producing a highly reactive organic free radical. The organic free radical combines with molecular oxygen and finally undergoes a peroxidative degeneration. The primary oxidation products of these unsaturated fatty acids are organic hydroperoxides exhibiting conjugated diene absorption (Folland and Koch, 1945). The extent of peroxidative destruction is dependent on the level of alpha-tocopherol and other antioxidants. The small amount of free radicals produced by natural biological processes are quenched by these natural antioxidants. Following carbon tetrachloride poisoning, these highly reactive free radicals are presumably produced at such a rapid rate that antioxidant activity is overwhelmed and cellular damage is produced. However, Keller et al. (1971) have observed no discernible rise in liver free radical levels following carbon tetrachloride poisoning when measured by electron spin resonance (ESR) spectroscopy. On the basis of these findings, these authors concluded that lipid peroxidation does not play an important role in carbon tetrachloride hepatotoxicity.

Support for the lipid peroxidation hypothesis and the activation of carbon tetrachloride to a toxic metabolite has come from several laboratories. Stuckler (1965) and Stuckler and Padnitt (1965) observed that

polysomes isolated from rat liver after carbon tetrachloride poisoning were disaggregated and had depressed protein synthesizing capacity as ascertained by incorporation of amino acids into protein *in vitro*. However, carbon tetrachloride *in vitro* produced no such effects. The metabolism of carbon tetrachloride appeared to be a prerequisite for the effect.

Using diene conjugation absorption as an indication of lipid peroxidation, Ghoshal and Recknagel (1965) showed that the loss of glucose-6-phosphatase activity from a microsomal preparation *in vitro* was paralleled by peroxidation of microsomal lipids. In this system, ascorbic acid was added to peroxidize the microsomal lipids. They showed that EDTA or alpha-tocopherol could act as inhibitors of lipid peroxidation and could prevent the loss of glucose-6-phosphatase activity. Under similar conditions, addition of carbon tetrachloride to 20 times the weight of the total lipid material was required to destroy glucose-6-phosphatase activity. If carbon tetrachloride fed to rats at a dose of one microliter per 100 g body weight could depress hepatic glucose-6-phosphatase activity, then clearly a toxicity based on solvent action alone could be eliminated (Recknagel and Ghoshal, 1966).

Typical conjugated diene absorption has been reported by Recknagel and Ghoshal (1966) in liver microsomal lipids 90 minutes after oral carbon tetrachloride treatment. More recently, Rao and Recknagel (1968) reported increased diene conjugation in liver microsomal lipids within 15 minutes after orally administered carbon tetrachloride. Enhanced lipid peroxidation at such an early time is particularly important in that it precedes the depression in microsomal enzyme activity, mitochondrial function, and triglyceride accumulation after carbon tetrachloride poisoning.

Alteration of carbon tetrachloride hepatotoxicity by drugs

Promethazine given to rats together or six hours after carbon tetrachloride has been reported to decrease hepatic necrosis as determined by histological methods (Rees et al., 1961). More recently, Slater (1965) reported that promethazine protected rats against the carbon tetrachloride induced decrease in hepatic microsomal inorganic pyrophosphatase. Promethazine in vitro produced a significant decrease in carbon tetrachloride induced microsomal lipid peroxidation suggesting that the protective effect may have been due to the antioxidant activity of this compound.

According to the lipid peroxidation hypothesis as stated above, carbon tetrachloride is activated to trichloromethyl free radicals through interaction with microsomal electron transport components. If this assumption is valid, then agents that alter microsomal electron transport activity should alter carbon tetrachloride toxicity. Garner and McLean (1969) have shown that induction of microsomal enzymes by phenobarbital is associated with an increased susceptiblity to carbon tetrachloride poisoning.

The drug 2-diethylaminoethyl-2,2-diphenylvalerate (SKF-525A) is a potent inhibitor of drug metabolizing enzymes associated with the microsomal electron transport chain. Seawright and McLean (1967) have shown that SKF-525A added to tissue slices prevents the carbon tetrachloride induced decrease in protein synthesis. Rao et al. (1970) have shown that phenobarbital treatment enhances liver microsomal lipid peroxidation in vivo as estimated by diene conjugation absorption of liver microsomal lipids following orally administered carbon tetrachloride. However, the administration of carbon tetrachloride under similar conditions to rats treated with SKF-525A resulted in a decrease in diene con-

jugation.

While many investigators support the lipid peroxidation hypothesis, others have reported a lack of correlative evidence between the antioxidant activity of drugs (alpha-tocopherol, butylated hydroxytoluene) and protection against carbon tetrachloride induced hepatic triglyceride accumulation (Carthorne et al., 1970). Cignoli and Castro (1971) reported that antioxidants offer protection against carbon tetrachloride induced necrosis, but were without effect on the carbon tetrachloride induced decrease in microsomal glucose-6-phosphatase activity. However, they did observe a correlation between the ability of carbon tetrachloride to enhance lipid peroxidation in vitro and the ability of this agent to produce acute liver injury in vivo. While carbon tetrachloride induced microsomal lipid peroxidation may not account for all the toxic manifestations following ingestion, it, nonetheless, appears to be the most acceptable hypothesis at this time.

Microsomal Electron Transport System

Mammalian liver contains an electron transport system found in the endoplasmic reticulum and is responsible for the catalysis of a number of mixed function oxidation reactions. In addition to the metabolism of endogenous compounds (steroid hormones, fatty acids, tyramine, thyroxin, tryptamine, etc.), this electron transport system also metabolizes a a variety of drugs by way of N-dealkylation, O-dealkylation, aromatic hydroxylation, and deamination, to name a few (see Conney, 1967).

Gillette (1963) proposed that the overall reaction of oxidative drug metabolism could be expressed as a hydroxylation reaction in which one atom of oxygen was incorporated into the drug substrate while the other atom was reduced to water. The term "mixed function oxidase" was

used to describe the overall reaction which can be written as follows:

$$\underset{\text{Substrate}}{AH} + \underset{\substack{\text{Hydrogen}\\\text{Donor}}}{DH} + O_2 \longrightarrow \underset{\substack{\text{Hydroxylated}\\\text{Substrate}}}{AOH} + D^+ + H_2O$$

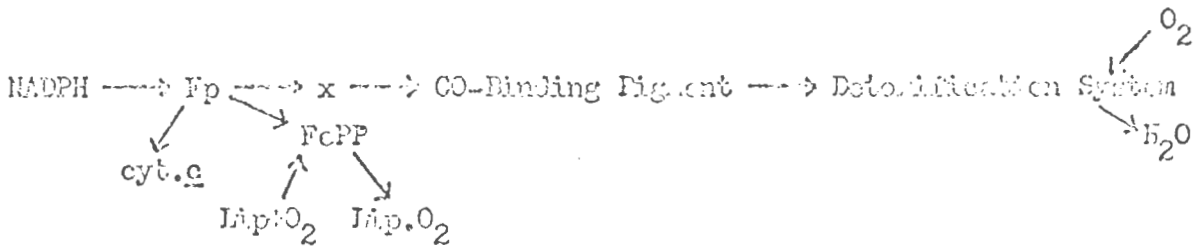
At that time it was proposed that NADPH reduced a component in liver microsomes which reacted with oxygen to form an "active oxygen" complex. The "active oxygen" was then transferred to the drug substrate by the action of an enzyme. Mason (1957) proposed that such hydroxylation reactions were coupled to a microsomal electron transport system that passed electrons from NADPH to oxygen.

The consumption of NADPH with the formation of a peroxide by an enzyme system in microsomes was reported by Gillette (1957) and referred to as an NADPH oxidase. While drug substrates did not enhance the rate of NADPH oxidation, agents inhibiting NADPH oxidation were found to block drug metabolism. A relationship between the NADPH oxidase activity and drug metabolism was supported by the observation of Conney and Burns (1962) that increases in drug metabolizing activity were concomitant with stimulation of NADPH oxidase activity.

Hochstein et al. (1964) and Orrenius et al. (1964) have described an NADPH-linked peroxidation of lipids in rat liver microsomes. The reaction is strongly inhibited by drugs undergoing oxidative demethylation and is slightly stimulated by carbon monoxide. In the absence of a substrate for the microsomal mixed function oxidase, reducing equivalents from NADPH are shunted from the microsomal electron transport chain to lipid peroxidation with the production of malonaldehyde, a cleavage product of lipid hydroperoxides. They suggested that the two reactions involved a common NADPH oxidizing enzyme. Ernster and Orrenius (1965) later suggested that the enzyme common to both systems was identical to NADPH cytochrome c reductase first described by Horecker

(1950). This conclusion supported earlier investigations implicating NADPH cytochrome c reductase as a component of the microsomal drug-metabolizing system (Phillips and Langdon, 1962; Masters et al., 1965). The ability of this enzyme to reduce cytochrome c in vitro provides a convenient method for the quantitation of enzyme activity. The in vivo electron acceptor for NADPH cytochrome c reductase is not known.

Liver microsomes also contain a hemoprotein which was first described by Klingenburg (1958) and is now generally referred to as CO-binding pigment or cytochrome P-450. Estabrook et al. (1963) have observed that carbon monoxide (CO) inhibits the hydroxylation of steroids by adrenal cortical microsomes and that the inhibition could be reversed by light. Orrenius and Ernster (1964) also demonstrated that carbon monoxide inhibits drug hydroxylation in liver microsomes. These studies have established CO-binding pigment or cytochrome P-450 as the terminal oxygen activating enzyme for mixed function oxidations. Sicekvitz (1965) reviewed the literature and suggested the following scheme for the passage of electrons from NADPH to oxygen:



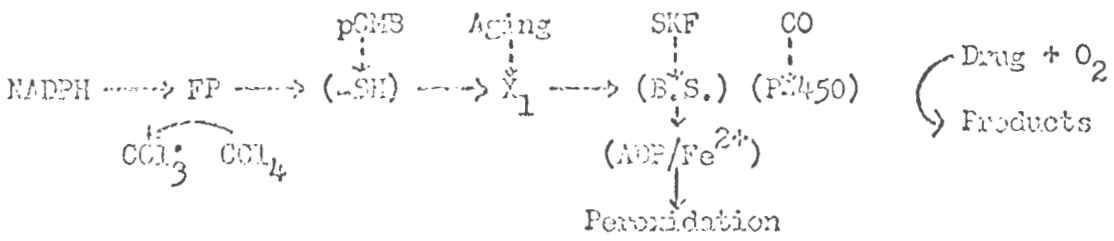
where Fp is a flavoprotein similar if not identical to NADPH cytochrome c reductase, and FePP is the NADPH-linked peroxidation of microsomal lipids catalyzed by an iron-pyrophosphate complex.

The interaction of drugs with hepatic microsomal cytochrome (assumed to be P-450) produces two distinct types of drug induced spectral changes, probably due to interaction of the drug with the hemoprotein (Remmer et al., 1966). The difference spectra are produced by comparison

of the absorption between suspensions of microsomes containing drug with suspensions without drug. Hexobarbital and other drugs producing Type I spectral changes cause a negative band at 420 nm and a positive band at 390 nm, whereas aniline and other Type II compounds produce a difference spectrum with a positive band at 430 nm and a negative band at 393 nm.

The treatment of rats with compounds that stimulate the metabolism of drugs produces alterations in the activity of microsomal electron transfer components. These inducers are of at least two types, typified by phenobarbital and 3-methylcholanthrene (Conney, 1967). Phenobarbital-like inducers stimulate various pathways of metabolism by liver microsomes including oxidation and reduction reactions, glucuronide formation, and de-esterification. In contrast, 3-methylcholanthrene-like inducers stimulate a more limited group of reactions (see Conney, 1967). While phenobarbital produces increases in both NADPH cytochrome c reductase activity and the amount of cytochrome P-450 (Kato, 1966), 3-methylcholanthrene produces increases in cytochrome P-450 alone (Von Der Decken and Hultin, 1969). In addition, the cytochrome P-450 induced by 3-methylcholanthrene has a slightly different absorption maximum, namely, at 448 nm (Alvarez et al., 1967).

Recently, Slater and Sawyer (1971b) presented a scheme for the sequence of carriers in the NADPH cytochrome P-450 electron transport chain which suggests that carbon tetrachloride can be metabolized to free radicals at the flavoprotein (NADPH cytochrome c reductase) site. The overall scheme for electron transport follows:



where FP is flavoprotein; pCMB is p-Chloromercuribenzoate; X_1 is a rate limiting component for drug metabolism; B.S. is drug binding site; and P-450 is the flavoprotein cytochrome P-450 also called CO-binding pigment. By using selective inhibitors of microsomal electron transport, Slater and Sawyer (1971b) concluded that carbon tetrachloride induced lipid peroxidation (flavoprotein site) is distinct from the endogenous pathway (ADP/Fe^{+2}).

Thus NADPH cytochrome c reductase and cytochrome P-450 appear to be well established as components of the electron transport chain, and as such, measurement of NADPH cytochrome c reductase activity and the content of CO-binding pigment will serve to evaluate changes in oxidative drug metabolism.

EXPERIMENTAL

Animals

Adult male Sprague-Dawley derived rats were obtained from the Charles River Breeding Laboratories (Wilmington, Massachusetts). The animals were housed in animal quarters maintained at 72°F with a 12-hour alternating period of light and dark. The bedding material was a commercial pine chip variety.

The rats were injected with either phenobarbital (50 mg/kg, intraperitoneally, for four days) or 3-methylcholanthrene in corn oil (40 mg/kg, intraperitoneally, for two days) or saline or corn oil vehicle as respective controls. Rats pretreated with phenobarbital or 3-methylcholanthrene will be referred to as induced, where appropriate.

Materials

Analytical reagent grade chemicals or equivalent were used throughout the investigation. Co-factors (NADP, NADPH, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, and cytochrome c), crystalline bovine serum albumin, and 3-methylcholanthrene were purchased from Sigma Chemicals. Drugs used in this investigation were purchased from their respective manufacturers. Reagent grade carbon tetrachloride was purchased from Mallinckrodt.

Carbon Tetrachloride Exposure

Twenty-four hours after the last dose of phenobarbital or 48 hours after the last dose of 3-methylcholanthrene, animals were exposed to

carbon tetrachloride vapors in a dynamic inhalation chamber consisting of a flat bottomed, cylindrical glass jar (12 by 18 inches) placed horizontally on a wooden platform. An air flow rate of 10 liters per minute was maintained throughout the exposure period. The exposure atmosphere was formed by passing air through a cylindrical glass jar containing carbon tetrachloride. The vapors formed were passed into the chamber to produce the final inhalation mixture. The concentration of carbon tetrachloride in the chamber air was determined with a Packard series 7400 gas chromatograph equipped with a flame ionization detector.

Animals were also exposed to atmospheres containing carbon tetrachloride and carbon monoxide or 7.5 per cent oxygen. Carbon monoxide was added to the exposure chamber by substituting air containing approximately 1000 ppm carbon monoxide for the normal 10 liter per minute air flow in the chamber. The 7.5 per cent oxygen atmosphere was prepared by mixing a 95 per cent nitrogen-air mixture with compressed air to produce the final concentration determined by the use of a Beckman model number 96260 oxygen adaptor connected to a Beckman model number 76 pH meter. The hypoxic (7.5 per cent) oxygen atmosphere was introduced into the chamber at 10 liters per minute. Carbon tetrachloride was then added as described above.

Collection of Biological Samples

Serum

Animals were lightly anesthetized with ether, the tails clipped, and approximately 5.0 ml of blood was collected in a test tube and allowed to clot. The clotted blood was then centrifuged and the serum was removed and frozen until assay.

Liver

Sections of liver for histological examination were fixed in Dietrich's solution and stained with hematoxylin and eosin.

Preparation of Liver Microsomes

Animals were sacrificed by cervical dislocation. The abdomen was opened and ice-cold saline was perfused through the liver *via* the hepatic portal vein. The liver was quickly excised, chilled on ice, and minced through the coarse screen of a Harvard tissue press (model number 141). Five grams of the mince was homogenized for 30 seconds in 4 volumes of 0.1 M phosphate buffer (pH 7.4) using a Teflon homogenizer. The Homogenate was centrifuged in a Sorvall RC2-B refrigerated centrifuge (0°C) at 10,000 RPM (20,000 x g max.) for 15 minutes to remove nuclei and mitochondria. The supernatant was centrifuged at 32,000 RPM (105,000 x g max.) in an IEC refrigerated ultracentrifuge for 90 minutes to obtain the microsomal fraction. The microsomal pellet was resuspended in 0.1 M phosphate buffer to yield a microsomal suspension equivalent to 250 mg liver per ml.

Microsomes to be used for the determination of lipid peroxidation as indicated by diene conjugation absorption were prepared as above except that 0.3 M sucrose containing 0.003 M EDTA was used for homogenization. The final microsomal pellet was immediately assayed for diene conjugates as described by Recknagel and Ghoshal (1966).

In Vitro Incubation Procedure

Aminopyrine demethylation

Conney *et al.* (1960) have reported increased aminopyrine demethylase activity in liver microsomes from phenobarbital pretreated rats. Therefore, the demethylation of aminopyrine *in vitro* by liver microsomal

suspensions from phenobarbital pretreated rats was determined as a correlate of altered drug metabolizing activity.

The incubations were carried out at 37°C under air using 25 ml Erlenmeyer flasks in a Dubnoff metabolic shaker for 30 minutes. The incubation mixture contained 45 μmoles semicarbazide, 60 μmoles glucose-6-phosphate, 5.0 I.U. glucose-6-phosphate dehydrogenase, 50 μmoles nicotinamide, 50 μmoles magnesium chloride, 4 μmoles NADP, 1.0 ml of 0.1 M phosphate buffer (pH 7.4), 0.5 ml of microsomal suspension (0.5 g liver equivalent), and 10 μmoles of aminopyrine in a total volume of 5.0 ml.

P-nitroanisole demethylation

Treatment of rats with 3-methylcholanthrene does not result in an increase in aminopyrine demethylation by liver microsomes (Conney et al., 1960). However, the induction of p-nitroanisole demethylase activity by 3-methylcholanthrene provides a convenient method for assessing alterations in drug metabolizing ability (Netter and Seidel, 1964).

Incubations were carried out under air at 37°C in 25 ml Erlenmeyer flasks as above. The incubation mixture contained 2 μmoles NADP, 20 μmoles glucose-6-phosphate, 80 μmoles nicotinamide, 120 μmoles $MgCl_2$, 5.0 I.U. glucose-6-phosphate dehydrogenase, 0.2 ml microsomal suspension (0.2 g liver equivalent), and 1.0 ml of 0.1 M phosphate buffer (pH 7.9). The final addition of 3 μmoles of p-nitroanisole brought the total volume to 3.9 ml.

Analytical Procedures

Determination of carbon tetrachloride in the inhalation chamber

A gas chromatograph (Packard series 7400) equipped with a flame ionization detector and a Heath Servo-Recorder model number EU-20B was

used to measure the concentration of carbon tetrachloride in the chamber air. The column (18 per cent Neopentyl Glycol Sebacate on 60/80 mesh Chromosorb WAW 6 ft. x 2 mm I.D. glass column) was maintained at a temperature of 105°C; the injector at a temperature of 145°C; and the detector at a temperature of 135°C. One µl of carbon tetrachloride was used as a standard. The concentration of carbon tetrachloride was determined as parts per million by using the equivalent of 1 mg of carbon tetrachloride/m^3 to give 159 ppm (Browning, 1965).

Serum glutamic-oxaloacetic and glutamic pyruvic transaminase

The administration of carbon tetrachloride to rats has been reported to produce a dose dependent increase in serum glutamic pyruvic transaminase (SGPT) (Balazs et al., 1961) and serum glutamic-oxaloacetic transaminase (SGOT) (Block and Cornish, 1958). These enzyme activities will be determined after carbon tetrachloride inhalation as an indication of hepatic injury. The assay system used to determine SGOT and SGPT was that of Reitman and Frankel (1957). The reactions themselves are:

aspartic acid + alpha-ketoglutaric acid ⟷ oxaloacetic acid + glutamic acid

alanine + alpha-ketoglutaric acid ⟷ pyruvic acid + glutamic acid

The substrate for the determination of SGPT or SGOT contained alpha-ketoglutarate (2 mM) and either dl-alanine (200 mM) or dl-aspartate (200 mM), respectively, adjusted to pH 7.4 by addition of NaOH and 0.1 M phosphate buffer (pH 7.4). A 1.0 ml portion of substrate was incubated with 0.2 ml of serum for 30 minutes to determine SGPT or 60 minutes to determine SGOT. At the end of the incubation period, 1.0 ml of 2,4-dinitrophenyl hydrazine reagent (1 mM dissolved in 1 N HCl) was added, and the solution left to stand for 20 minutes. Then, 10.0 ml of

0.4 N NaOH was added, the samples shaken and read against a water blank at 520 nm exactly 30 minutes later. A blank was prepared for each sample by adding 2,4-dinitrophenylhydrazine reagent immediately to a test tube containing 1.0 ml of substrate and 0.2 ml of serum. The absorbance of each sample was corrected for the blank and compared to a standard curve (Reitman and Frankel, 1957). Results are expressed as the number of Reitman-Frankel units per ml of serum.

Estimation of microsomal NADPH cytochrome c reductase activity

Microsomal NADPH cytochrome c reductase activity was determined by the method of Dallner (1963). The assay is based on the rate of reduction of cytochrome c through the measurement of the increase in absorbance at 550 nm.

The assay system contained 0.6 μmoles NADPH, 0.3 μmoles cytochrome c and 0.99 μmoles KCN in 0.1 M phosphate buffer (pH 7.4) in a total volume of 3.0 ml. These reagents were placed in the reference and sample cuvettes of a Beckman model DB-G spectrophotometer equipped with a constant temperature bath (25°C) and a Beckman linear-log potentiometric recorder (model 1005). A 0.1 ml aliquot (containing approximately 0.1 mg protein) of the microsomal suspension was introduced into the sample cuvette and mixed. The linear change in absorbance over a five minute period at 550 nm was then recorded. The extinction coefficient used in these determinations was 18.5×10^3 at 550 nm for reduced minus oxidized cytochrome c (Margoliash, 1954). Results were expressed as nmoles of cytochrome c reduced per mg microsomal protein.

Estimation of CO-binding pigment

The method used for the determination of microsomal CO-binding pigment was that described by Dallner (1963). A Beckman model DB-G

spectrophotometer and recorder were used as described above.

A 0.5 ml aliquote of the microsomal suspension (0.5 g liver equivalent) was mixed with 2.5 ml of 0.1 M phosphate buffer (pH 7.4) in each cuvette. A few milligrams of sodium dithionate ($Na_2S_2O_4$) was added to each cuvette and mixed. The sample cuvette was gassed with carbon monoxide for three minutes, and the spectrum scanned from 500 nm to 400 nm at a rate of 40 nm per minute. The content of CO-binding pigment was reported as the difference in absorbance between 450 nm and 500 nm per mg microsomal protein.

Estimation of protein

Protein content of liver microsomal suspensions was determined by the colorimetric method of Lowry et al. (1951). A 1.0 ml aliquot of microsomal suspension (1.0 g liver equivalent) was added to a test tube containing 4.0 ml of 0.5 N KOH solution. The tubes were heated in a water bath until all material was dissolved. Standards were prepared using crystalline bovine serum albumin in 0.5 N KOH. A blank carried through the entire procedure consisted of 5.0 ml of 0.5 N KOH. After the samples were completely dissolved, a 0.1 ml aliquot was removed and placed in a second tube containing 1.0 ml of NaOH solution. Five ml of Reagent A (prepared by the addition of 1.0 ml of 1 per cent cupric sulphate solution plus 1.0 ml of 2.7 per cent potassium tartrate to 100 ml of 2 per cent sodium carbonate solution) was added, the sample mixed, and the tubes allowed to stand at room temperature for 20 minutes. At this time, 0.5 ml of Reagent B (prepared by dilution of commercial Folin-phenol reagent to 1 N with distilled water) was added and each sample rapidly shaken. The samples were allowed to stand at room temperature for 40 minutes for color development. The absorbence was then deter-

mined against the blank in a Beckman model DB-G spectrophotometer at 500 nm.

Estimation of formaldehyde

The enzymatic demethylation of drugs by hepatic microsomal fractions has been shown to result in the release of formaldehyde (McMahon and Easton, 1962). The procedure described by these authors was used for the estimation of formaldehyde produced by the *in vitro* N-demethylation of aminopyrine.

After a 30-minute incubation period described above, the reaction was stopped by pouring the flask contents into a test tube containing 4.0 ml of 10 per cent $ZnCl_2$ (prepared with CO_2-free distilled water). After mixing, 2.0 ml of saturated barium hydroxide solution was added to each tube. The tubes were centrifuged and 5.0 ml of the clear supernatant was added to a tube containing 2.0 ml of double strength Nash reagent (0.04 M acetylacetone in 0.4 M ammonium acetate-0.1 M acetic acid solution). The tubes were shaken and the color developed by heating in a water bath at 60°C for 30 minutes. The samples were then read against blanks at 410 nm in a Beckman model DB-G spectrophotometer. Aminopyrine demethylase activity was reported as moles of formaldehyde formed per mg microsomal protein per hour.

Estimation of p-nitrophenol

The enzymatic demethylation of p-nitroanisole produces formaldehyde and p-nitrophenol (Netter and Seidel, 1964). The amount of p-nitrophenol liberated by the *in vitro* enzymatic demethylation of p-nitroanisole was used to determine p-nitroanisole demethylase activity.

At the end of the 30-minute incubation period, 10.0 ml of ice-cold acetone was added to the incubation flasks. After centrifugation

(10,000 RPM for 10 minutes in a refrigerated Sorvall RC2-B centrifuge), the supernatant solution was read versus a tissue blank at 410 nm on a Beckman DB-G spectrophotometer. The amount of p-nitrophenol formed was determined by comparison with standard p-nitrophenol solutions. Enzyme activity was expressed as μg p-nitrophenol formed per mg microsomal protein per hour.

Estimation of microsomal lipid peroxidation by diene conjugation absorption

Liver microsomal lipids exhibit an increase in absorption in the region 230-240 nm when they undergo peroxidative decomposition (Rao and Recknagel, 1968). A modification of the method described by these authors was used to estimate microsomal lipid peroxidation following carbon tetrachloride exposure.

The liver microsomal pellet (5.0 g liver equivalent) obtained after high speed centrifugation described above was quantitatively transferred with 10.0 ml absolute methanol to a large screw top test tube. A 20.0 ml portion of chloroform was added, and the mixture was warmed to 50°C for one minute and shaken vigorously on a Kuehler rolling shaker (model 2-3000) for ten minutes. The mixture was then filtered through Whatman No. 2 filter paper to isolate the chloroform-methanol lipid extract. Ten ml of saline solution (4 g NaCl per 100 ml) was then added to the lipid extract. The samples were gently shaken and centrifuged at 2,000 RPM in an IEC model HN-S centrifuge for ten minutes. A 10.0 ml aliquot of the lower chloroform lipid phase was transferred to tared beakers and evaporated under vacuum at 25°C. The recovered lipid was weighed and dissolved in spectroquality cyclohexane to a final concentration of 1.0 mg per ml. The absorbance was then determined in a Beckman model DB-G spectrophotometer over the range 220 to 260 nm. Estima-

tion of lipid peroxidation was reported as the difference between the mean absorbance at 240 nm for microsomal lipids from carbon tetrachloride exposed rats and their respective controls. The mean optical density obtained by difference is the diene conjugation absorption for peroxidized lipid at a final concentration of 0.1 per cent, which when multiplied by 10, is reported as delta $E^{1\%}_{1\ cm}$ (Reo and Recknagel, 1968).

Statistical methods

Student's t test was used to compare differences between means. The "t" statistic was calculated on an Olivetti Underwood Programma 101 desk top computer. The formula employed is as follows:

$$t = \frac{\bar{X}_1 - \bar{X}_2}{\sigma \sqrt{\frac{1}{N_1} + \frac{1}{N_2}}}$$

Where

$$\sigma = \sqrt{\frac{N_1 S_1^2 + N_2 S_2^2}{N_1 + N_2 - 2}}$$

$$S^2 = \frac{\sum(X^2)}{N} - \left(\frac{\sum X}{N}\right)^2$$

RESULTS AND DISCUSSION

Effect of Carbon Tetrachloride Exposure on SGPT and SGOT

The rises in serum enzymes immediately following exposure to 2450 ppm of carbon tetrachloride (CCl_4) are presented in figure 1. In the phenobarbital (PB) pretreated animals which were exposed, SGOT and SGPT values were elevated two-fold when compared to saline treated controls that were similarly exposed. However, the 3-methylcholanthrene (3MC) pretreated group had significantly lower ($p<.05$) values of SGOT and SGPT than the corn oil treated controls.

The effects of pretreatment were further intensified when SGPT and SGOT values were obtained 21 hours after the termination of exposure (figure 2). Exposure to carbon tetrachloride resulted in a 100-fold increase in the SGPT value of the phenobarbital pretreated group when compared to saline animals similarly exposed and a 28-fold increase in SGOT. However, the SGPT and SGOT values for the 3-methylcholanthrene pretreated groups were only 28 per cent and 57 per cent, respectively, of the corn oil group when both groups were exposed to carbon tetrachloride.

These data support the finding of Garner and McLean (1969) that phenobarbital pretreatment enhances carbon tetrachloride hepatotoxicity. However, pretreatment with 3-methylcholanthrene was associated with a protective effect on the carbon tetrachloride induced rise in serum enzymes.

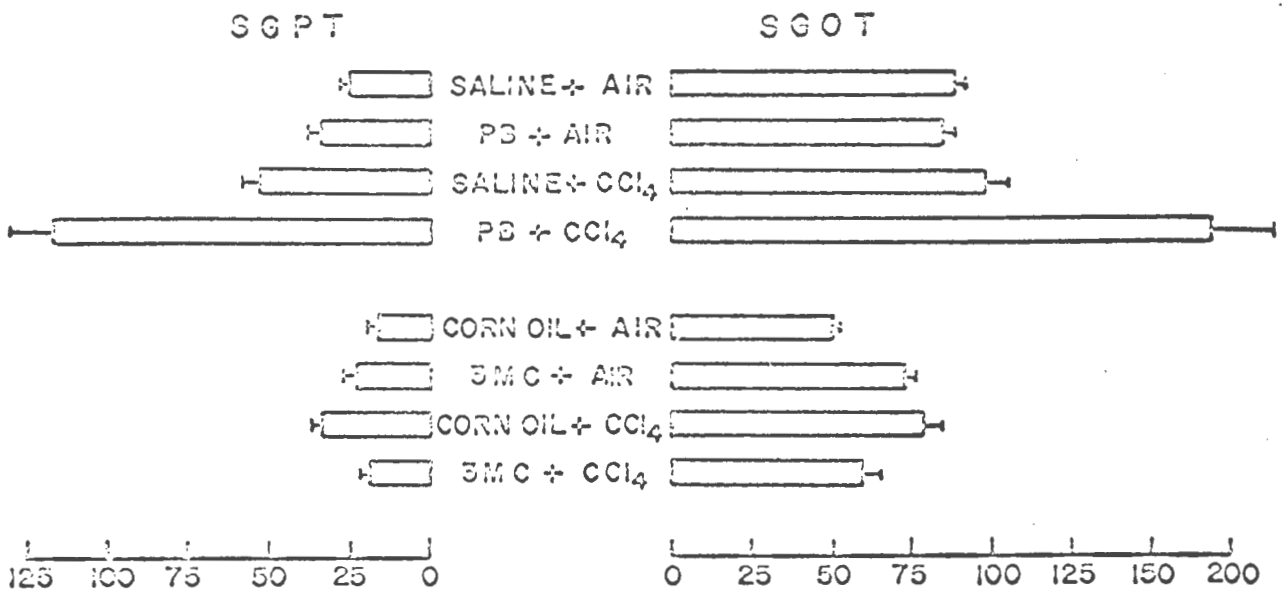


FIGURE 1. SGPT and SGOT Determined Immediately After Termination of a Three-hour Exposure to CCl_4 Vapors (2450 ppm). Results are expressed as the number of Reitman-Frankel units per ml of serum ± S.E. Five animals in PB + air group, seven in PB + CCl_4 group, and six animals in each of the other groups.

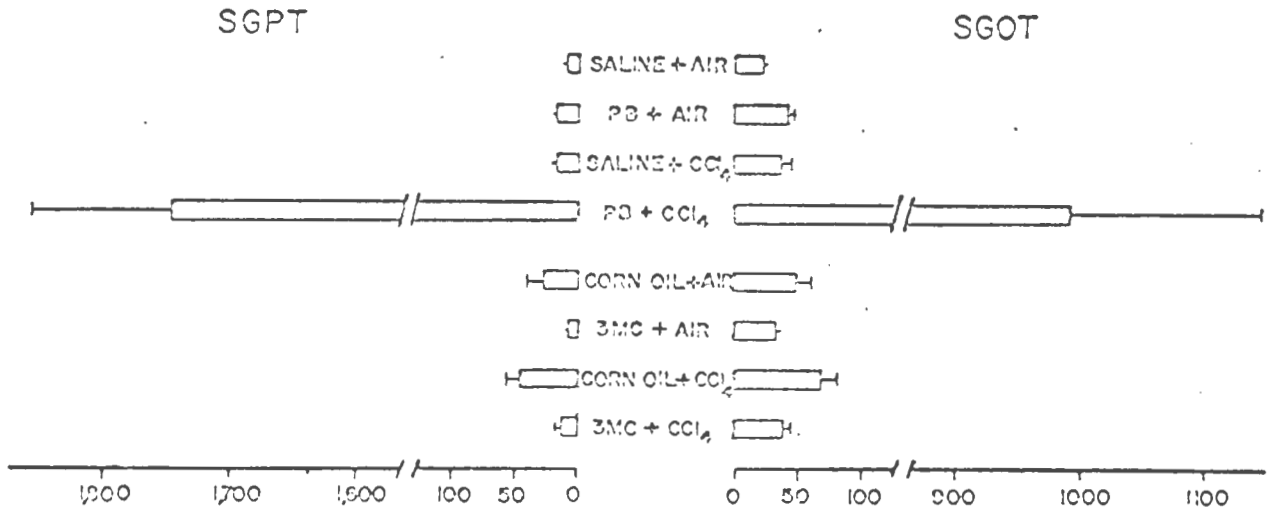


FIGURE 2. SGPT and SGOT Determined 21 Hours After Termination of a Three-hour Exposure to CCl_4 Vapors (2650 ppm). Results are expressed as the mean number of Reitman-Frankel units per ml of serum ± S.E. for a group of four animals.

Histological Evidence of Carbon Tetrachloride Induced Liver Damage

Microscopic examination of the liver following carbon tetrachloride exposure reveals midzonal focal necrosis as early as six hours after poisoning (Wigglesworth, 1964). At 12 hours, the centrolobular cells exhibit prenecrotic changes, and ballooning is evident in cells of the midzonal region. Marked centrolobular necrosis is evident in up to half the lobules at 24 hours after exposure. In the present study specimens of liver tissue were stained with hematoxylin and eosin and examined by a qualified pathologist.*

Histological examination of the livers from the air-exposed animals in the 24-hour study revealed no morphological abnormalities in the animals receiving either saline or corn oil. The phenobarbital pretreated rats demonstrated a more coarse granular cytoplasm in the parenchymal cells. There were some vacuoles in the parenchymal cells of the 3-methylcholanthrene pretreated rats. In the groups exposed to carbon tetrachloride, the livers showed varying degrees of damage. In the saline treated rats there was centrolobular necrosis in most but not all lobules. The cells of the centrolobular areas showed hydropic degeneration of various degrees of intensity and ballooning. A few cells in these areas showed pyknotic nuclei or nuclear lysis. The parenchymal cells of the periphery of the affected lobules were well preserved. Essentially the same type of lesion was present in the livers of animals injected with corn oil, although injury was slightly more severe and widespread. In the phenobarbital pretreated group, injury was much more severe with submassive necrosis of the liver. Fat droplets, hydropic

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degeneration and ballooning was present in most cells. In the animals pretreated with 3-methylcholanthrene, lesions ranged from minimal alterations in some animals (consisting of fat droplets but no necrosis) to centrolobular necrosis and hydropic degeneration (in about one half of the liver lobules) in other rats. In the rats showing the most marked lesions, the injury was confined to only a few cells in the centrolobular areas and appeared to be much less intense than the morphological alterations found in animals receiving corn oil and carbon tetrachloride.

The histological finding of less liver damage due to carbon tetrachloride inhalation in the 3-methylcholanthrene pretreated animals than in the corn oil controls is of special interest since Reuber et al. (1968, 1970) demonstrated that 3-methylcholanthrene in the diet of rats greatly increased liver cirrhosis due to carbon tetrachloride administration. This difference is probably due to the fact that these authors administered the compounds for 12 weeks.

Effect of Carbon Tetrachloride Exposure on Levels of Hepatic Microsomal Constituents in the Rat

The impairment of liver microsomal drug metabolizing enzymes has been shown to be accompanied by a decrease in liver microsomal CO-binding pigment (Castro et al., 1968; Sawickler et al., 1967). In the present study, exposure of rats to 2450 ppm of carbon tetrachloride reduced CO-binding pigment content in all four groups of animals when compared to their respective air exposed controls (table 1). However, the greatest decrease (61 per cent) was found in the phenobarbital pretreated group. The decrease in the corn oil group (38 per cent) was similar to the decrease in the 3-methylcholanthrene group (39 per cent). This exposure did not significantly decrease the NADPH cytochrome c reductase activity

Table 1. Effect of CCl_4 Inhalation (2450 ppm) on Microsomal Electron Transport Components in Induced and Control Rats Determined Immediately After Termination of a Three-hour Exposure

Group	N[a]	NADPH Cytochrome c Reductase[b]	Per cent Decrease	p[c]	N[a]	CO-Binding Pigment[a] x 10^{-4}	Per cent Decrease	p[c]
Saline-air	6	93.8 ± 4.3	---	---	6	127 ± 8.2	---	---
Saline-CCl_4	6	82.2 ± 6.1	12	N.S.[e]	6	106 ± 7.4	16	<.05
PB-air	5	174.2 ± 11.4	---	---	3	269 ± 30.8	---	---
PB-CCl_4	7	164.6 ± 11.0	6	N.S.	7	104 ± 7.5	61	<.005
Corn oil-air	6	115.9 ± 5.3	---	---	6	194 ± 9.5	---	---
Corn oil-CCl_4	6	85.8 ± 6.5	26	<.005	6	121 ± 11.4	38	<.005
3MC-air	6	118.9 ± 7.6	---	---	6	324 ± 13.1	---	---
3MC-CCl_4	6	95.3 ± 6.6	20	<.025	6	196 ± 11.8	39	<.005

[a]Number of animals

[b]nmoles of cytochrome c reduced/minute/mg protein

[c]Two-tailed Student's t test

[d]Difference in absorbance between 450 and 500 nm per mg protein

[e]Not significant

of the saline and phenobarbital pretreated animals and only slightly reduced activity in the corn oil and 3-methylcholanthrene pretreated groups.

Exposure to carbon tetrachloride decreased aminopyrine demethylase activity in saline and phenobarbital pretreated rats and p-nitroanisole demethylase activity in corn oil and 3-methylcholanthrene pretreated rats (table 2). While the per cent decrease due to carbon tetrachloride exposure was greater after phenobarbital pretreatment, the opposite relationship was seen after 3-methylcholanthrene pretreatment.

These results suggest that phenobarbital pretreatment enhances the carbon tetrachloride induced decrease in microsomal constituents while 3-methylcholanthrene pretreatment exerts a protective effect.

Effect of Carbon Tetrachloride Exposure on Microsomal Diene Conjugation Absorption

Liver microsomal lipids exhibit diene conjugation absorption as early as five minutes after orally administered carbon tetrachloride (Rao and Recknagel, 1968). Maximal diene conjugation occurs at one half hour after administration. In a preliminary experiment, induced and control rats were administered 3.0 ml/kg of carbon tetrachloride intragastrically. Enhanced diene conjugation absorption was observed at 30 minutes after poisoning (table 3). Phenobarbital pretreatment enhanced diene conjugation following carbon tetrachloride when compared to saline pretreated carbon tetrachloride exposed controls. However, the opposite effect was observed with 3-methylcholanthrene pretreated rats when compared to respective controls.

To observe this phenomenon under inhalation conditions, groups of induced and control animals were exposed to an atmosphere containing approximately 4400 ppm carbon tetrachloride for 30 minutes. The animals

Table 2. Effect of CCl_4 Inhalation on Microsomal Demethylase Activity in Induced and Control Rats Determined Immediately After Termination of a Three-hour Exposure

Group	N[a]	Aminopyrine[b] Demethylase	Per cent Decrease	p[c]	p-Nitroanisole[d] Demethylase	Per cent Decrease	p[c]
Saline-air	6	130.3 ± 22.0	—	—	—	—	—
Saline-CCl_4	6	85.5 ± 8.9	35	N.S.[e]	—	—	—
PB-air	5	394.0 ± 51.6	—	—	—	—	—
PB-CCl_4	7	166.4 ± 17.6	58	<.005	—	—	—
Corn oil-air	6	—	—	—	1.59 ± 0.08	—	—
Corn oil-CCl_4	6	—	—	—	0.38 ± 0.015	76	<.005
3MC-air	6	—	—	—	9.83 ± 1.27	—	—
3MC-CCl_4	6	—	—	—	4.93 ± 0.75	50	<.005

[a]Number of animals

[b]nmoles formaldehyde formed/mg protein/hour

[c]Two-tailed Student's t test

[d]µg p-nitrophenol formed/mg protein/hour

[e]Not significant

Table 3. Microsomal Diene Conjugation Absorption Determined 30 Minutes After Oral Carbon Tetrachloride

Group	N[a]	Delta $E^{1\%}_{1\ cm}$	p[b]
Saline-CCl_4[c]	3	1.2	<.05
PB-CCl_4	3	2.2	<.05
Corn oil-CCl_4	3	1.5	<.05
3MC-CCl_4	3	1.2	<.05

[a]Number of animals

[b]Two-tailed Student's t test compared to air-exposed controls

[c]CCl_4 administered orally, 3.0 ml/kg

Table 4. Microsomal Diene Conjugation Absorption Determined Immediately After a 30-minute Exposure to Carbon Tetrachloride Vapors (4400 ppm)

Group	N[a]	Delta $E^{1\%}_{1\ cm}$	p[b]
Saline-CCl_4	3	1.2	<.05
PB-CCl_4	3	1.9	<.05
Corn oil-CCl_4	3	0.9	<.05
3MC-CCl_4	3	0.6	<.05

[a]Number of animals

[b]Two-tailed Student's t test compared to air-exposed controls

were immediately sacrificed and microsomal diene conjugation absorption was determined. The resulting data (table 4) was similar to that obtained after oral carbon tetrachloride. Phenobarbital pretreatment resulted in an increase in lipid peroxidation as a response to carbon tetrachloride, while 3-methylcholanthrene pretreatment resulted in a decrease.

Although free radicals generated *in vitro* can produce lipid metabolites from methyl oleate similar to those found in the liver after carbon tetrachloride intoxication (Gordis, 1969), free radicals generated from carbon tetrachloride by liver microsomal enzymes need not be essential for lipid peroxidation. Hochstein *et al.* (1964) and Orrenius *et al.* (1964) have described an NADPH-linked peroxidation of lipids in rat liver microsomes. The reaction is strongly inhibited by drugs undergoing oxidative demethylation and is slightly stimulated by carbon monoxide. In the absence of a substrate for the microsomal mixed function oxidase, reducing equivalents from NADPH are shunted from the microsomal electron transport chain with the production of malonaldehyde, a product of lipid peroxidation.

Any agent preventing reducing equivalents from leaving the electron transport chain at CO-binding pigment could conceivably activate a shunt responsible for lipid peroxidation. Carbon tetrachloride itself may be such an agent since it combines with CO-binding pigment producing a Type I spectral change (McLean, 1967).

Slater and Sawyer (1971a) have concluded that the stimulation of malonaldehyde production by carbon tetrachloride is dependent upon its homolytic cleavage to free radical products. They also conclude (Slater and Sawyer, 1971b) that the point of interaction between carbon tetrachloride and the mixed function oxidase system providing for the stimu-

lation of malonaldehyde formation is at the NADPH cytochrome c reductase flavoprotein.

Whether carbon tetrachloride produces an increase in lipid peroxidation by activating a lipid peroxidation shunt or by formation of toxic free radicals from interaction with intermediates in microsomal electron transport remains undetermined at this time. In either event, an increase in NADPH cytochrome c reductase and CO-binding pigment by phenobarbital pretreatment would account for the increase in the hepatotoxic effect of carbon tetrachloride and enhanced lipid peroxidation as reported by Rao et al. (1970). However, after 3-methylcholanthrene induction an increase in CO-binding pigment without a concomitant increase in NADPH cytochrome c reductase was associated with decreased carbon tetrachloride induced hepatotoxicity. In this case, the increase in CO-binding pigment could prevent lipid peroxidation through enhanced utilization of reducing equivalents in the metabolism of endogenous substrates. Hence, the protective effect afforded by hexobarbital and aniline on the carbon tetrachloride perfused rat liver (Rubin et al., 1970) may be due to utilization of reducing equivalents through CO-binding pigment.

On the basis of the data presented, it was proposed that phenobarbital pretreatment enhances microsomal electron transport and lipid peroxidation responsible for the potentiation of carbon tetrachloride hepatotoxicity. Three-methylcholanthrene pretreatment creates an imbalance in NADPH cytochrome c reductase and CO-binding pigment favoring the utilization of reducing equivalents at the terminal electron acceptor resulting in a protective effect. If this hypothesis is valid, interruption of electron transport at CO-binding pigment should enhance lipid peroxidation and hepatotoxicity following carbon tetrachloride exposure.

Several investigators have reported on the in vitro inhibition of

drug metabolism by carbon monoxide in isolated hepatic microsomal fractions (Cooper et al., 1965; Kampffmeyer and Kiese, 1965; Kato, 1966). Lewis (1967) has reported that atmospheres containing 40 to 90 per cent carbon monoxide inhibit CO-binding pigment dependent insecticide metabolism in houseflies. More recently, Montgomery and Rubin (1971) have reported that acute exposure to carbon monoxide resulted in the inhibition in vivo of drug metabolism in rats as measured by prolonged response to hexobarbital and zoxazolamine. However, these authors did not conclude whether the effect was due to direct inhibition of CO-binding pigment or to induced tissue hypoxia. These findings were the foundation for additional experiments to determine whether or not carbon monoxide could enhance in vivo lipid peroxidation and hepatotoxicity due to carbon tetrachloride by virtue of its affinity for CO-binding pigment. Rats were exposed to carbon monoxide (approximately 1000 ppm) for 60 minutes to reach equilibrium saturation. Carbon tetrachloride was then introduced into the chamber for the final 30 minutes of exposure. A control experiment was also performed to determine the effect of hypoxia (7.5 per cent oxygen) on carbon tetrachloride hepatotoxicity.

In the carbon monoxide-carbon tetrachloride group, SGPT and SGOT determined 24 hours after exposure were elevated three-fold when compared to animals exposed to carbon tetrachloride alone (table 5). The effect is not likely due to decreased tissue oxygen as hypoxia did not potentiate the response. The effect on microsomal lipid peroxidation determined immediately after exposure was as predicted by the hypothesis: carbon monoxide enhanced diene conjugation absorption following carbon tetrachloride exposure while hypoxia was without effect (table 6). These data support a hypothesis for the protective effect of 3-methylcholanthrene based on enhanced utilization of reducing equivalents through the terminal

Table 5. Effect of Carbon Monoxide (CO) or Hypoxia on SGPT and SGOT Determined 24 Hours After Termination of a 30-minute Exposure to Carbon Tetrachloride

Experiment Number	Group[a]	N[b]	SGPT[c]	p[d]	SGOT[c]	p[d]
1. 5140 ppm CCl_4	Air	5	16 ± 3	—	45 ± 5	—
	CO[e]	5	17 ± 1	N.S.[f]	40 ± 1	N.S.
	CCl_4-air	5	220 ± 26	—	406 ± 100	—
	CCl_4-CO	6	700 ± 102	$<.005$	1466 ± 160	$<.005$
2. 3799 ppm CCl_4	Air	5	16 ± 3	—	45 ± 5	—
	Hypoxia[g]	4	21 ± 1	N.S.	50 ± 5	N.S.
	CCl_4-air	6	100 ± 31	—	198 ± 38	—
	CCl_4-hypoxia	5	127 ± 23	N.S.	214 ± 33	N.S.

[a]Animals were exposed to 958 ppm carbon monoxide or hypoxia for 60 minutes followed by exposure for an additional 30 minutes to carbon tetrachloride vapors combined with carbon monoxide or hypoxia.

[b]Number of animals

[c]Reitman-Frankel units per ml serum

[d]Two-tailed Student's t test

[e]958 ppm CO

[f]Not significant at $p<.05$

[g]7.5 per cent oxygen

Table 6. Effect of Carbon Monoxide (CO) or Hypoxia on Microsomal Diene Conjugation Absorption Determined 30 Minutes After Exposure to Carbon Tetrachloride Vapors (4400 ppm)

Group[a]	N	Delta $E^{1\%}_{1\ cm}$	p[b]
CCl_4-air	3	1.2	<.05
CCl_4-hypoxia[c]	3	1.1	<.05
CCl_4-CO[d]	3	2.6	<.05
CO alone	3	-0.4	<.05
Hypoxia alone	3	-0.2	N.S.[e]

[a]Animals were exposed to 958 ppm carbon monoxide or hypoxia for 60 minutes followed by exposure for an additional 30 minutes to carbon tetrachloride vapors combined with carbon monoxide or hypoxia.

[b]Two-tailed Student's t test compared to air-exposed controls

[c]7.5 per cent oxygen

[d]958 ppm CO

[e]Not significant

electron acceptor.

SUMMARY AND CONCLUSIONS

(1) Phenobarbital pretreatment significantly enhanced the rise in serum enzymes SGOT and SGPT used as parameters of carbon tetrachloride hepatotoxicity while 3-methylcholanthrene pretreatment produced the opposite effect. Histological evaluation confirmed the differential effect of these two inducers of microsomal enzymes on carbon tetrachloride hepatotoxicity.

(2) Phenobarbital pretreatment enhanced the diene conjugation absorption of liver microsomal lipids following carbon tetrachloride exposure. However, 3-methylcholanthrene pretreated rats exposed to carbon tetrachloride had lower diene conjugation absorption than corn oil treated controls exposed to carbon tetrachloride.

(3) The carbon tetrachloride induced decrease in CO-binding pigment and microsomal demethylase activity was greater in phenobarbital treated animals than in animals treated with 3-methylcholanthrene.

(4) The enhancement of carbon tetrachloride toxicity by exposure to carbon monoxide supports the hypothesis that the protective effect of 3-methylcholanthrene pretreatment is due to an imbalance in NADPH cytochrome c reductase and CO-binding pigment favoring the utilization of reducing equivalents at the terminal electron acceptor.

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