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THE UNIVERSITY OF OKLAHOMA

GRADUATE COLLEGE

LIPID PEROXIDATION BY ISOLATED RAT LIVER PARENCHYMAL CELLS: FACTORS THAT AUGMENT AND INHIBIT LIPID PEROXIDATION

A DISSERTATION

SUBMITTED TO THE GRADUATE FACULTY

in partial fulfillment of the requirements for the

degree of

DOCTOR OF PHILOSOPHY

BY

CHARLES C. WEDDLE, JR.

Oklahoma City, Oklahoma

1975

LIPID PEROXIDATION BY ISOLATED RAT LIVER PARENCHYMAL CELLS:

FACTORS THAT AUGMENT AND INHIBIT LIPID PEROXIDATION

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To Nancy
and
Charles, III.

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CHAPTER I

INTRODUCTION

This study is concerned with the production and the effects of endogenously formed free radicals on the membranous components of isolated rat liver parenchymal cells. There are several different enzymatic systems that are believed to produce free radicals *in vitro* and/or *in vivo* which are known to initiate subcellular membrane perturbations; two of these are 1) the NADPH oxidase system of rat liver microsomes and mitochondria *in vitro*, and 2) the enzymic system in liver tissue which metabolizes haloalkanes, especially carbon tetrachloride. The latter system is also located in the endoplasmic reticulum and is active both *in vivo* and *in vitro*. Because each of these systems is believed to be unique, the introduction will describe each system separately.

A. General Aspects of Free Radicals and of Free Radicals Generated by the NADPH Oxidase Enzyme System.

Lipid peroxidation is a process resulting in oxidative chain cleavage of polyunsaturated fatty acids. This phenomenon is a consequence of a reaction between oxygen and unsaturated lipid mediated by free radical intermediates, producing semi-stable peroxides. Since animal tissues

are rich in polyunsaturated fatty acids, lipid peroxidation and free radical reactions involving other cell components probably constitute a progressive mechanism of cellular membrane damage in all metabolizing animal cells. This is supported by the gradual accumulation (in animal tissues) of lipofuscin and ceroid pigments which are believed to be end-products of membrane lipid peroxidation (1). Many in vitro studies of lipid peroxidation in model systems have been performed but not all of the basic mechanisms and properties of such systems are known. Lipid peroxidation of pure lipids is thought to be an autocatalytic series of reactions involving a free radical mechanism (2,3). This mechanism is represented as a series of four steps.

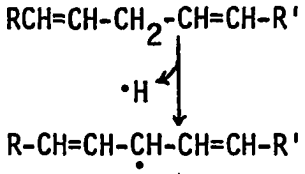
1) Initiation: formation of a free radical on a polyunsaturated lipid by hydrogen abstraction at a methylene group of a methylene-interrupted (1,4) pentadiene portion of the lipid structure; this methene radical (H-C$^{\cdot}$), which is in the unconjugated diene configuration, shifts to the more stable conjugated 1,3 diene radical configuration.

2) Propagation: formation of a peroxy radical by reaction of the diene free radical with molecular oxygen. Subsequent abstraction of a H atom from another unsaturated lipid forms a hydroperoxide and also another lipid free radical. This process may be repeated as a chain reaction until all polyunsaturated lipids are oxidized or until a free radical scavenger terminates the reaction as described below.

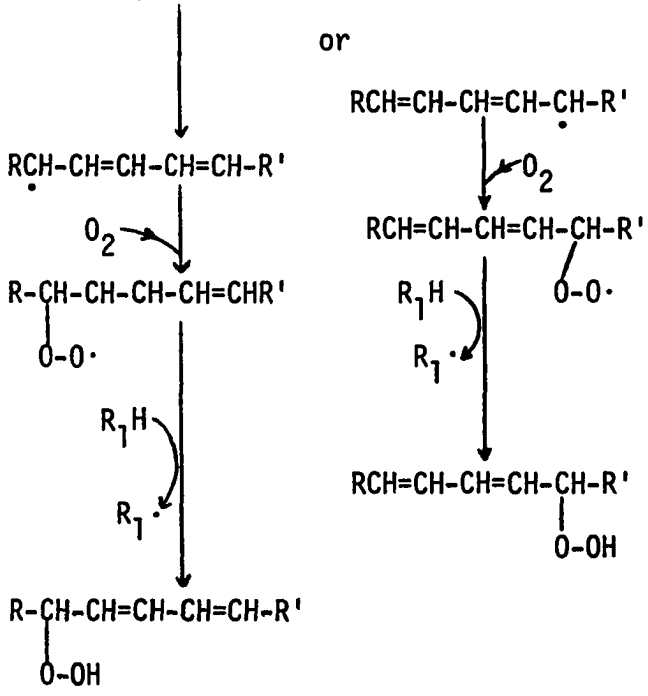
3) Lipid hydroperoxide breakdown: homolytic chain cleavage with formation of more free radicals, carbonyl compounds and other substances.

4) Chain termination - radical products react with each other or with other substances to form stable products.

Initiation

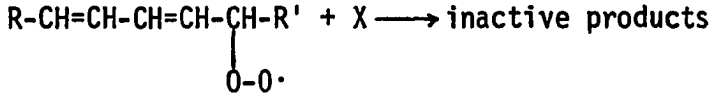


Propagation



Peroxide breakdown products include: aldehydes, ketones, alcohols, carboxylic acids, and polymers.

Chain termination:

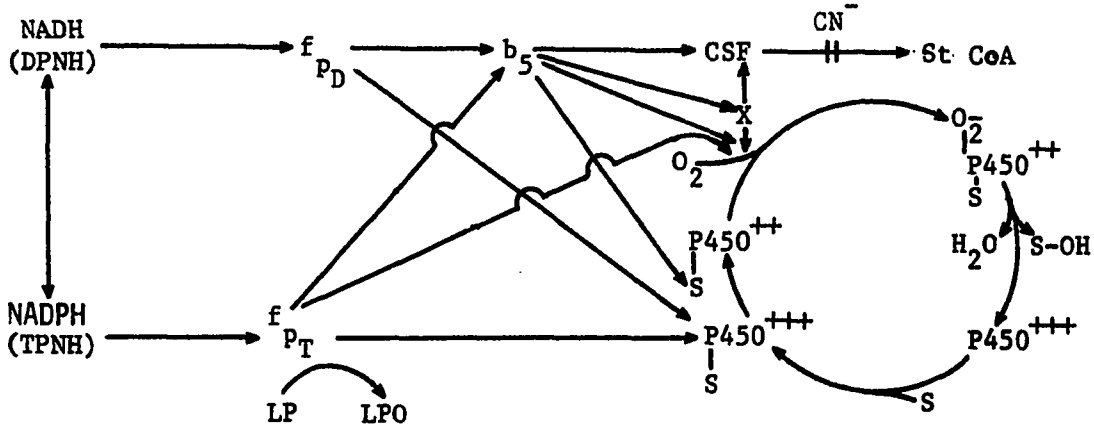


R_1H may be an unsaturated fatty acid with a methylene group of a methylene interrupted (1,4) pentadiene portion present and X is either another free radical or compound which may react with a free radical to form products which may then be stable and inactive.

Lipid peroxidation *in vitro* has been associated with an electron transport system found in microsomes prepared from several tissues. This electron transport system is believed to be related to the mixed function oxidase system (5), and is involved in the metabolism of normal components such as the hydroxylation of steroids and in the metabolism of substances foreign and often toxic (termed "xenobiotic") to the metabolic network: aromatic hydrocarbons, halogenated hydrocarbons, and various drugs. In general, mixed-function oxidases perform the following reaction:

$$NADPH + H^+ + R\text{-}H + O_2 \longrightarrow NADP^+ + R\text{-}OH + H_2O$$

The components and their relationships required for mixed function oxidase activity have been established by: a) studies on isolated components; b) studies of the effects of various electron acceptors and inhibitors; c) studies on the induction or destruction of certain components of the system by drugs; and d) the appearance of various enzymatic activities in the liver of fetal and developing animals. These studies have produced the following reaction scheme which was described by Correia and Mannering (6).



This mechanism is derived from an earlier study by Cohen and Estabrook (7) who investigated the cooperative interaction between NADH and NADPH (TPNH)-linked electron transport pathways by studying the demethylation of ethylmorphine, aminopyrine, and codeine. There are several different pathways for NADH-NADPH interaction:

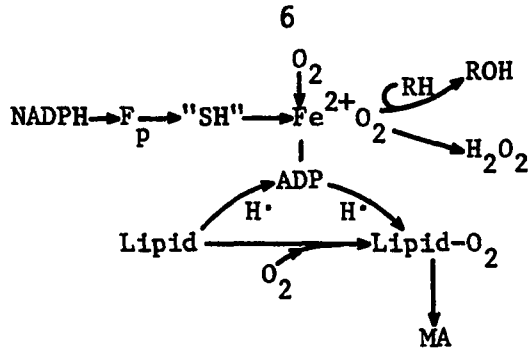
Flavoprotein-mediated electron transport:

1) electron flow from
   a) NADPH-cytochrome c reductase flavoprotein (fp_T) to cytochrome b_5.
   b) NADH to fp_T.

Electron transport at the cytochrome P-450 level:

1) electron flow from
   a) NADH to cytochrome P-450 via cytochrome b_5. (two pathways available)
   b) NADPH to cytochrome P-450 via fp_T.
   c) NADH to cytochrome P-450 via NADH-cytochrome b_5 reductase (fp_D).

In addition, lipid (LP) and NADPH-cytochrome c reductase (fp_T) can react to form lipid peroxides (LPO). This mechanism for lipid peroxidation in vitro was first described by Hochstein and Ernster (5) and more recently investigated by McCay et al. It was found that liver microsomes, in the presence of NADPH, ferric ion, and adenine dinucleotide phosphate, catalyze the rapid formation of a 2-thiobarbituric acid (TBA)-reacting compound, believed to be malondialdehyde, a product formed during the peroxidation of unsaturated lipids. From their study, Hochstein and Ernster proposed the first mechanism for this reaction:



Several compounds have been shown to effectively inhibit the formation of this thiobarbituric acid-reacting substance. These include the metal-chelating agent, ethylenediamine-tetraacetic acid (EDTA), transition metal ions such as cobalt, manganese, and cerium; antioxidants such as diphenyl-p-phenylenediamine, ethoxyquin (Santoquin) and d-α-tocopherol (vitamin E); and an inhibitor of the microsomal electron transport system such as para-chloromercuribenzoate. The process has been shown *in vitro* to be heat labile, i.e., pre-heating microsomes inactivates the oxidation of NADPH. From these results Hochstein and Ernster proposed that the formation of an intermediate ($ADP\text{-}Fe^{2+}O_2$) in the NADPH oxidase system catalyzed the lipid peroxidation.

The NADPH-linked lipid peroxidation of liver microsomes appears to be associated with the microsomal drug metabolizing system. Orrenius *et al.* (8) demonstrated that drugs undergoing oxidative demethylation such as codeine and aminopyrine strongly inhibited the NADPH-mediated peroxidation in microsomes *in vitro*. This was the first evidence for the presence of a common NADPH-oxidizing enzyme in microsomes. Because drugs are known to be metabolized similarly *in vivo*, the inhibition of lipid peroxidation by the metabolism of drugs *in vitro* suggested that NADPH-linked lipid peroxidation may exist *in vivo*, a phenomenon still unre-

solved. More specifically, Pederson and Aust (9) demonstrated that either purified NADPH-cytochrome c reductase or deoxycholate solubilized NADPH-cytochrome P-450 reductase was capable of oxidizing NADPH in presence of ADP-Fe^{3+}, O_2, and EDTA,and producing malondialdehyde from isolated microsomal phospholipid. This was later verified by Fong et al. (10) who demonstrated, however, that oxidation of NADPH by a purified NADPH-cytochrome P-450 reductase lysed lysosomal membranes and produced malondialdehyde during the lytic process in the absence of EDTA, the lysis and malondialdehyde production being inhibited by EDTA. These studies indicated that,during the oxidation of NADPH in the presence of ADP-Fe^{3+}, a flavin enzyme of the drug metabolizing system was involved in the peroxidation of membrane-bound lipids.

McCay et al. have investigated 1) the cellular location, properties, and requirements for NADPH oxidase activity; 2) the polyunsaturated fatty acids of microsomal phospholipids that are susceptible to this enzyme-catalyzed radical attack; 3) the formation of lipid peroxide intermediates; 4) the identification of malondialdehyde as the thiobarbituric acid-reacting substance; and 5) the destructiveness of this enzyme system on not only microsomes, but erythrocytes, mitochondria, and lysosomes (10-17). May and McCay showed that polyunsaturated fatty acids from microsomal membrane phospholipids of rat liver were consumed by the NADPH-oxidase system (11). In particular, they demonstrated that arachidonic and docosahexaenoic acids, esterified to the β-position of phospholipids, were consumed by the oxidase reaction. They subsequently investigated the conditions necessary for optimum NADPH oxidase activity, the effect of various inhibitors, and the determination of the stoichio-

metry of the system (12). NADPH was found to be required for the oxidase activity; the maximum activity for the reaction system was obtained with a physiological NADPH concentration, 0.3 mM NADPH. The stoichiometry of the reaction showed the molar ratios of fatty acid peroxidized to NADPH to oxygen to be 1:1:4. The enzymic production of malondialdehyde from polyunsaturated fatty acids could be inhibited by the in vitro addition of EDTA, Co^{2+}, Mn^{2+}, or parahydroxymercuribenzoate, or when microsomes are prepared in a d-α-tocopherol-supplemented solution. Tam and McCay provided evidence for the production of phospholipid peroxide intermediates which produce small, but measurable quantities of malondialdehyde during the peroxidative process (13). The identification of the chromogenic compound formed by the enzyme reaction as malondialdehyde was later demonstrated by Poyer and McCay (14). The production of this compound was also found to be dependent on catalytic quantities of Fe^{3+}.

The information cited above was accumulated from studies with rat liver microsomes which peroxidized their own phospholipids during the oxidation of NADPH. Also, this microsomal NADPH-oxidase system has been shown to hemolyze erythrocytes (15), rupture lysosomal membranes in vitro (16), and inactivate mitochondria (17). The lytic action of the microsomal NADPH oxidase system on these cellular membranes could be inhibited by various radical scavenging agents such as d-α-tocopherol, ethoxyquin, and diphenyl-p-phenylenediamine. This suggested that the NADPH-oxidase system produced a radical-like component capable of attacking other biological membranes. Subsequently, Fong et al. (10) provided evidence that the peroxidation of lysosomal membranes was caused by hydroxyl free radicals produced during NADPH-oxidase activity, and that

the superoxide anion ($O_2^{\cdot}$) was possibly an intermediate in the formation of the hydroxyl free radical. Pfeifer and McCay (17) reported that mitochondria alone were capable of oxidizing NADPH in the presence of Fe^{3+} and molecular oxygen,with the concommitant production of malondialdehyde. As a result, the mitochondria lost part or all of their capacity to oxidize Kreb Cycle intermediates and were unable to phosphorylate ADP.

Many attempts have been made to detect the formation of lipid peroxide intermediates and their products in vivo: lipid peroxides, diene conjugates, and malondialdehyde. Of those mentioned, only diene conjugates have been detected in significant quantities. The lipid peroxides have been shown to be metabolized in vitro to malondialdehyde (18). In addition, malondialdehyde (MDA) is rapidly metabolized in vivo (19) and by tissue suspensions in vitro. The metabolism of malondialdehyde in vitro can be performed by mitochondria and is an energy-requiring process (utilizing ATP) (20). In 1970, Horton and Packer (21) showed that liver tissue contains a mitochondrial aldehyde oxidase of low specificity capable of metabolizing malondialdehyde and several other low molecular weight aldehydes. As a result of the ability of liver cells to metabolize lipid peroxides and malondialdehyde, the only substantial evidence for lipid peroxidation in vivo is the detection of diene conjugates and the occurrence of lipofuscin pigments, which are thought to be polymerized end products of the reaction of peroxidized lipids with proteins.

B. Free Radicals Generated by Haloalkane Metabolism.

The NADPH oxidase enzyme system promotes free radical formation in the presence of ADP-Fe^{3+} in vitro, but there are several substances that are capable of free radical formation without catalytic amounts of Fe^{3+} when metabolized in vivo or in vitro by the NADPH oxidase system. These include bromotrichloromethane ($BrCCl_3$), carbon tetrachloride (CCl_4), fluorotrichloromethane ($FCCl_3$), chloroform ($CHCl_3$), ethanol, and 1,1,2,2-tetrachloroethane ($Cl_2HC\text{-}CHCl_2$), the latter occurring in mice only. Many of these compounds are known to initiate lipid peroxidation in vivo and in vitro, but some are more potent hepatotoxins than others. The degree of hepatotoxicity of some of these compounds has been rationalized in terms of the relative bond dissociation energies of either the halogen moieties of $Br\text{-}CCl_3$, $Cl\text{-}CCl_3$, $F\text{-}CCl_3$, or the hydrogen moiety of $H\text{-}CCl_3$, i.e., as the bond energy increases the hepatotoxicity decreases. The relative dissociation bond energy of these compounds can be represented as $FCCl_3$ and $CHCl_3 > CCl_4 > BrCl_3$. The cleavage of the moieties from the parent compound is believed to be homolytic, i.e., bond splitting, so that the products each contain a single unpaired electron ($CCl_4 \rightarrow \cdot Cl + \cdot CCl_3$). Evidence for the relative reactivities of these compounds has been accumulated from previously unpublished in vitro studies of Slater and Sawyer (18) and from information cited in a review article by Recknagel and Glende (22). As one might expect, $BrCCl_3$ is more hepatotoxic than $CHCl_3$. Many other compounds are known to be hepatotoxic, such as aromatic hydrocarbons, ethionine, orotic acid, and thioacetamide, but there is very little evidence that they are metabolized to free radical intermediates.

Of those compounds mentioned, carbon tetrachloride has been the most extensively investigated of the free radical producing substances. Carbon tetrachloride is known to promote a variety of hepatic abnormalities *in vivo* and *in vitro*. Within five minutes after CCl_4 administration to rats, hepatic lipid peroxidation has been detected by the formation of diene conjugates *in vivo* (23). Also at that time the binding of labeled carbon in $^{14}CCl_4$ to microsomal proteins and lipids has been demonstrated by Rao and Recknagel (24). A few hours later, endogenous NADPH and cytochrome P-450 content (25,26), protein synthesis (27), and aminopyrine demethylase (28) and glucose-6-phosphatase (G-6-Pase activities (29) were significantly depressed. Also, the liver began to accumulate neutral lipid, while the activity of microsomal NADPH-cytochrome c reductase, (30), triglyceride biosynthesis (28), and mitochondrial oxidation of octanoate, succinate, β-hydroxybutyrate, and glutamate were unaffected for several hours (31). Twenty hours after CCl_4 administration, mitochondrial function was significantly depressed. Electron micrographic studies confirmed these biochemical perturbations; there were no significant alterations in mitochondrial structure during the first few hours of poisoning, but there was noticeable swelling of the microsomal cisternae within 60 minutes after CCl_4 administration (27,32-34). Some subtle changes in mitochondrial nucleotide content have been shown by Slater and Delaney (35).

Another characteristic of CCl_4 hepatotoxicity *in vivo* is its ability to lower the total glycogen content of starved rats (36). Twenty-four hours after CCl_4 dosing, the activity of the I-form of glycogen transferase was decreased, while the activity of glycogen phos-

phorylase was increased with respect to untreated, starved rats. This was found to be related to a decreased activity rather than a decreased synthesis of glycogen transferase phosphatase and of phosphorylase phosphatase, as demonstrated by pretreatment of animals with cycloheximide, i.e., phosphorylase phosphatase activity decreased, but that of transferase phosphatase was apparently unaffected. Thus, the effect of CCl_4 on the activity of glycogen transferase phosphatase must be unrelated to the inhibition of protein synthesis of this enzyme. The net result of CCl_4 administration to rats is a decrease in glycogen synthetic ability of the liver.

Many of the biochemical changes produced *in vivo* by CCl_4 have been observed *in vitro* with rat liver microsomes. These include lipid peroxidation (22,37-41), loss of cytochrome P-450 content (28), glucose-6-Pase (42), and aminopyrine demethylase activity (26). The loss of cytochrome P-450 and microsomal enzyme activities have been attributed to lipid peroxidation (43); however, Slater has unpublished evidence that CCl_4 destroys cytochrome P-450 in the absence of lipid peroxidation (44). It is believed that any process that produces structural changes in membranes, i.e., destruction of lipid, is capable of producing functional alterations of those membranes or the contents which they encapsulate. It certainly appears that this has been demonstrated with hepatic microsomes, but unfortunately, there are no *in vitro* studies of either mitochondria or lysosomes with CCl_4 that correlate functional changes with membranous lipid alterations of these subcellular organelles. For example, Artizzu *et al.* demonstrated that CCl_4 caused a decrease of P/O ratio, a slackening of respiratory control, an increase in ATPase

activity, and a swelling of mitochondria *in vitro*, but they provided no evidence that these functional changes were related to structural alterations caused by CCl_4 (45). Comporti *et al.* showed that mitochondria are partially protected from CCl_4 damage *in vitro* by EDTA, which they attributed to the chelating effect for calcium in the extramitochondrial solution (37). Also, Castro *et al.* showed that lysosomes could be lysed *in vitro* after the addition of CCl_4 (46), and Baccino *et al.* demonstrated that this lytic action of CCl_4 on lysosomes *in vitro* could not be prevented by the addition of agents either known to retard the *in vivo* onset of hepatic necrosis, i.e., promethazine, or those shown to protect mitochondrial damage by CCl_4 *in vitro*, i.e., EDTA (47). Although CCl_4 produced marked functional alterations of these two subcellular organelles *in vitro*, the severity of these effects of CCl_4 *in vivo* is less evident (48).

In summation, *in vivo* and *in vitro* studies of CCl_4 on hepatic subcellular organelles suggest that the initial major structural and functional alterations appear to be localized in the microsomes. As previously mentioned, the loss of glucose-6-Pase and aminopyrine demethylase activities and cytochrome P-450 content are related to lipid peroxidation of microsomal membranes; a process believed to be initiated by the metabolism of CCl_4. A more detailed discussion of CCl_4 free-radical-mediated lipid peroxidation *in vivo* and *in vitro* will be presented later.

Many of the hepatotoxic effects can be enhanced by starvation (39,49,50) and/or phenobarbital (22,51-56) or DDT (22,51) pretreatment of animals prior to CCl_4 administration. Starvation of rats prior to CCl_4 dosing has been shown to decrease the hepatocellular glycogen

stores and the quantity of smooth endoplasmic reticulum (SER). Krishnan and Stenger (49) also demonstrated that the hepatocellular rough endoplasmic reticulum (RER) was more susceptible to CCl_4 poisoning than was the SER of the starved animals. They suggested that because the SER has been implicated as the membrane component capable of detoxifying drugs, the depletion of the SER and consequently the loss of hepatocellular drug detoxification could afford an even greater susceptibility of the RER to the toxin. This implies that because the RER is unable to detoxify CCl_4, the RER is more susceptible to the toxin.

The administration of phenobarbital to animals induces the proliferation of the SER of liver parenchymal cells (58). Also the drug-hydroxylating enzymes, NADPH-cytochrome c reductase, and cytochrome P-450 are induced to levels 3- and 5- fold above those of control animals(3-5 times control activities of non-induced animals) (59). In addition, the rate of protein synthesis in ER is increased (60). After the administration of phenobarbital for 5 days, the total quantity of liver lipid increased, but there was no change in the proportions of lipids in the liver microsomal fraction as compared to protein (61). More specifically, the phospholipids of this membrane fraction increased 2-3 fold after phenobarbital pretreatment of rats (62,63). The phenobarbital stimulation or augmentation of CCl_4 toxicity is believed to be due to the induction of the xenobiotic metabolizing (detoxifying) system for CCl_4 of the ER. As a result, there would be a more rapid conversion of CCl_4 to its hepatotoxic metabolite which is capable of catastrophic structural and functional perturbations of RER and related cellular components.

In addition to compounds that are capable of either stimulating

or accelerating the hepatotoxic effects of CCl_4, there are agents known to alter many of these effects induced by CCl_4 *in vivo* and *in vitro*. Some of these compounds include antioxidants such as d-α-tocopherol, N,N'-diphenyl-p-phenylenediamine, ethoxyquin, promethazine, reduced ubiquinone-4, butylated hydroxytoluene (BHT), and propyl gallate; inhibitors of the microsomal drug metabolizing system such as 2-diethylamino ethyl, 2,2-diphenylvalerate (SKF525-A), 2,4-dichloro-6-phenyl phenoxyethyl diethylamine (Lilly 18947), and others; compounds which act by unknown mechanisms such as reduced glutathione, diethyl dithiocarbamate, and animals fed diets either supplemented with cystamine or depleted of protein. A complete description of the protective effects of each compound or condition previously cited is not pertinent to this study. However, the action of a few of these agents will be discussed due to their relevance to this study. D-α-tocopherol, N,N'-diphenyl-p-phenylenediamine, and ethoxyquin have somewhat different effects *in vivo* and very similar effects *in vitro* after CCl_4 administration either *in vivo* or *in vitro*.

D-α-tocopherol was the first antioxidant reported to protect rats against CCl_4 lethality. Hove demonstrated that α-tocopherol administered either by mouth or in the diet increased the survival of the CCl_4-treated rats 40 to 60 percent, compared to non-vitamin pretreated animals (64). Cawthorne *et al.* have obtained similar results with orally administered α-tocopherol (65), and Gallagher demonstrated the same protective effect against CCl_4 lethality with α-tocopherol given intraperitoneally (66). Both investigators reported that the number of doses of the vitamin and its time of administration prior to CCl_4 dosing influenced the survival of the rats poisoned with CCl_4. Besides these studies, many

investigators have examined the effects of α-tocopherol on CCl_4-linked accumulation of triglyceride, necrosis, decrease in cytochrome P-450 content, decrease in protein synthesis, and the endogenous production of diene conjugates of microsomes of rat liver. Cawthorne et al. (65) and Green et al. (67) reported that administration of d-a-tocopherol either orally, by diet, or intraperitoneally did not prevent CCl_4-induced liver necrosis and triglyceride accumulation in female rats. Conversely, McLean (68) showed that male rats pretreated with d-α-tocopherol administered orally prevented CCl_4-induced hepatic triglyceride accumulation, but had no effect on CCl_4-induced hepatic necrosis. Meldolesi reported that d-α-tocopherol administered intraperitoneally prevented both hepatic necrosis and triglyceride accumulation in male rats treated with CCl_4 (69). Thus the protective effects of the vitamin on CCl_4-induced hepatic necrosis and triglyceride accumulation may be a result of 1) the route of d-α-tocopherol administration, 2) the sex of the animal, or 3) both. Carbon tetrachloride is known to depress the activity of the hepatic drug metabolizing system and to destroy the cytochrome P-450 component of that system. D-α-tocopherol was unable to prevent these phenomena from occurring (25,70). Hepatic protein synthesis is also diminished after poisoning the animal with CCl_4, and Alpers et al. demonstrated that neither the depression of protein synthesis nor the disaggregation of hepatic polyribosomes could be altered by intraperitoneal injections of d-α-tocopherol in vivo (71). Similarly, the protective effects of the vitamin in vivo have been reported on CCl_4-linked production of diene conjugates of rat liver microsomes. Comporti et al. showed that the endogenous formation of lipid diene conjugates, intermediates in the process of lipid peroxidation,

formed as a result of CCl_4 administration were decreased 40 percent after pretreatment of rats with α-tocopherol (72). More recently, Benedetti et al. reported that the amount of endogenous diene conjugates formed after CCl_4 dosing was progressively decreased with increasing amounts of d-α-tocopherol (73).

The prevention of some of the toxic effects of CCl_4 in vivo has been demonstrated with animals pretreated with N,N'-diphenyl-p-phenylenediamene. Several investigators have shown that N,N'-diphenyl-p-phenylenediamine administered in vivo protected rats against hepatic necrosis (65,66,70,74) and triglyceride accumulation after the administration of CCl_4 (65,71,74,75), while N,N'-diphenyl-p-phenylenediamene was shown to be unable to protect against the loss of hepatic cytochrome P-450 (70), the depression of drug metabolizing activity (25,70), and the inhibition of protein synthesis (71). In addition, Comporti et al. (41) and Cignoli and Castro (76) demonstrated that N,N'-diphenyl-p-phenylenediamine did not prevent the loss of hepatic glucose-6-Pase activity after CCl_4 administration. There is a definite controversy concerning the action of N,N'-diphenyl-p-phenylenediamine on the production lipid diene conjugates induced by CCl_4 in vivo; Dianzani et al. reported that N,N'-diphenyl-p-phenylenediamene prevented diene conjugate formation in vivo (77), while Comporti et al. (41) reported no protective effect of N,N'-diphenyl-p-phenylenediamine on that process. In general, the action of N,N'-diphenyl-p-phenylenediamine in vivo on a majority of the hepatotoxic effects of CCl_4 are accepted, the exception being the prevention of diene conjugate formation.

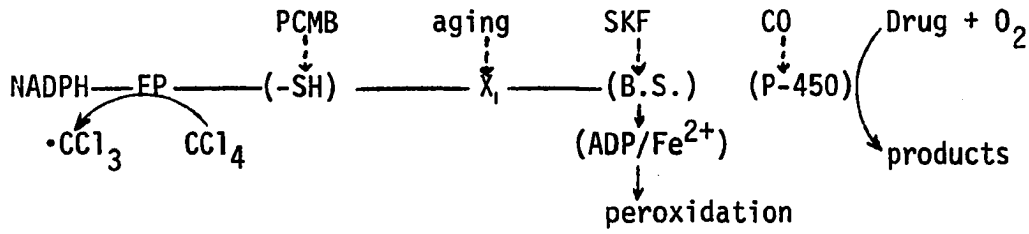
Ethoxyquin has been shown to prevent many of the toxic effects

of CCl_4 *in vivo*. Later, Cawthorne *et al*. (65) showed that ethoxyquin protected against the loss of hepatic glucose-6-Pase, aminopyrine demethylase, hexabarbitone oxidase, and aniline hydroxylase activities, but it did not inhibit the loss of cytochrome P-450 after CCl_4 dosing.

Studies with the previously mentioned antioxidants *in vitro* have demonstrated their ability to protect against microsomal lipid peroxidation (judging by certain criteria) initiated by the addition of CCl_4. Gram and Fouts reported that rat liver homogenates to which d-α-tocopherol was added did not undergo lipid peroxidation upon the addition of CCl_4 (78). Slater and Sawyer demonstrated that the addition of α-tocopherol decreased lipid peroxidation initiated by CCl_4 *in vitro* (79). If N,N'-diphenyl-p-phenylenediamine was added to rat liver homogenates prior to incubation with CCl_4, no lipid peroxidation occurred (41,77). Slater and Sawyer demonstrated the preventative effect of N,N'-diphenylenediamine against lipid peroxidation with hepatic microsomes-plus-supernatant stock suspension (79). The effect of ethoxyquin *in vitro* on lipid peroxidation stimulated by the addition of CCl_4 has not been reported. The studies which have been reported, however, prove that antioxidants appear to be effective *in vitro* against lipid peroxidation induced by CCl_4.

Carbon tetrachloride produces catastrophic disturbances within the liver which can be enhanced or partially altered *in vivo* or *in vitro* by several compounds or dietary conditions. These structural and functional changes of liver tissue in susceptible animals have provided sufficient evidence for the emergence of two theories on the mechanism of CCl_4 metabolism. Slater and Sawyer proposed the first mechanism for CCl_4 metabolism (39). In their hypothesis, CCl_4 was metabolized by the

NADPH-cytochrome c reductase flavoprotein of the hepatic drug metabolizing system. The mechanism is represented as follows:



The evidence for the support of this mechanism has been gathered from CCl_4-stimulated lipid peroxidation studies of hepatic microsomes *in vitro* (39). The production of malondialdehyde was stimulated by concentrations of SKF-525A and parachloromercuribenzoate (PCMB) known to inhibit drug metabolism. Also, carbon monoxide (CO) stimulated the production of malondialdehyde in the presence of CCl_4 and microsomes, but carbon monoxide was shown to depress drug metabolism in the absence of CCl_4. Because carbon monoxide and SKF-525A are known to bind to cytochrome P-450 and because PCMB is known to inhibit drug metabolism by possibly binding to an -SH group between FP and cytochrome P-450, it appears that the stimulation of malondialdehyde production by CCl_4 in this system requires a segment of the drug metabolizing system closer to the flavoprotein (NADPH-cytochrome c reductase) of this pathway. The possible involvement of the NADPH-cytochrome c reductase flavoprotein (FP) was indicated by the inhibition of CCl_4-stimulated malondialdehyde production with cytochrome c.

The second mechanism proposed for CCl_4 metabolism involves the interaction of CCl_4 with cytochrome P-450 of the NADPH-cytochrome P-450 electron transport system. The support for this mechanism is as follows:

1) Administration of CCl_4 *in vivo* in doses which do not cause necrosis will result in a protective effect against a subsequent dose of CCl_4. These protective doses of CCl_4 depress the microsomal cytochrome P-450 content and mixed function oxidase activity, while the NADPH-cytochrome c reductase activity remains unaffected. Thus, when those animals are then given a challenging dose of CCl_4, they are resistant to the toxin. The protection afforded by the initial low dosage of CCl_4 has been attributed to the depressed levels of cytochrome P-450 and the mixed function oxidase activity associated with it, both of which are thought to be required for CCl_4 metabolism (26). Coincident with the depression of cytochrome P-450 content is the prolongation of hexobarbital sleeping time and the diminution of the rat's capacity to convert $^{14}CCl_4$ to $^{14}CO_2$ (80).

2) Pretreatment of rats with SKF-525A, a compound known to bind strongly to cytochrome P-450, markedly depressed the production of diene conjugates *in vivo* after CCl_4 dosing (56).

3) At birth the newborn rat is resistant to CCl_4. This resistance is believed to be associated with a low cytochrome P-450 content of the liver, because the NADPH-cytochrome c reductase activity is present at an adult level in the liver (81).

4) CCl_4 forms a type I binding spectrum with cytochrome P-450 (82,83). This binding spectrum is formed by substances which bind to the cytochrome P-450 apoprotein. Although this does not necessarily indicate that cytochrome P-450 is the catalytic site for CCl_4 metabolism, the binding of a variety of drugs to the cytochrome P-450 apoprotein is a prerequisite for the metabolism of the xenobiotic (84).

5) 3-Methylcholanthene (3-MC) is known to induce the metabolism of drugs that produce Type II binding spectra with cytochrome P448 (85). Castro et al. demonstrated that 3-MC pretreatment decreased the metabolism of $^{14}CCl_4$ as measured by ^{14}C incorporation into the lipids of hepatic microsomes (86).

6) Castro et al. demonstrated that 3-amino-1,2,4 triazole (AT), an inhibitor of heme synthesis and consequently of cytochrome P-450 synthesis prevented irreversible binding of $\cdot CCl_3$ and $\cdot Cl$ radicals to rat liver microsomal lipids in vivo. In addition, AT reduced diene conjugate formation in rat liver of CCl_4 pretreated animals (87).

7) Additions of various steroid hormones and drugs in vitro, inhibit the in vitro pro-oxidant action of CCl_4, supposedly by competing with CCl_4 for the cytochrome P-450 binding site (18, pp. 284).

It is quite apparent from radioactive binding studies of $^{14}CCl_4$ and $^{14}C^{36}Cl_4$ by Reynolds (88) and Reynolds and Yee (89), the electron paramagnetic resonance of Burdino et al. (90), and the antioxidant effects on lipid peroxidation in vitro (18, pp. 140), that CCl_4 is homolytically cleaved to the free radicals $\cdot CCl_3$ and $\cdot Cl$. The involvement of free-radical-mediated lipid peroxidation by CCl_4 metabolites is analogous to that of the NADPH-oxidase system, previously described. The only difference in the two processes occurs at the initiation step of the peroxidative mechanism, i.e., abstraction of the methylene hydrogen atom. For the CCl_4 system, it is believed that the $\cdot CCl_3$ radical abstracts the hydrogen atom from the methylene carbon and produces $CHCl_3$ and a polyunsaturated fatty acid free radical. Once the peroxidative process is initiated, the polyunsaturated fatty acids of the phospholipids of rat liver

microsomes should be lost after CCl_4 administration. Indeed, Horning et al. (91) showed that 35 percent of the arachidonic acid (C20:4) of the total phospholipid fraction of rat liver was lost after dosing the animal with CCl_4. Later, Comporti et al. (40) showed a slight loss of arachidonic acid from the total phospholipid fraction of ER and a 26 percent loss of this fatty acid from the total phospholipids of whole liver following the administration of CCl_4. Similarly, Recknagel and Ghoshal (38) reported a 20 percent loss of arachidonic acid relative to palmitic acid from the phospholipids of rat liver microsomes in vitro 90 minutes after dosing the animal with CCl_4. At present, the only substantial evidence for CCl_4-stimulated lipid peroxidation is either the production of diene conjugates in vivo or the apparent production of malondialdehyde in vitro and possibly in vivo. Recently Slater stated that small amounts of malondialdehyde could be detected in vivo after administration of CCl_4 to rats (92). Because malondialdehyde is readily metabolized in the whole animal (19) and by mitochondria in vitro (20), it has been extremely difficult to detect substantial quantities of the thiobarbituric acid reacting chromagen during lipid peroxidation in vivo.

In summation, there exist two types of enzyme-mediated lipid peroxidation; the NADPH-oxidase free radical-generating system and the microsomal enzyme system that is believed to homolytically cleave some haloalkanes. The production of malondialdehyde by the free radicals of the latter system can be enhanced by pretreatment of animals with phenobarbital, but there are no reports concerning the enhancement of the activity of the NADPH oxidase system by phenobarbital. The production of malondialdehyde by either system can be inhibited by a variety of anti-

oxidants or drugs. The production of malondialdehyde by an NADPH-oxidase system *in vivo* has not been demonstrated, while the formation of malondialdehyde induced by CCl_4 *in vivo* has been detected in small amounts by Slater. Other than the one study cited by Slater, there have been no other studies that conclusively showed the apparent production of malondialdehyde by CCl_4 *in vivo*. Likewise, there is very little data (except possibly from microsomes and/or whole liver phospholipid studies), to substantiate that the production of malondialdehyde by CCl_4 results in a loss of polyunsaturated fatty acids. Finally, the question of whether or not cytochrome P-450 is involved in the promotion of lipid peroxidation during the metabolism of CCl_4 is as yet unanswered.

Because of the various uncertainties and controversies which have arisen as a result of the various studies described above, the objectives of this study were:

1) To determine whether or not isolated rat liver parenchymal cells show evidence of lipid peroxidation when incubated in the presence of either NADPH and ADP-FE^{3+} or NADPH and/or CCl_4.

2) To determine the concentration of CCl_4 which will yield maximum thiobarbituric acid-reacting chromagen (malondialdehyde).

3) To study the effect of d-α-tocopherol, N,N'-diphenyl-p-phenylenediamine, or ethoxyquin on the lipid peroxidative process initiated by either of the free radical producing systems.

4) To study the effect of phenobarbital pretreatment of animals on the lipid peroxidative capacity of isolated rat liver cells from those animals.

5) To determine whether the administration of allylisopropyl-

acetamide (AIA), a porphyric agent which results in a loss of cytochrome P-450, had any effect on lipid peroxidation initiated by either free radical producing system.

6) To analyze the microsomal, mitochondrial, and nuclei-plasma membrane fractions of the isolated cells exposed to the factors described under 1) above for the loss of fatty acids from their membrane lipids. This would provide information as to which of the subcellular organelles of incubated liver cells was attacked by free radicals produced from exposure to CCl_4.

CHAPTER II

MATERIALS AND METHODS

Materials

Animals

Adult male albino rats (280-350 g), derived from the Holtzman-Sprague Dawley strain, were bred and maintained in the animal facilities of the Oklahoma Medical Research Foundation for these experiments. These animals received distilled water and were fed ad libitum either a commercial pellet diet or a synthetic diet described below.

Materials for Diets

Casein, vitamins (except α-tocopherol acetate), cod liver oil, and Alphacel (a pure, powdered cellulose added for bulk) were obtained from Nutritional Biochemicals Corporation, Cleveland, Ohio. Stripped lard (α-tocopherol and other volatile materials removed by molecular distillation) and d-α-tocopherol acetate were obtained from Distillation Products Industries, Rochester, New York.

Experimental Diets

α-Tocopherol-deficient diets.

The experimental diet used was that of Young and Dinning as modified by Caputto et al. (108). The salt mixture and vitamin mixture

were prepared according to the method of Hubbell et al. (109).

Stock Diet (Pellet diet)

Composition of Vitamin Mixture

Inositol	22.5 g
Choline Chloride	22.5 g
Nicotinamide	4.5 g
Pyridoxine HCl	112.5 g
Thiamine	112.5 g
Riboflavin	112.5 mg
Calcium Pantothenate	225.0 mg
Folic Acid	112.5 mg
2-Methylnapthoquinone	5.6 mg
Vitamin B_{12}	1.0 mg
Biotin	1.1 mg
Dextrose	100.0 g

Composition of Salt Mixture

$CaCO_3$	54.300 %	KH_2PO_4	21.200 %
KCl	11.200 %	NaCl	6.900 %
$MgCO_3$	2.500 %	$MGSO_4$	1.600 %
$FeSO_4 \cdot 7H_2O$	0.090 %	$MnSO_4H_2O$	0.035 %
$Alk(SO_4)_2 \cdot 12H_2O$	0.017 %	KI	0.008%

Composition of Basal Diet

	Percent Composition (w/w)
Casein, vitamin free	17.0
Sucrose	37.3

Composition of Basal Diet (Continued)

	Percent Composition (w/w)
Corn Starch	36.0
Lard	3.0
Cod Liver Oil	3.0
Salt Mixture	3.0
Vitamin Mixture	0.7

The basal diet was mixed with Alphacel in a ratio of 10 to 1, respectively.

Stock Diet (Pellet Diet)

Rats that were not maintained on an experimental diet were fed a commercial pellet diet from Rockland Laboratories, Teklad Incorporated, Monmouth, Illinois. This diet had the following ingredients: soybean meal, ground yellow corn, fish meal, pulverized barley, wheat midlings, ground wheat, dehydrated alfalfa meal, pulverized oats, feeding oat meal, dried skim milk, 1% animal fat, vitamin A palmitate, irradiated dried yeast, niacin, calcium panthothenate, riboflavin supplement, menadione, vitamin B_{12}, 1% calcium carbonate, 0.5% dicalcium phosphate, 1% sodium chloride, and traces of manganese oxide, copper oxide, cobalt carbonate, iron carbonate, zinc oxide, and calcium iodate. The manufacturers guaranteed the following analyses: crude protein not less than 24%; crude fat, not less than 4%; and crude fiber, not more than 6%.

Reagent Chemicals

All chemicals and solvents were reagent quality unless otherwise specified and were obtained from the following sources. Nicotinamide adenine dinucleotide phosphate, reduced (NADPH), nicotinamide adenine dinucleotide phosphate (NADP), nicotine adenine dinucleotide, reduced

(NADH),sodium D-glucose-6-phosphate dehydrogenase (D-glucose-6-phosphate: NADP-oxidoreductase), and hyaluronidase (ovine, type III) were obtained from Sigma Chemical Company, St. Louis, Missouri.

The following chemicals were purchased from J.T. Baker Chemical Company, Phillipsburg, New Jersey: cyclohexane (GC-Spectroquality), carbon tetrachloride, sodium chloride, potassium chloride, and magnesium sulfate.

Sodium monohydrogen phosphate and potassium dihydrogen phosphate were obtained from Mallinckrodt Chemical Works, St. Louis, Missouri.

Ultra pure sucrose was obtained from Schwarz/Mann, Orangeburg, New York.

N,N'-diphenyl-p-phenylenediamine (DPPD) was purchased from B.F. Goodrich Company, Akron, Ohio.

Santoquin (ethoxyquin) was purchased from Monsanto Chemical Company, St. Louis, Missouri.

The following chemicals were obtained from Fisher Scientific Company, Fair Lawn, New Jersey: methylene chloride (dichloromethane), ferric chloride, sodium bicarbonate, chloroform, methanol, trichloroacetic acid (TCA), and tris (hydroxymethyl) amino methane.

2-thiobarbituric acid (TBA) was obtained from Eastman Organic Chemicals, Rochester, New York.

Adenosine 5'-diphosphate (ADP) was obtained from P-L Biochemicals, Milwaukee, Wisconsin.

Trypan blue stain was obtained from Curtin Scientific Company, Tulsa, Oklahoma.

Collagenase (CLS II) was obtained from Worthington Biochemical

Corporation, Freehold, New Jersey.

Allylisopropyl acetamide (AIA) was a gift from Mr. Wayne Levin, Department of Biochemistry, Hoffman-La Roche Inc., Nutley, New Jersey.

The following compounds were obtained from local sources: pentobarbital, phenobarbital, and Wesson oil.

The oxygen: carbon dioxide mixture (95:5) was obtained from Ohio Medical Products, Madison, Wisconsin, while carbon monoxide was obtained from Matheson Company, Chicago, Illinois.

Instruments and Equipment

The liver cells were prepared by perfusing the rat liver in situ in a plexiglass chamber which I designed and constructed (Fig. 1).

Spectrophotometric measurements were made using either a Beckman DU-2 from Beckman Instruments Company, South Pasadena, California, or a Cary Model 14 Recording Spectrophotometer, Applied Physics Corporation, Pasadena, California. Fluorometric measurements were made using a Model A-3 Farrand Photoelectric Fluorometer, Farrand Optical Company, Inc., New York.

Centrifugation was performed using a Sorvall Model RC2-B centrifuge, Ivan Sorvall, Inc., Newtown, Connecticut, an International Refrigerated Centrifuge Model PR-2 or a non-refrigerated model, International Equipment Company, Boston, Massachusetts, or a Spinco Model L ultracentrifuge, Beckman Instruments Company, Spinco Division, Palo Alto, California. Either Beckman or Sorvall centrifuge rotor heads were used for differential centrifugation.

Gas-liquid chromatographic analysis was performed using a Perkin-Elmer Model 881 Gas-Liquid chromatograph, Perkin-Elmer Corporation,

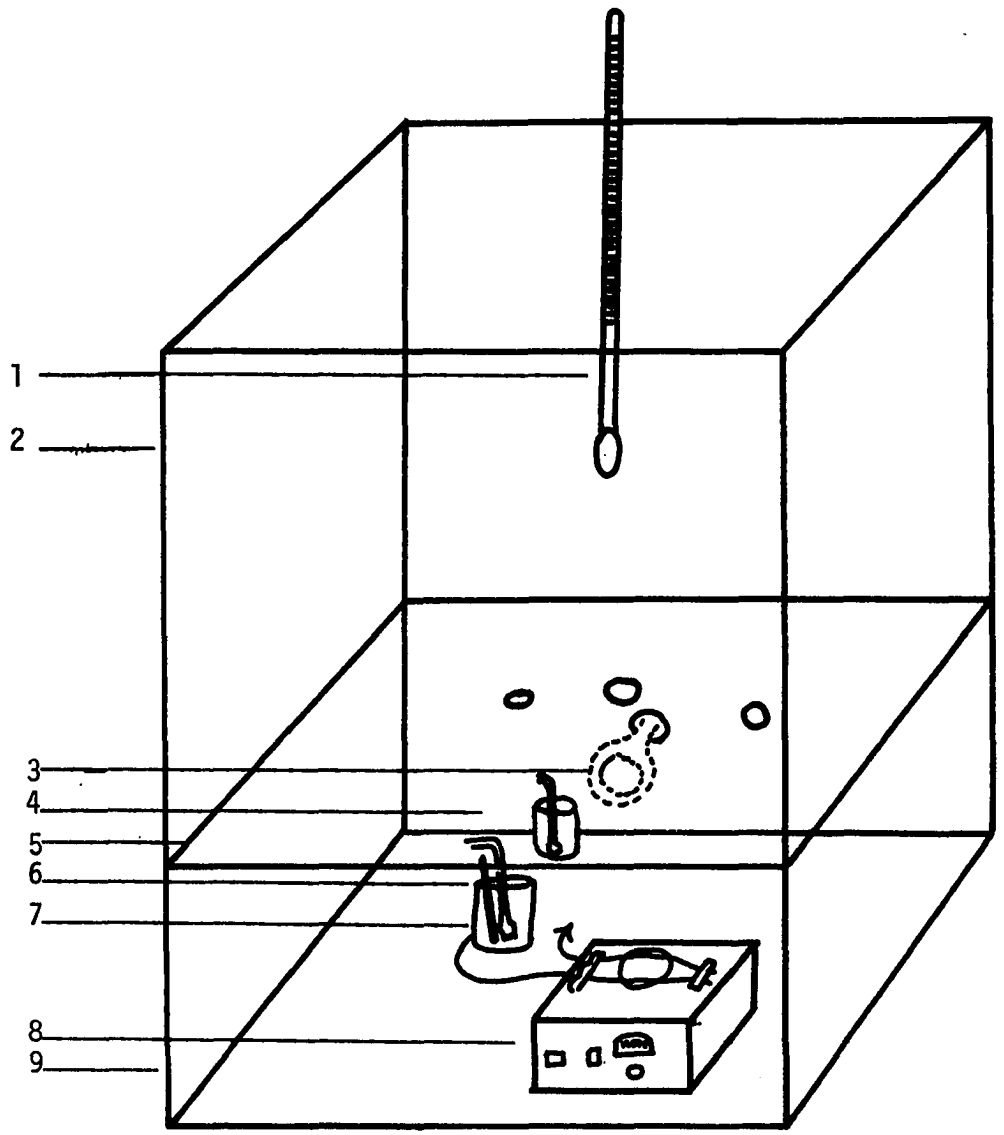


Figure 1. Rat liver perfusion apparatus. The chamber is constructed of plexiglass, 0.25 inches thick, and is held together by Pleximent. 1 - thermometer, 2 - upper chamber, 3 - 150 watt light bulb controlled by a rheostat, 4 - fresh, gassed, enzyme free Hank's-bicarbonate buffer, 5 - platform for animal, 6 - thermometer, 7 - perfusate reservoir, 8 - Holter pump, and 9 - lower chamber.

Norwalk, Connecticut.

Incubations were performed in either a Dubnoff shaker, Precision Scientific Company, Chicago, Illinois, or a Gilson Respirometer, Gilson Medical Electronics, Inc., Middleton, Wisconsin, both containing a constant temperature water bath.

Oxygen consumption was done in a Gilson Oxygraph Model K1C, Gilson Medical Electronics, Inc., Middleton, Wisconsin.

Methods

Treatment of Animals

All animals were starved 22-24 hours and then were anesthetized with an intraperitoneal injection of pentobarbital, 60 mg/kg body weight, twenty minutes prior to cannulating the hepatic portal vein.

Injections of phenobarbital were administered intraperitoneally, 75 mg/kg body weight in 0.9% saline for five days. Ethoxyquin (Santoquin) or N,N'-diphenyl-p-phenylenediamine (DPPD) was given intraperitoneally, 400 mg/kg body weight in Wesson oil, for two or three days respectively. Wesson oil alone was also given in equivalent amounts for three days to control animals. Allylisopropylacetamide (AIA) was injected subcutaneously, 200 mg/kg body weight in deionized, distilled water, two hours prior to the rat liver perfusion.

Isolation of Rat Liver Parenchymal Cells

Rat liver parenchymal cells were isolated by minor modifications of the *in situ* enzymatic procedure described by Berry and Friend (93). Immediately prior to cannulation of the hepatic portal vein, the perfusion medium, Hank's-bicarbonate buffer (Ca^{2+} and glucose-free) (94), was gassed for 30 minutes with O_2:CO_2 (95:5), dispersed by scintered glass

filters, and was maintained at 37° C. (Bovine serum albumin was not added to the perfusate). The hepatic portal vein was then cannulated, using a perfusion flow rate of 2.0 ml/min., and then the perfusate flow rate was adjusted to 2.6 ml/min. until the inferior vena cava was cannulated. After cannulating the vena cava, the perfusate flow rate was increased to 20-25 ml/min. The initial 60 ml of perfusion medium was collected without recirculation. Then the perfusate was recirculated, and the connective tissue was digested by addition of collagenase (0.02 percent) and hyaluronidase (0.05 percent) to the perfusion medium. After 20-25 minutes, the liver was excised, minced, and fresh, gassed, enzyme-free perfusion medium was added. Calcium chloride solution was gently stirred into the tissue suspension to obtain a final concentration of 1.0 mM. This addition was made in order to obtain a greater cell yield and to obtain liver cells with a higher potassium content and a greater respiratory activity than that obtained in liver cells isolated without adding $CaCl_2$ as demonstrated by Howard *et al.* (95). The cells were incubated with shaking in a 37°C water bath for 12 minutes, filtered through silk sieve cloth (100 mesh) to remove aggregated cells and undigested tissue. The filtrate was then centrifuged in 50 ml glass centrifuge tubes at 30 x g for 2 minutes in a Sorvall RC-2 centrifuge. The cells were washed first with cold, gassed Hank's bicarbonate solution (Ca^{2+}-and glucose-free) and secondly with Hank's solution containing additional 10 mM sodium phosphate, pH 7.4 (S buffer). The cells were resuspended with 8-14 ml of S buffer.

The cells were routinely counted with a Bright-Line hemocytometer, and the cell concentration of the original suspensions ranged from

1.4 - 2.2 x 10^8 cells/ml. Aliquots (0.1 ml) of cells were also removed for the determination of the protein content by the method of Lowry et al. (96), and for measurement of packed cell volume, a procedure designed by our laboratory.

Packed Cell Volume Protein Determination

Several 0.1 ml samples were removed with Clay-Adams micropipets from different dilutions of a single, whole cell suspension. The pipets were flame sealed and gently agitated to remove air bubbles from the column of liquid. They were then centrifuged at 1700 rpm for 2 minutes in a PR-2 International centrifuge fitted with swinging buckets. The packed cell volume was determined with a Bausch and Lomb measuring magnifier at 7x power. The protein concentration of each cellular dilution was analyzed by the method of Lowry et al. (96). Then a plot of the packed cell volume versus the quantity of protein content was performed in subsequent experiments. When this method is used, a separate packed cell volume plot is required for cells obtained from rats receiving compounds such as phenobarbital that affect either cellular volume and/or protein content.

Criteria Investigated for Parenchymal Cell Viability

There are several analyses performed with isolated hepatocytes that are considered criteria of cellular viability; trypan blue stain exclusion, gluconeogenesis, endogenous lactate dehydrogenase release, and oxygen consumption. All cell preparations were routinely analyzed for trypan blue exclusion (0.05-0.1% in 0.9% saline), a means of analyzing plasma membrane integrity. Preparations in which more than 15 percent

of the cells absorbed the dye were discarded.

The gluconeogenic ability of these cells was investigated by using L-alanine (20 mM) as the glucose precursors and then incubating the cells in an incubation lung. The incubation lung consisted of a rubber stoppered, scintillation counting vial into which O_2:CO_2 (95:5) entered through a 21 gauge needle and exited through a 25 gauge needle inserted in the stopper. The cells were incubated with shaking in Hank's-bicarbonate buffer (Ca^{2+}-and glucose-free) at 37°C in a water bath for zero, 30, 60, and 90 minutes. After incubation, the vials were heated in a 90-100° C water bath for 2 minutes and then centrifuged at 8300 x g. Aliquots of the supernatant fraction were analyzed for glucose content by the method of Lowry et al. (96).

The release of endogenous lactate dehydrogenase (LDH) from the cells was analyzed by fluorometrically determining the amount of NADH produced by the reaction: lactate $\xrightarrow[\text{LDH}]{\text{NAD+ NADH}}$ pyruvate. To determine the endogenous level of LDH, a liver cell suspension was homogenized with a motor driven zero-tolerance, Teflon-glass homogenizer, and the homogenate was analyzed for LDH content. Then a suspension of hepatocytes was incubated with shaking at 37° in a water bath for 0, 15, 30, and 35 minute intervals, at which time aliquots were removed and centrifuged 2 minutes at 30 x g in a Sorvall RC-2, centrifuge. The supernatant fraction was analyzed for LDH content, and the amount released was represented as a percentage of the total endogenous LDH content of the broken hepatocytes.

Oxygen consumption by 2.1 x 10^6 isolated rat liver parenchymal cells was analyzed by a Gilson oxygraph. The effects of dextrose (a few

grains), succinate (5 mM), and potassium cyanide (0.5 mM) on oxygen consumption were also determined.

Analysis of Lipid Peroxidation

Malondialdehyde, a minor but easily detected product of lipid peroxidation, was spectrophotometrically determined by the thiobarbituric acid reaction. To 1.0 ml of the incubation system, 0.5 ml 35% TCA and 1.0 ml 0.5% thiobarbituric acid (TBA) were added. The tubes were boiled 20 minutes, cooled, and 1.0 ml 70% TCA was added. Chloroform (2.0 ml) was added to clarify the reaction mixture, and then the tubes were centrifuged 10 minutes at 1700 rpm to collect the chloroform in a layer at the bottom of the tube. The chromagen content was determined in the upper layer by measuring the optical density at 532 nm. These values can be converted to nmoles of malondialdehyde by multiplying by 22.4.

Cytochrome P-450 Analysis

Cytochrome P-450 of whole cells was analyzed by the method of Omura and Sato (97). Aliquots of suspended cells (0.15, 0.25, and 0.45 ml) were brought to a final volume of 1.15, 1.25, or 1.35 ml with S buffer in each of two cuvettes. Dithionite was added to both the reference and sample cuvettes, and carbon monoxide was bubbled slowly for 1.5 minutes through the solution in the sample cuvette. Cytochrome P-450 was determined by scanning the difference in optical density between 500 nm and 400 nm.

Conditions of Isolated Rat Liver Parenchymal Cell Incubation

1) <u>Studies of Lipid Peroxidation Initiated by NADPH, an NADPH Regenerating System, or NADH</u>:

Liver cells (3- 4.5 x 10^6/ml) were suspended in S buffer and preincubated 17 minutes with shaking in a 37° C water bath. Then either NADPH, NADPH regenerating system, or NADH (all 0.6 mM in S buffer), and/or ADP-Fe^{+3} (0.4 mM ADP, 1.2 x 10^{-5} M Fe^{+3} in 0.15 M Tris, pH 7.4) was added. The incubation was terminated at specified time intervals by the addition of 0.5 ml 35% TCA for each milliliter of reaction system.

2) Studies of Lipid Peroxidation Initiated by the Addition of CCl_4.

Parenchymal cells (3-4.5 x 10^6/ml) were suspended in S buffer and preincubated 2 minutes in a Dubnoff metabolic shaker at 37° C. Then CCl_4, freshly homogenized in S buffer, was added to the systems in final concentrations of 0.37, 1.11, 3.33, 10.00, or 30.00 µl/ml of the cell suspension system. The systems were incubated an additional 15 minutes and then either NADPH, or NADPH regenerating system, or NADH, and/or ADP-Fe^{+3} were added. The systems were incubated for specified time periods, and the reactions were terminated as previously stated.

Either dichloromethane or cyclohexane was suspended in S buffer as described for CCl_4.

All incubation systems were analyzed for TBA reacting chromagen as previously mentioned.

Determination of Fatty Acid Composition of Membrane Lipids

To determine whether the malondialdehyde produced during liver cell incubations was derived from membranous polyunsaturated fatty acids, the cells were homogenized and fractionated into several subfractions after incubation, and the fatty acid compositions of the microsomal,

mitochondrial, and nuclear-plasma membrane cell debris fractions were determined. The initial incubation was performed by incubating 11 ml liver cell suspensions (approximately 10^8 cells) in 50 ml Erlenmeyer flasks equipped with center wells. The flasks were then attached to the Gilson Respirometer flask adaptors.

The hepatocytes were placed in that portion of the flask that surrounds the center well and then agitated at 108 rpm for 5 minutes at 37^{o} C. All systems were closed to the atmosphere. A control cell system was treated similarly except that the flask was capped with parafilm and kept on ice. Then 50 µl of CCl_4 was added to the center wells which contained filter paper wicks and allowed to diffuse via the gas phase into the flasks containing the liver cells for another 5 minutes under the conditions previously described. Then all flasks were detached from the respirometer or removed from the ice bath, and NADPH and/or ADP-Fe^{+3} were added. The flasks were gassed with pure O_2 for 10 seconds after which they were either attached to the respirometer and incubated at 37^{o} C or stoppered and placed at 5^{o} C for 30 minutes. After incubation, 1.0 ml of each system was removed for malondialdehyde analysis. The remainder of the incubation systems was transferred to test tubes, placed in ice, and centrifuged in a refrigerated PR-2 International centrifuge at 1700 rpm for 2 minutes.

The supernatant solutions were usually discarded, and the pellets were resuspended in 5.0 ml of cold 0.25 M sucrose-0.05 M Tris-HCL, pH 7.4-7.5. Each cellular suspension was homogenized by 3 complete strokes with a motor driven, zero-tolerance, Teflon glass homogenizer, and the resulting homogenate was transferred to Beckman cellulose nitrate

tubes (1.6 x 7.6 cm). An additional 5.0 ml of the sucrose buffer was added to each tube, which were then centrifuged in an SS 21 rotor at 1700 rpm in a Sorvall, RC-2 centrifuge. The supernatant solutions were decanted from the pellets containing nuclei-plasma membranes and cell debris and centrifuged at 8300 rpm for 10 minutes. The resulting supernatant solutions were saved for the isolation of microsomes. The pellets containing the mitochondria were resuspended with fresh sucrose buffer and were repelleted by centrifugation at 8300 rpm for 10 minutes. The supernatant solutions were discarded and the mitochondrial pellets were resuspended in 10 ml of sucrose buffer. Aliquots were removed for protein determination, and the remainder was extracted by the method of Folch, et al. (98) to obtain the total lipids for fatty acid determination.

The initial 8300 rpm supernatant solution was centrifuged in a Beckman 40 rotor at 40,000 rpm for 60 minutes. The supernatant solution was discarded, and the microsomal pellets were resuspended in 11.0 ml of 0.1 M Tris-HCl, pH 7.4-7.5. The suspensions were recentrifuged under the same conditions. The microsomal pellets were homogenized as described for the mitochondrial pellets, and aliquots were removed for protein determination prior to the Folch extraction.

The original 1700 rpm pelleted fraction (nuclei and plasma membranes) of each tube was frozen and stored at -20^{0} C. Later they were homogenized with 10.0 ml of 0.1 M Tris-HCl buffer, pH 7.4-7.5, and aliquots were removed for protein determination. The remainder was extracted to obtain total lipids for fatty acid determination as previously cited.

Exact amounts of an internal standard, arachidic acid (C20:0), were added to each extraction vessel in order to facilitate the quantitation of the fatty acids of the respective membrane fractions. The material undergoing Folch extraction was allowed to stand at 5^{o} C overnight. Then the chloroform layer, which was completely separated by that time, was collected and evaporated to dryness under reduced pressure. A few drops of absolute ethanol were added to each flask containing the concentrated lipid, and the material was then evaporated to dryness to facilitate the removal of traces of water. The lipid was then transferred to screw capped tubes using $CHCl_3$ to dissolve the lipids. The $CHCl_3$ was removed by evaporation under vacuum after the transfer, and the lipids were methylated with BF_3-CH_3OH by the method of Morrison and Smith (99). The fatty acid methyl esters of the total lipid extract of each subcellular fraction were separated on a 6 foot, 0.25 inch (I.D.) aluminum column containing Supelco SP 222 PS support, and analyzed by a Perkin-Elmer Model 881, gas-liquid chromatograph.

CHAPTER III

RESULTS

A. Lipid Peroxidation of Isolated Rat Liver Parenchymal Cells Initiated by an NADPH-Dependent Enzyme System

The oxidation of NADPH by a microsomal enzyme system in rat liver has been shown to initiate microsomal lipid peroxidation *in vitro*, producing malondialdehyde as a minor product. The enzyme production of malondialdehyde is related to the loss of polyunsaturated fatty acids of microsomal lipids, and the formation of malondialdehyde can be inhibited or greatly depressed by either the *in vitro* addition of some antioxidants or metallic ions or by the pretreatment of rats with d-α-tocopherol prior to the isolation of liver microsomes. The action of antioxidants suggested that this enzyme system generated free radicals. As previously mentioned, this NADPH-oxidase system has been thoroughly investigated *in vitro*, but there is no evidence that this system exists and functions similarly *in vivo*. At present, there appear to be no procedures for selectively inducing this enzyme system and for detecting its potential for exerting cellular damage *in vivo*. For those reasons and many others to be discussed, we have approached the problem by using isolated rat liver parenchymal cells. This procedure allowed us to: 1) study the stimulation of lipid peroxidation on a homogeneous

population of intact, functional liver cells, 2) control the cellular environment, 3) control the time of cellular exposure to the environment, and 4) rapidly separate the extracellular medium from the cells for analysis of specific compounds.

As mentioned in Chapter II, the cells were routinely treated with trypan blue stain, and only those preparations in which 85 percent or more of the cells excluded the dye were used. The cell count of each preparation was also determined and used as a basis for calculating the production of malondialdehyde. The ability of cells isolated by this procedure to produce glucose from added alanine was determined for two different cell preparations. Basal glucose production was 5 µg/mg prot./60 min. and with the addition of 20 mM alanine the production increased to 18 µg/mg prot./60 min. The cells appear to utilize endogenous precursors in the absence of added substrate. In the presence of alanine, glucose production is almost linear with time of incubation for 60 minutes. In addition to examining the functional integrity of the cell preparations, we examined their structural integrity by determining the release of endogenous lactate dehydrogenase (LDH) during incubation at 37°C. Table 1 shows that an initial release of 10-15 percent of cellular lactate dehydrogenase occurs in the suspension buffer after isolation and while the cells are still kept in ice. Only another 9-10 percent is released after a 45 minute incubation period. Thus, most of the endogenous LDH remains inside the cell under these conditions of incubation.

Oxygen consumption of isolated hepatocytes was followed on a Gilson oxygraph as shown in Figure 2. The cells consumed oxygen for

TABLE 1

THE RELEASE OF LACTATE DEHYDROGENASE FROM ISOLATED RAT LIVER PARENCHYMAL CELLS DURING INCUBATION AT 37°C

Incubation Time		Lactate Dehydrogenase Released
Min.		% of total activity
0	#1	10.49
	#2	15.09
15	#1	11.71
	#2	18.27
30	#1	14.63
	#2	22.62
45	#1	19.76
	#2	24.73

The incubation conditions are described in "Methods".

The percentage of Lactate Dehydrogenase released into the buffer is based on the activity of LDH found after mechanical lysis of the cells.

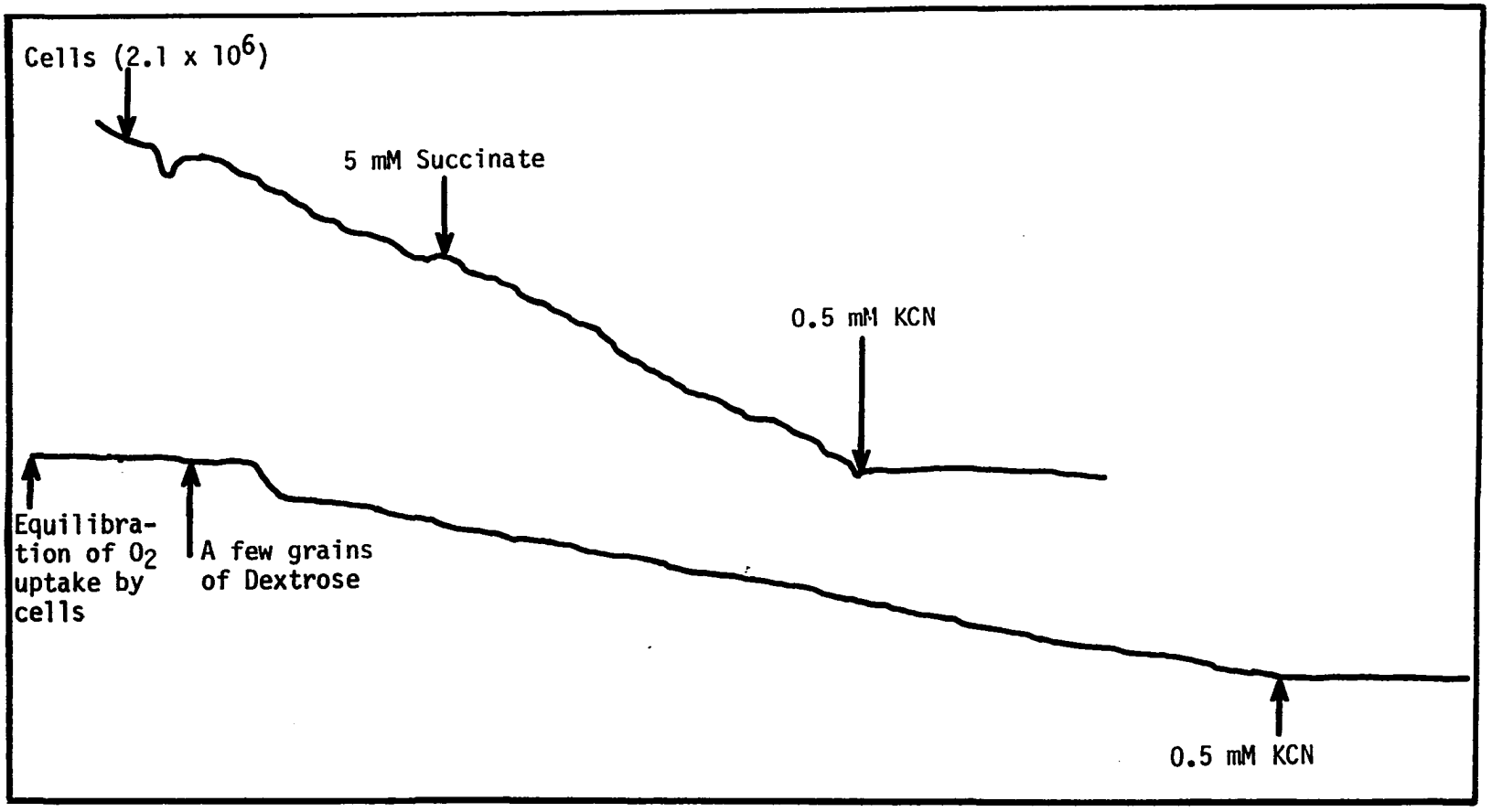


Figure 2. Oxygen consumption of hepatocytes isolated from control rats. The oxygen consumption was analyzed by a Gilson oxygraph. Each tracing was made after the addition of fresh liver cells.

several minutes, and the uptake of O_2 by these cells was completely inhibited after the addition of potassium cyanide. In another type of assay, hepatocytes were allowed to consume oxygen until the rate of consumption dropped to low levels. Then either succinate or dextrose was added. Only dextrose stimulated an additional oxygen uptake by the cells. This would be an expected result since dextrose is known to easily diffuse into liver cells and undergo oxidation, while succinate, a highly charged compound, might not enter the cell with such ease. Thus, the isolated hepatocytes consume O_2, a process that is stimulated by dextrose and inhibited by potassium cyanide.

Finally, Dr. Robert Nordquist made electron micrographs of freshly isolated liver cells in order to assess the integrity of the subcellular morphology. Figure 3 shows the cellular morphology of a liver cell isolated from a 24 hour-fasted rat previously fed a standard laboratory ration (normal cell). All subcellular organelles appear to be intact. Figure 4 is an electron micrograph of a liver cell from a phenobarbital pretreated rat isolated after 24 hours of starvation (phenobarbital treated cell). This cell contains large vacuoles which are interpreted to be lipid. The same vacuolizations can be detected in liver biopsies taken from either phenobarbital treated or saline treated animals as demonstrated in our laboratory. Thus the presence of endogenous lipid vacuoles in the isolated hepatocytes is probably not due to either our cell isolation procedures or fixation procedures used by the electron microscopist. The phenobarbital treated cells also contain a greater content of smooth endoplasmic reticulum than cells isolated from untreated animals, as expected.

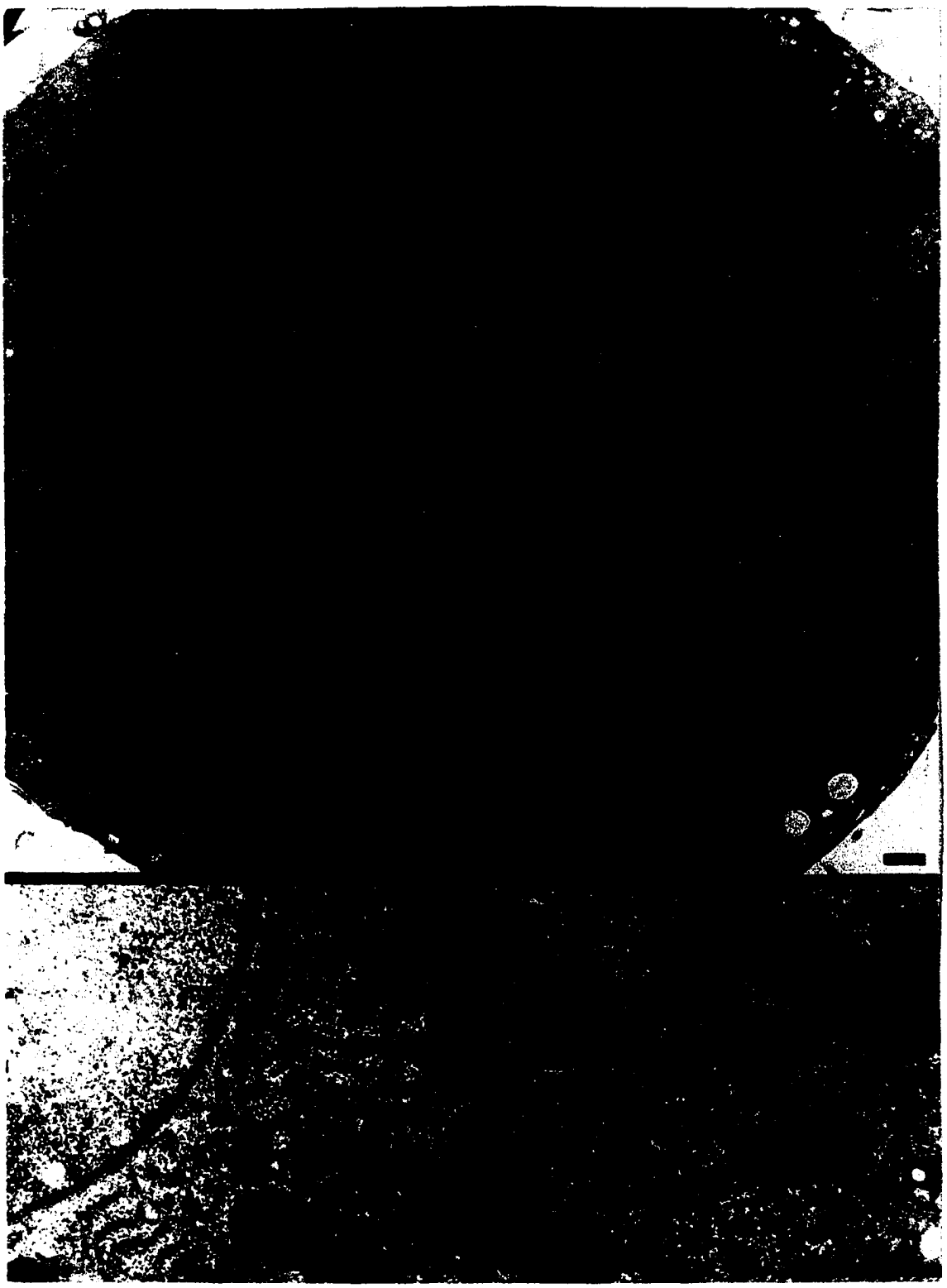


Figure 3. An electron micrograph of a freshly isolated hepatocyte from a control rat. Bar denotes one micron.

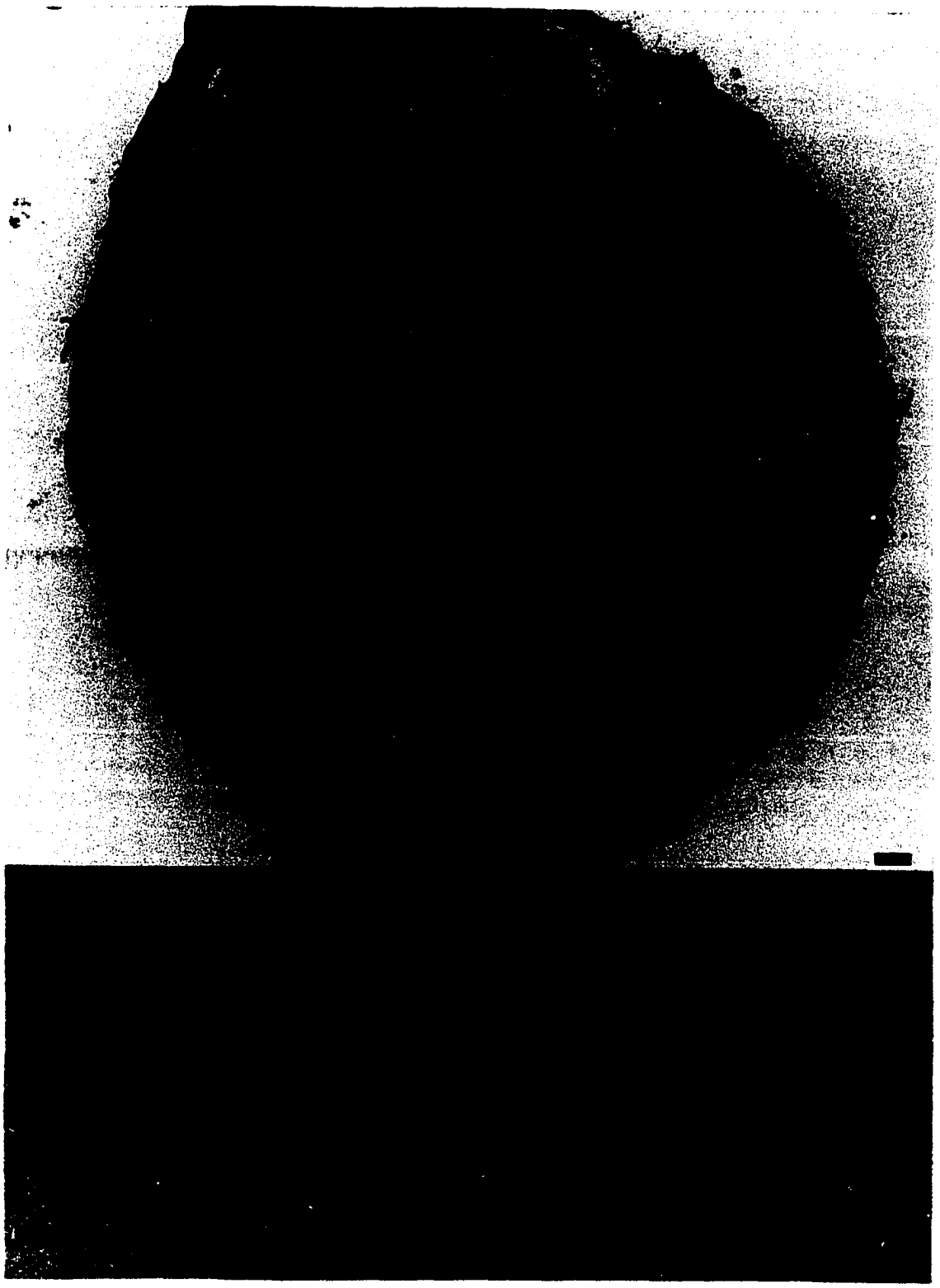


Figure 4. An electron micrograph of a freshly isolated hepatocyte from a phenobarbital-treated rat. Bar denotes one micron.

As described in Chapter II, many preparations of hepatocytes were analyzed for total cellular protein content by a packed cell procedure. Figure 5 represents a plot of the protein content, as determined by the method of Lowry et al. (96), versus the packed cell volume for liver cells isolated from fasted, normal rats. If one measures the packed cell volume of liver cells from phenobarbital treated animals and extrapolates the protein content of a 0.1 ml aliquot from Figure 5, one will obtain a greater packed cell protein content from the same quantity of cells. For example when the ratio of the packed cell protein content for normal cells is 0.98 mg/10^6 cells (average of 13 experiments), a ratio of 1.5 mg/10^6 cells is obtained from phenobarbital treated animals (average of 7 experiments). Thus, the packed cell volume of phenobarbital treated cells is approximately 50 percent larger than that of normal cells. This suggests that phenobarbital treatment produces an increase in cellular volume as well as cellular protein content, while the cell yield remains unchanged.

In order to determine if liver cells were capable of undergoing lipid peroxidation, they were incubated at 37° C in S buffer for different time periods. Figure 6 shows the time course study of hepatocytes isolated from normal and phenobarbital-treated animals. Table 2 gives the amounts of malondialdehyde produced as calculated from Figure 6 and adjusted for the malondialdehyde content already present at zero time. (Zero time is that period at which the cells have been pre-incubated for 17 minutes.) The data indicate that isolated liver cells do undergo lipid peroxidation during incubation and that the amount of malondialdehyde produced is enhanced if the cells are isolated

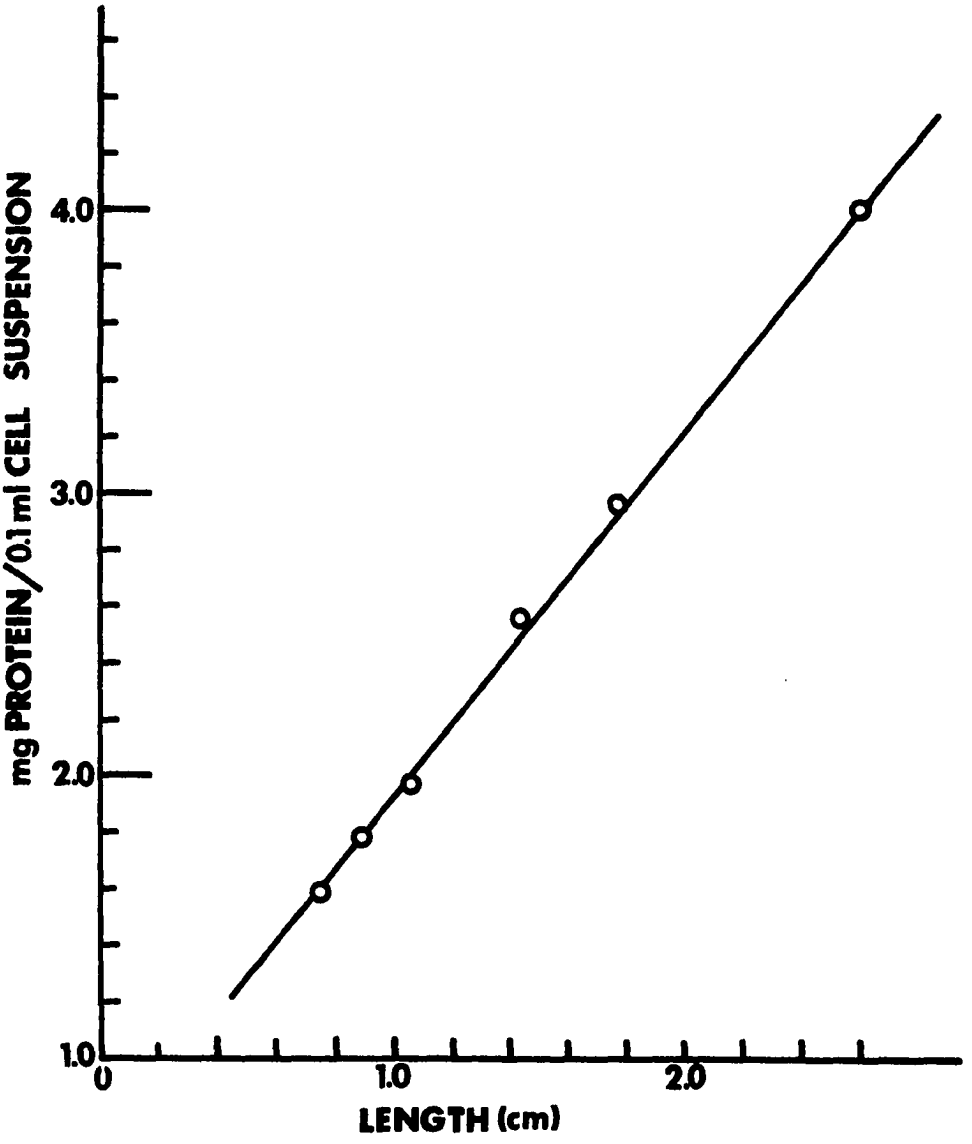


Figure 5. Relationship of the packed cell volume of liver cells from control rats to their protein content. The procedure is described in "Methods".

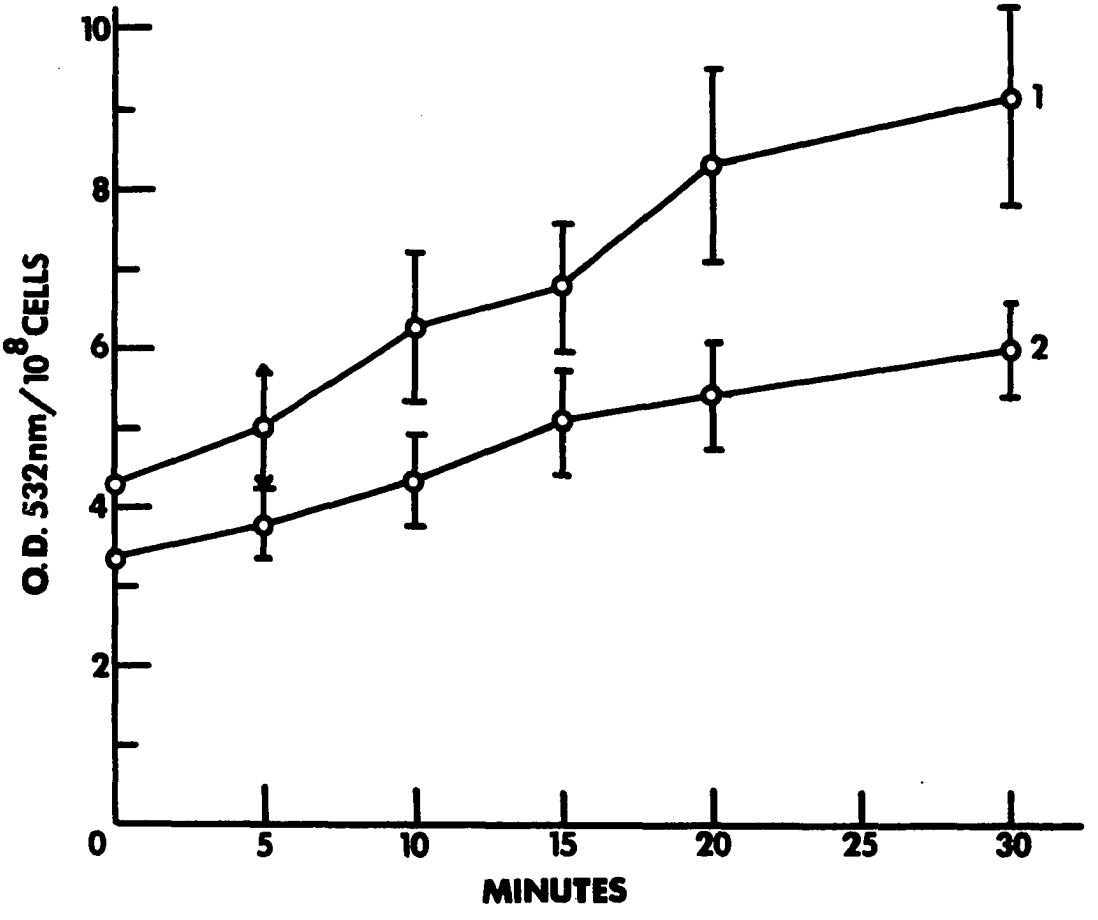


Figure 6. A time course study of malondialdehyde production by liver cells isolated from control and phenobarbital-treated rats during incubation at 37°C. The incubation procedure is described in "Methods".

1. Phenobarbital-treated liver cells
2. Control liver cells.

TABLE 2

MALONDIALDEHYDE PRODUCTION BY LIVER CELLS ISOLATED FROM CONTROL AND PHENOBARBITAL-TREATED RATS DURING INCUBATION AT 37°C

Animal Pretreatment	Malondialdehyde Formation	
	$\Delta O.D._{532\ nm}/10^8$ cells[a]	
	5 min	30 min
None (6)[b]	0.4 ± 0.1	2.6 ± 0.2
Phenobarbital (5)	0.9 ± 0.3	4.9 ± 0.9

The incubation conditions are described in "Methods".

The data is calculated from Figure 6. The incubation system contained hepatocytes (2-3 x 10^6 cells/ml) in S buffer, final volume, 1.0 ml. The systems were preincubated for 17 minutes before the timed measurements were begun.

[a] The values for the $O.D._{532\ nm}/10^8$ cells at the beginning of these measurements (which began after a 17 minute preincubation of the cells in S buffer) was subtracted from the final $O.D._{532\ nm}/10^8$ cells values at the 5 and 30 minute time points of each cell type respectively. $O.D._{532\ nm}/10^8$ cells at "zero" time for untreated hepatocytes equaled 3.4 ± 0.4. $O.D._{532\ nm}/10^8$ cells at "zero" time for liver cells isolated from phenobarbital-treated rats equaled 4.3 ± 0.5.

[b] The data represents an average of 6 and 5 $\pm$ S.E.M. separate experiments for hepatocytes isolated from control and phenobarbital-treated rats respectively.

from rats treated with phenobarbital. Figure 7 is an electron micrograph of a control cell incubated alone in S buffer at 37°C for 47 minutes. Similar results are obtained with phenobarbital cells (micrograph not shown). There are essentially no noticeable morphologic changes when these micrographs are compared to Figures 3 and 4, freshly isolated cells from similarly treated rats. Because these cells alone were capable of producing malondialdehyde and because there is no evidence for an NADPH-oxidase system capable of promoting such a process *in vivo*, the effect of adding NADPH and ADP-Fe^{3+} to the isolated cell incubations was investigated. Since it developed that addition of these components to the cells did stimulate lipid peroxidation, it was of interest to determine the concentration of NADPH that would yield maximum lipid peroxidation of normal cells in the presence of a constant concentration of ADP-Fe^{3+} (0.4 mM ADP and 1.2×10^{-5} M Fe^{3+}) used by McCay *et al.* (11). Figure 8 shows the plot of lipid peroxidation versus the concentration of NADPH. The resulting curve resembles classic Michaelis-Menten kinetic plots of enzyme-substrate reactions. The maximum lipid peroxidation of hepatocytes is produced with 0.6 mM NADPH. The amount of malondialdehyde produced at this final concentration of NADPH in this experiment was less than that usually found by us for normal cells isolated from rats bred in the Oklahoma Medical Research Foundation. This finding may have been the consequence of using rats obtained from a commercial source rather than from our rat colony, because peroxidative processes in liver are affected by the diets on which the animals are raised. The final concentration of NADPH used in all subsequent incubations of hepatocytes was 0.6 mM. When either an NADPH-generating system or NADH is substituted for NADPH, the final

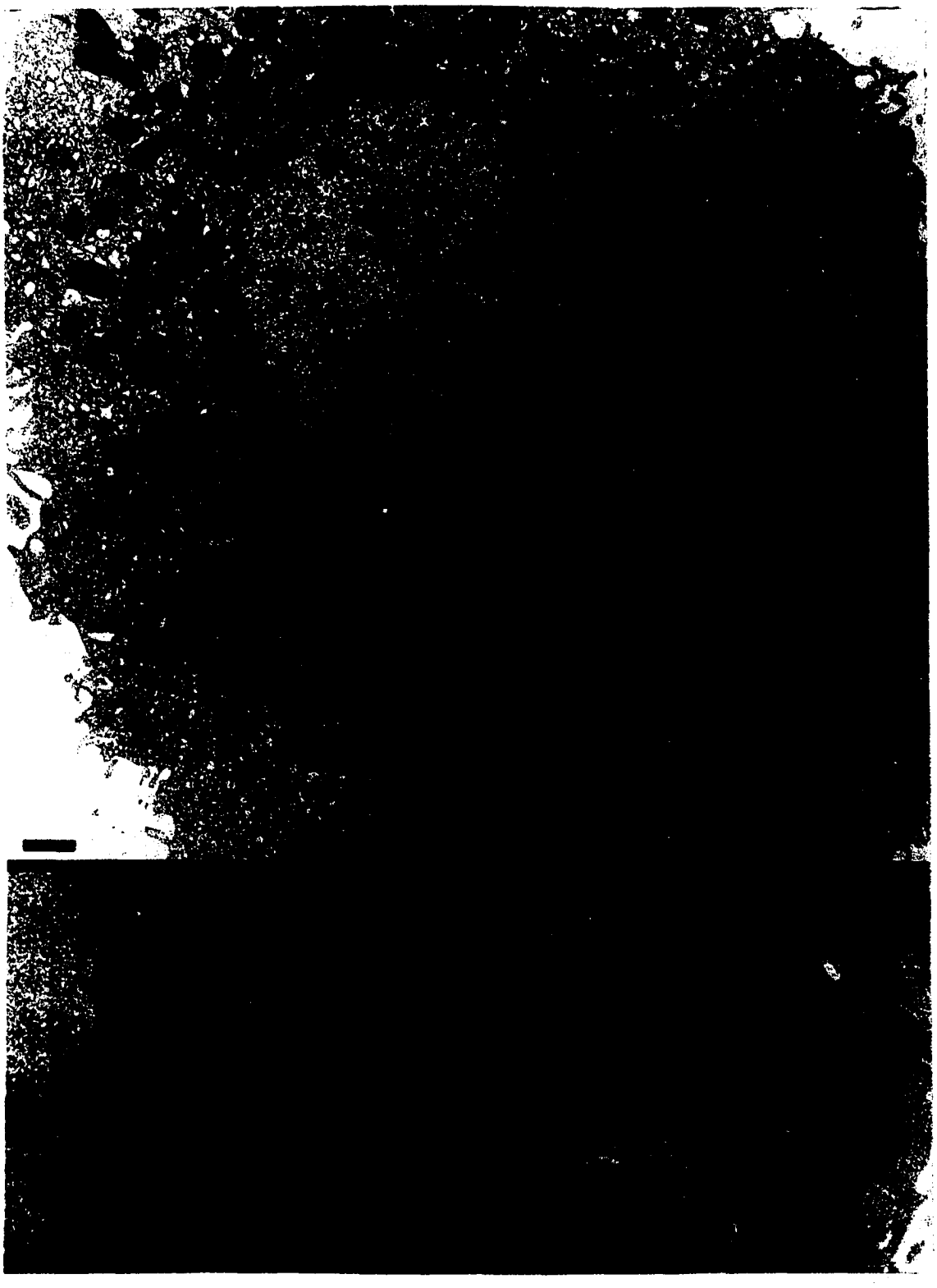


Figure 7. An electron micrograph of a liver cell isolated from a control rat and incubated without additions at 37°C. Bar denotes one micron.

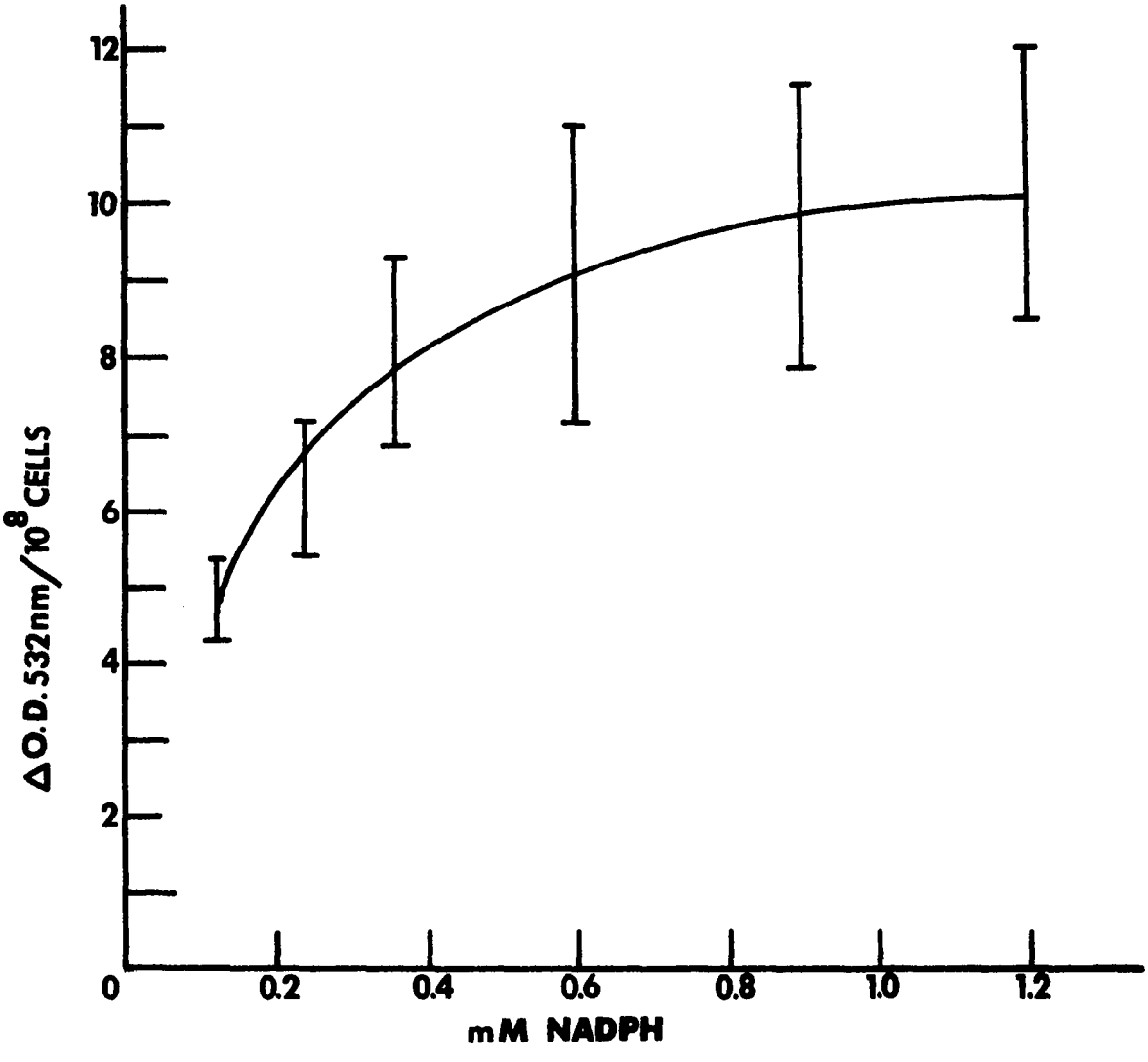


Figure 8. The effect of different concentrations of NADPH on the production of malondialdehyde. Liver cells (3-4.5 x 10^6/ml) were suspended in S buffer and preincubated 17 minutes with shaking in a 37°C water bath. Then different concentrations of NADPH and a constant concentration of ADP-Fe^{3+} (0.4 mM ADP, 1.2 x 10^{-5} M Fe^{3+} in 0.15 M Tris, pH 7.4) were added. After 30 minutes of incubation with the nucleotides, malondialdehyde was determined as stated in "Methods". The malondialdehyde produced by liver cells incubated alone was subtracted from the absolute values of $O.D._{532\ nm}/10^8$ cells/30 min of cells incubated with NADPH prior to plotting the points.

concentrations of these nucleotides was also 0.6 mM. Figures 9 and 10 illustrate the time courses of lipid peroxidation of rat liver parenchymal cells isolated from either normal or phenobarbital-treated rats respectively, after the addition of NADPH and/or ADP-Fe^{3+}. Figure 9 shows that either NADPH or ADP-Fe^{3+} is a minor stimulator of lipid peroxidation of normal hepatocytes, but when the nucleotides are added together, there is a marked augmentation of lipid peroxidation. Lipid peroxidation was produced in normal hepatocytes equally well with an NADPH-generating system and ADP-Fe^{3+}, but NADH was only 25 percent as effective in the presence of ADP-Fe^{3+} as either NADPH or the NADPH-generating system (data not shown). Figure 10 demonstrates similar results for hepatocytes isolated from phenobarbital-treated rats after the addition of NADPH, but ADP-Fe^{3+} produces a marked stimulation of lipid peroxidation with respect to cells incubated alone. After the addition of NADPH and ADP-Fe^{3+} there is an even greater production of the thiobarbituric acid-reacting chromagen by phenobarbital-treated cells than that produced by normal cells. Thus, there appears to be an NADPH-dependent enzyme system in intact, isolated hepatocytes capable of stimulating lipid peroxidation, and it is similar to that demonstrated by McCay et al. with rat liver microsomes.

The liver cells were obviously producing a thiobarbituric acid-reacting chromagen which absorbed light at 532nm. This corresponds to the optical absorption of the malondialdehyde-thiobarbituric acid chromagen. Because Poyer and McCay demonstrated that malondialdehyde was produced from the peroxidation of polyunsaturated fatty acids of rat liver microsomes, it was necessary to determine the origin of the thiobarbituric acid-reacting species produced by incubated hepatocytes.

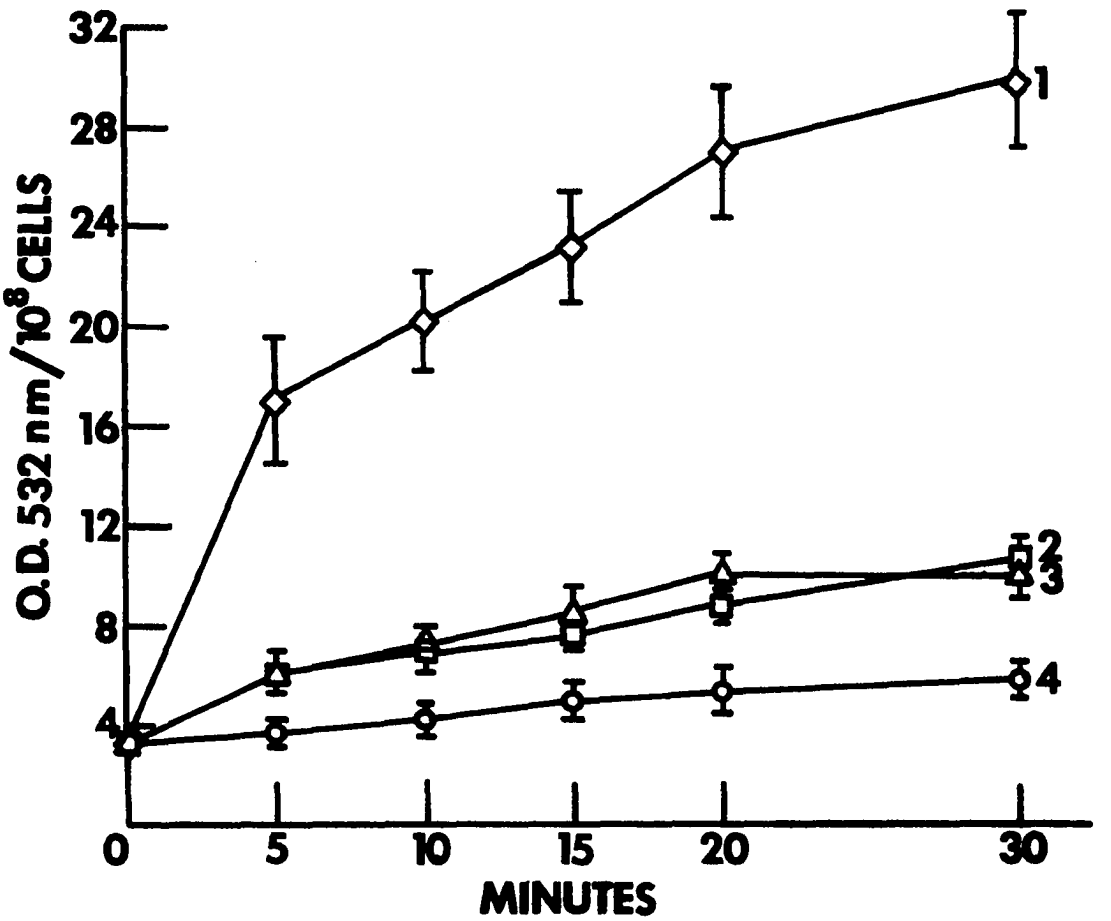


Figure 9. A time course study of malondialdehyde production by liver cells isolated from control rats incubated with NADPH and/or ADP-Fe^{3+}. The incubation procedure is described in "Methods". The "zero" time point corresponds to a 17 minute preincubation of the liver cells prior to the addition of the nucleotides.

1. cells + NADPH + ADP-Fe^{3+}
2. cells + NADPH
3. cells + ADP-Fe^{3+}
4. cells alone

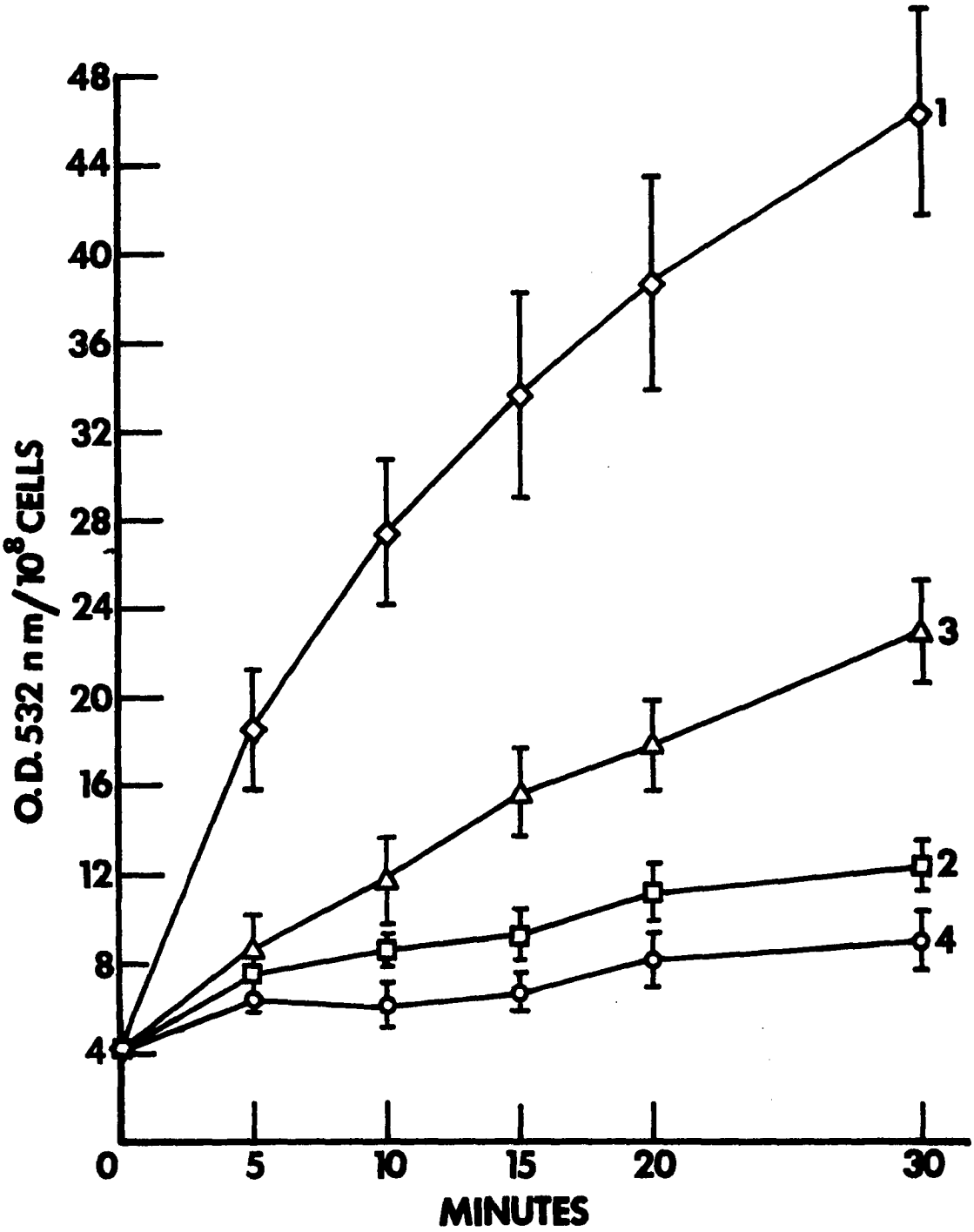


Figure 10. A time course study of malondialdehyde production by liver cells isolated from phenobarbital-treated rats incubated with NADPH and/or ADP-Fe^{3+}. The incubation procedure is described in "Methods". Refer to Figure 9 for a description of the "zero" point.

1. cells + NADPH + ADP-Fe^{3+}
2. cells + NADPH
3. cells + ADP-Fe^{3+}
4. cells alone

If the thiobarbituric acid-reacting species of hepatocytes was being produced from the peroxidation of membranous phospholipids, then the loss of membranous lipids of different subcellular organelles would suggest that the thiobarbituric acid-reacting species was malondialdehyde. Liver cells isolated from phenobarbital-treated rats were used for this study because phenobarbital is known to increase the phospholipid content of the endoplasmic reticulum (ER) of liver cells and to increase the cellular volume of hepatocytes as demonstrated by the packed cell volume procedure. As a result, the liver cell should contain more ER, plasma membrane surface area, and possibly more mitochondria. The experimental conditions are discussed in Chapter II. Table 3 gives the lipid peroxidation data for the four experiments cited in Tables 4, 5, and 6. Lipid peroxidation is minimal for cells incubated in ice, while the production of malondialdehyde is significantly greater for cells incubated either alone at 37° C or with NADPH at 37° C. The latter two systems produced equivalent amounts of malondialdehyde. The addition of NADPH and ADP-Fe^{3+} to the phenobarbital-treated cells produced markedly larger amounts of malondialdehyde than that produced by any other systems investigated. Table 4 shows the fatty acid content of the endoplasmic reticulum of these cells after incubation with either NADPH or NADPH and ADP-Fe^{3+}. The addition of ADP-Fe^{3+} alone was not done because of the limited number of flasks that could be incubated in each experiment. There are no fatty acid alterations caused by the addition of either NADPH or NADPH and ADP-Fe^{3+} in any of the microsomal lipids isolated from

TABLE 3

MALONDIALDEHYDE PRODUCTION BY LIVER CELLS ISOLATED FROM PHENOBARBITAL-TREATED RATS

Additions to Cell Suspension	Malondialdehyde Formation
	$\underline{O.D._{532\ nm}}/10^8$ cells/30 min[a]
None[b]	3.3 ± 0.2
None	16.3 ± 0.2
NADPH	16.9 ± 1.1
NADPH and ADP-Fe^{3+}	57.4 ± 3.1

The incubation conditions are described in "Methods".

[a] This data represents an average ± S.E.M. of 4 cell preparations.

[b] These cells were incubated at 0°C.

TABLE 4

FATTY ACID CONTENT OF MICROSOMAL LIPIDS FROM INCUBATED LIVER CELLS FROM PHENOBARBITAL-TREATED RATS

Additions	Fatty Acid Content						Protein Content
	16:0	18:0	18:1	18:2	20:4	22:6	
	mg/10^8 cells[a]						mg/10^8 cells
None-0°	0.67	1.07	0.33	0.60	1.11	0.19	11.58
None-37°	0.69	1.11	0.34	0.60	1.08	0.20	11.72
NADPH	0.77	1.24	0.38	0.70	1.24	0.22	12.74
NADPH + ADP-Fe^{3+}	0.87[b]	1.41	0.44	0.77	1.27	0.21	13.47

The incubation conditions are described in "Methods".

[a] Average value for cells from 4 different rats.

[b] Underlined values are significantly higher than those for cells incubated at 37° without additions $p < 0.05$.

hepatocytes incubated under any experimental conditions investigated. This was an unexpected result since it is contrary to the results shown for the lipid peroxidation of rat liver microsomes reported by May and McCay (11). The same results are obtained for the fatty acid contents of mitochondria isolated from those cells as shown in Table 5. This too was somewhat unexpected, because Pfeifer and McCay demonstrated that rat liver mitochondria are capable of oxidizing NADPH in vitro and are capable of producing malondialdehyde with a concomitant loss of mitochondrial polyunsaturated fatty acids. The fatty acid content of the total lipids of the nuclei-plasma membrane fraction are given in Table 6. There are significant losses of arachidonic acid (C 20:4) and docosahexenoic acid (C 22:6) in the incubation system containing NADPH and ADP-Fe^{3+}. Thus, the nuclei-plasma membrane fraction is the only fraction that exhibits an appreciable loss of membrane fatty acids during lipid peroxidation initiated by the addition of NADPH and ADP-Fe^{3+}. Because we were able to demonstrate a loss of polyunsaturated fatty acids of at least one membrane fraction during the incubation of hepatocytes isolated from phenobarbital treated rats with NADPH and ADP-Fe^{3+}, it is probable that the thiobarbituric acid-reacting material formed during the incubation is malondialdehyde.

As stated in the introduction, the enzymic peroxidation of microsomal phospholipids of rat liver can be inhibited or greatly diminished either by the in vitro addition of some antioxidants or by the pretreatment of rats with antioxidants prior to the isolation of liver microsomes. Three antioxidants, ethoxyquin, N,N'-diphenyl-p-phenylenediamine, and d-α-tocopherol, have been shown to protect rat liver

TABLE 5

FATTY ACID CONTENT OF MITOCHONDRIAL LIPIDS FROM INCUBATED LIVER CELLS ISOLATED FROM PHENOBARBITAL-TREATED RATS

Additions	Fatty Acid Content						Protein Content
	16:0	18:0	18:1	18:2	20:4	22:6	
	mg/10^8 cells[a]						mg/10^8 cells
None-0°	1.01	1.53	0.49	0.97	1.53	0.31	16.90
None-37°	0.99	1.49	0.54	1.09	1.57	0.30	15.85
NADPH	1.06	1.55	0.60	1.19	1.64	0.32	15.39
NADPH + ADP-Fe^{3+}	1.21	1.77	0.63	1.10	1.29	0.28	16.43

The incubation conditions are described in "Methods".

[a] Average value for cells from 4 different rats.

TABLE 6

FATTY ACID CONTENT OF NUCLEI AND PLASMA MEMBRANE LIPIDS FROM INCUBATED LIVER CELLS FROM PHENOBARBITAL-TREATED RATS

Additions	Fatty Acid Content						Protein Content
	16:0	18:0	18:1	18:2	20:4	22:6	
	mg/10^8 cells[a]						mg/10^8 cells
None-0°	2.65	3.31	1.70	2.89	2.99	0.58	54.05
None-37°	2.42	3.11	1.53	2.91	2.78	0.57	48.15
NADPH	2.55	3.22	1.66	3.12	2.85	0.55	49.17
NADPH + ADP-Fe^{3+}	2.07	2.54[b]	1.42	2.48	2.00	0.39	41.18

The incubation conditions are described in "Methods".

[a] Average value for cells from 4 different rats.

[b] Underlined values are significantly lower than those for cells incubated at 37° without additions $p < 0.05$.

microsomes against lipid peroxidation in vitro as mentioned in Chapter I. Because lipid peroxidation does occur in isolated rat hepatocytes and because the process can be stimulated by the addition of NADPH and ADP-Fe^{3+}, it was necessary to investigate the action of these three antioxidants on the liver cell systems previously described. If these antioxidants inhibit lipid peroxidation of liver cell systems in a manner similar to that reported for lipid peroxidation of rat liver microsomes in vitro, then the lipid peroxidation in the whole cell systems is probably free-radical mediated. Table 7 shows the effect of phenobarbital, ethoxyquin, N,N'-diphenyl-p-phenylenediamine, and dietary d-α-tocopherol on the amount of malondialdehyde produced from incubated hepatocytes. Neither phenobarbital nor Wesson oil (data not presented), the vehicle used for the administration of ethoxyquin and N,N'-diphenyl-p-phenylenediamine, has any inhibitory effect on the amount of malondialdehyde formed compared to that of normal cells, while ethoxyquin, N,N'-diphenyl-p-phenylenediamine, and d-α-tocopherol all significantly depress the amount of malondialdehyde produced. After 30 minutes of incubation, 1) phenobarbital significantly increases the amount of malondialdehyde formed as compared to that of hepatocytes isolated from non-treated rats, 2) Wesson oil pretreatment of rats does not inhibit lipid peroxidation (data not presented), while 3) N,N'-diphenyl-p-phenylenediamine, ethoxyquin, and d-α-tocopherol are very effective inhibitors of lipid peroxidation. The effects of these compounds on lipid peroxidation in isolated rat hepatocytes change after the addition of NADPH and/or ADP-Fe^{3+} as shown in Table 8. The action of these agents on liver cells at 5 minutes of incubation as compared to that of untreated rats is 1) phenobarbital pretreatment

TABLE 7

THE EFFECT OF DIFFERENT ANIMAL PRETREATMENTS ON THE STIMULATORY ACTION OF NADPH AND/OR ADP-Fe^{3+} ON THE PRODUCTION OF MALONDIALDEHYDE BY ISOLATED LIVER CELLS

Animal Pretreatment[a]	Malondialdehyde Formation	
	Δ O.D.$_{532\ nm}$/10^8 cells	
	5 min	30 min
None (5-6)[b]	0.4 ± 0.1[c]	2.6 ± 0.2
Phenobarbital (6)	0.9 ± 0.3	4.9 ± 0.9
Ethoxyquin (3)	-0.7 ± 0.3	0.9 ± 0.3
N,N'-diphenyl-p-phenylenediamine (3)	-0.6 ± 0.1	0.1 ± 0.2
d-α-tocopherol (dietary) (2-3)	-0.3 ± 0.2	0.1 ± 0.4

The incubation conditions are described in "Methods".

[a] All pretreatments were performed as described in Chapter II.

[b] The numbers in parentheses denote the number of individual experiments performed to obtain the average O.D.$_{532\ nm}$/10^8 cells ± S.E.M.

[c] The amount of malondialdehyde production is calculated by subtracting the value found for O.D.$_{532\ nm}$/10^8 cells at "zero" time from those values obtained at 5 and 30 minutes of incubation.

TABLE 8

THE EFFECT OF DIFFERENT ANIMAL PRETREATMENTS ON THE STIMULATORY ACTION OF NADPH AND/OR ADP-Fe^{3+} ON THE PRODUCTION OF MALONDIALDEHYDE BY ISOLATED LIVER CELLS

Animal Pretreatment	Malondialdehyde Formation					
	Δ O.D.$_{532}$ nm/10^8 cells					
	5 min			30 min		
	NADPH	ADP-Fe^{3+}	NADPH + ADP-Fe^{3+}	NADPH	ADP-Fe^{3+}	NADPH + ADP-Fe^{3+}
None (5-6)[b]	2.3 ± 0.3[a]	2.0 ± 0.4	11.5 ± 1.5	4.7 ± 0.8	4.1 ± 0.3	23.9 ± 2.6
Phenobarbital (6)	2.6 ± 0.4	3.4 ± 0.7	10.3 ± 0.2	3.4 ± 0.6	13.9 ± 1.2	37.3 ± 3.5
Ethoxyquin (3)	2.0 ± 0.3	1.5 ± 0.2	5.2 ± 0.2	1.4 ± 0.2	5.5 ± 0.9	11.3 ± 0.4
N,N'-diphenyl-p-phenylenediamine (3)	0.9 ± 0.3	0.5 ± 0.2	0.9 ± 0.3	0.2 ± 0.2	-0.6 ± 0.4	-0.1 ± 0.2
d-α-tocopherol (dietary) (2-3)	1.2 ± 0.1	c	5.9 ± 2.0	2.6 ± 0.4	6.1 ± 1.0	18.4 ± 5.4

[a] The data represents an average of several different experiments ± S.E.M.

[b] The numbers in parentheses denote the number of separate experiments performed.

[c] There were insufficient numbers of isolated cells in the preparation from the d-α-tocopherol-treated animal to include this incubation system.

The data is calculated by subtracting the O.D.$_{532}$ nm/10^8 cells of the same cell suspension containing no additions for the same time periods from those values obtained after the addition of NADPH and/or ADP-Fe^{3+}.

of rats does not enhance malondialdehyde production caused by the addition of either NADPH or NADPH and ADP-Fe^{3+}, 2) Wesson oil pretreatment of rats appears to have no effect on lipid peroxidation (data not presented), 3) ethoxyquin and dietary d-α-tocopherol depress malondialdehyde formation approximately 50 and 36 percent respectively for the NADPH and ADP-Fe^{3+} system, while N,N'-diphenyl-p-phenylenediamine depresses it 98 percent, and 4) d-α-tocopherol inhibits lipid peroxidation initiated by the addition of NADPH, but it is much less effective when ADP-Fe^{3+} is present, while N,N'-diphenyl-p-phenylenediamine diminshes the production of malondialdehyde stimulated by either NADPH or ADP-Fe^{3+}. After 30 minutes of incubation of rat liver cells with NADPH and/or ADP-Fe^{3+}, the following information is obtained with cells isolated from untreated rats 1) phenobarbital stimulates lipid peroxidation initiated by either ADP-Fe^{3+} or NADPH and ADP-Fe^{3+}, 3.4 and 1.6 fold respectively, 2) ethoxyquin depresses the production of malondialdehyde stimulated by either NADPH or NADPH and ADP-Fe^{3+}, 70 and 53 percent respectively, 3) d-α-tocopherol effectively depresses malondialdehyde formation initiated by NADPH, 46 percent, but again is not effective when ferric ion is present, 4) N,N'-diphenyl-p-phenylenediamine totally inhibits lipid peroxidation of all systems studied, and 5) Wesson oil pretreatment of rats has no effect on malondialdehyde production by any system investigated (data not presented). In summation, 1) phenobarbital stimulates malondialdehyde production at 30 minutes after the addition of NADPH and ADP-Fe^{3+}, 2) ethoxyquin inhibits lipid peroxidation at 5 and 30 minutes after the addition of either NADPH or NADPH and ADP-Fe^{3+}, 3) either N,N'-diphenyl-p-phenylenediamine or d-α-tocopherol significantly

inhibits all systems studied at 5 minutes after the addition of substrates, while N,N'-diphenyl-p-phenylenediamine is the only effective inhibitor of lipid peroxidation 30 minutes after the addition of NADPH and/or ADP-Fe^{3+}, and 4) pretreatment of rats with Wesson oil has no effect on the production of malondialdehyde at either 5 or 30 minutes after the addition of NADPH and/or ADP-Fe^{3+} to the hepatocytes. Because NADPH and ADP-Fe^{3+} have been shown to stimulate the destruction of polyunsaturated fatty acids of the lipids of the nuclei-plasma membrane fraction, the action of these compounds previously described must be related to their effects on this subcellular fraction.

A complete time course study of lipid peroxidation of rat liver parenchymal cells isolated from ethoxyquin pretreated rats is presented in Figure 11. This graph shows that ethoxyquin pretreatment significantly depresses the formation of malondialdehyde of all systems investigated as compared to that produced by normal cells, except for lipid peroxidation stimulated by the addition of ADP-Fe^{3+}. This again demonstrates the selectivity of this antioxidant against lipid peroxidation stimulated by the addition of certain substrates.

As previously cited in Chapter I, the NADPH-oxidase enzyme system is primarily located in the endoplasmic reticulum of rat liver. Also, it was postulated that the drug metabolizing system of this membrane was responsible for oxidizing NADPH, and in the presence of ferric ion the enzyme system was capable of initiating lipid peroxidation of several different subcellular organelles. Subsequently, the components of the drug metabolizing system were purified and analyzed with respect to their role in the NADPH-oxidase enzyme system. Pederson and Aust

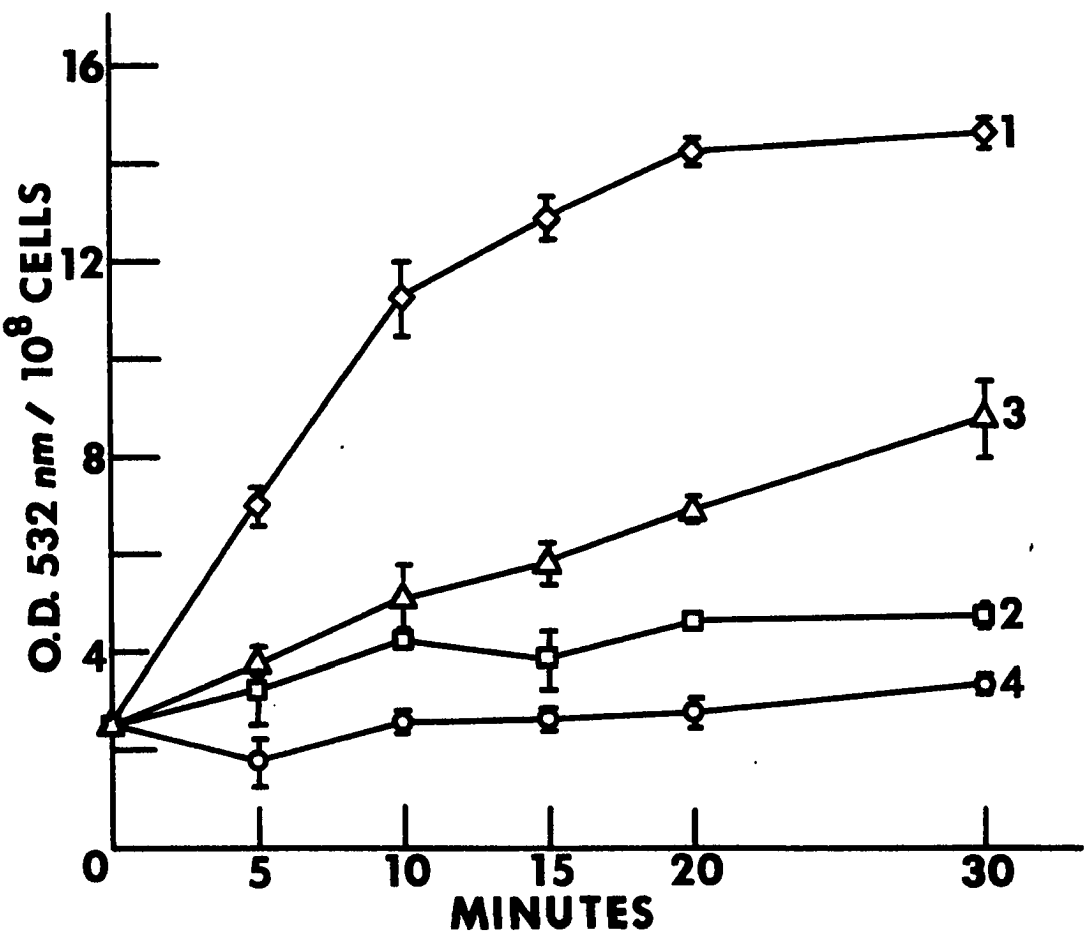


Figure 11. A time course study of malondialdehyde production by liver cells isolated from rats pretreated with ethoxyquin incubated with NADPH and/or ADP-Fe^{3+}. The rats were pretreated with ethoxyquin and incubation procedure is also described in Chapter II. Refer to Figure 9 for a description of the "zero" time point.

1. cells + NADPH + ADP-Fe^{3+}
2. cells + NADPH
3. cells + ADP-Fe^{3+}
4. cells alone

demonstrated that partially purified NADPH-cytochrome c reductase isolated from rat liver microsomes was capable of initiating lipid peroxidation during the oxidation of NADPH in the presence of EDTA (9). Fong et al. showed that purified cytochrome P-450 reductase of rat liver microsomes promoted lipid peroxidation under the same conditions, but in the absence of EDTA (10). Furthermore, Levin et al. (100) demonstrated that lipid peroxidation of rat liver microsomes in vitro resulted in the loss of microsomal cytochrome P-450 heme. Because Pederson and Aust demonstrated that partially purified cytochrome c reductase was capable of initiating microsomal lipid peroxidation, Levin et al. considered the loss of cytochrome P-450 to be a consequence of lipid peroxidation, i.e. cytochrome P-450 was probably not responsible for lipid peroxidation. In an attempt to examine this type of effect in an intact cell, rat liver parenchymal cells were isolated from animals pretreated with a porphyric agent, allylisopropylacetamide (AIA); a compound which has been demonstrated to decrease endogenous cytochrome P-450 heme content of rat liver about 60 percent (101). These animals must be pretreated for 5 days with phenobarbital prior to the subcutaneous administration of allylisopropylacetamide. Figure 12 represents an average of 5 time course studies of lipid peroxidation done with these cells. Table 9 provides 1) the data for lipid peroxidation of hepatocytes, isolated from phenobarbital and allylisopropylacetamide treated rats, incubated alone, 2) lipid peroxidation of hepatocytes, isolated from phenobarbital treated rats, incubated alone, and 3) the cytochrome P-450 content of liver cells isolated from rats pretreated with either phenobarbital and allylisopropylacetamide or phenobarbital. The pretreatment of rats with pheno-

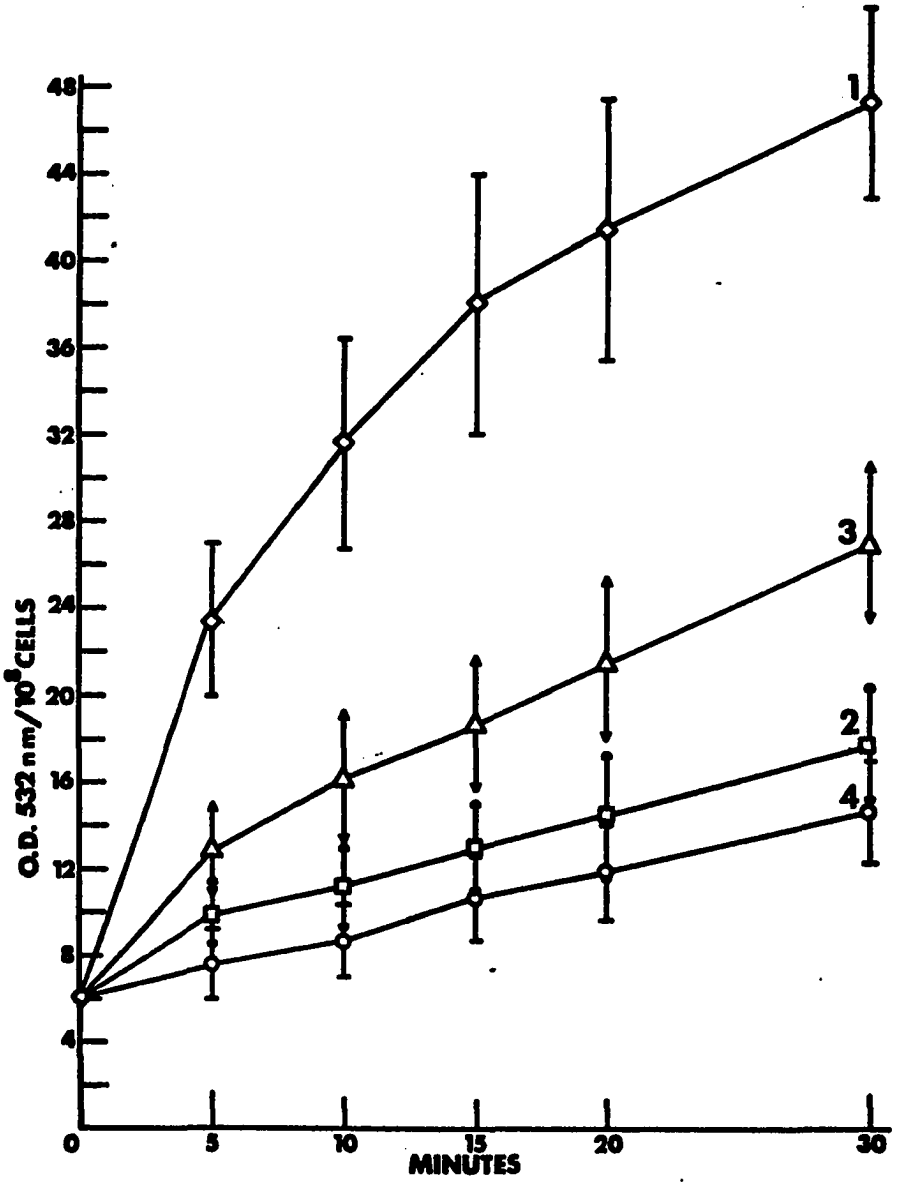


Figure 12. A time course study of malondialdehyde production by liver cells isolated from rats pretreated with phenobarbital and allylisopropylacetamide incubated with NADPH and/or ADP-Fe^{3+}. The rats were pretreated with phenobarbital and allylisopropylacetamide and the liver cells were isolated as described in "Methods". The incubation procedure is also described in "Methods".

1. cells + NADPH and ADP-Fe^{3+}
2. cells + NADPH
3. cells + ADP-Fe^{3+}
4. cells alone

TABLE 9

MALONDIALDEHYDE PRODUCED DURING INCUBATION OF LIVER CELLS AS RELATED TO THE INITIAL CYTOCHROME P-450 CONTENT IN FRESHLY PREPARED LIVER CELLS ISOLATED FROM RATS PRETREATED WITH EITHER PHENOBARBITAL OR PHENOBARBITAL AND ALLYLISOPROPYLACETAMIDE

Animal Pretreatment	Additions to Cell Suspension	Cytochrome P-450 [b1] Content		Malondialdehyde Formation	
				Δ O.D.$_{532\ nm}$/10^8 cells[c]	
				5 min	30 min
Phenobarbital (6)[a]	None	0.05	(3)[b2]	0.7 ± 0.3	4.9 ± 0.9
Phenobarbital and Allylisopropylacetamide (5)	None	0.02	(2)	1.5 ± 0.4	8.6 ± 1.4

[a] The number in parentheses denotes the number of separate experiments performed.

[b1] The cytochrome P-450 data is expressed as O.D.$_{450\ nm}$/10^8 cells/ml of cuvet contents.

[b2] The number in the parentheses denotes the number of separate cytochrome P-450 analyses. The determination of cytochrome P-450 is described under "Methods".

[c] The data is calculated as described in Table 3. The treatment of the animals with phenobarbital and allylisopropylacetamide is described under "Methods".

barbital and allylisopropylacetamide increases lipid peroxidation approximately two fold after 5 and 30 minutes of incubation as compared to cells isolated from phenobarbital pretreated rats. The cytochrome P-450 content of liver cells isolated from rats pretreated with phenobarbital and allylisopropylacetamide is 60 percent less than that of hepatocytes isolated from phenobarbital pretreated rats. These results show that a decrease of endogenous cytochrome P-450 content results in an increase of endogenous lipid peroxidation during the incubation of hepatocytes, and this information tends to support the in vitro studies of lipid peroxidation with purified enzymes, i.e., cytochrome P-450 is not the initiator of NADPH, ADP-Fe^{3+}-stimulated lipid peroxidation, nor is it required to promote the process.

The additions of NADPH and/or ADP-Fe^{3+} to liver cells isolated from either phenobarbital or phenobarbital and allylisopropylacetamide treated rats stimulated lipid peroxidation, and the data is given in Table 10. Except for the somewhat stimulating effect of ADP-Fe^{3+} on lipid peroxidation of hepatocytes isolated from phenobarbital and allylisopropylacetamide pretreated rats, there are no significant differences between the responses of hepatocytes isolated from either phenobarbital or phenobarbital and allylisopropylacetamide treated rats to either NADPH or NADPH and ADP-Fe^{3+}. Although the cytochrome P-450 heme content of the liver cells isolated from phenobarbital and allylisopropylacetamide treated rats is 60 percent less than that of hepatocytes isolated from phenobarbital treated rats, the loss of cytochrome P-450 apparently has no effect on the NADPH-dependent enzyme system of the intact liver cells.

TABLE 10

INCREASED MALONDIALDEHYDE PRODUCTION AFTER THE ADDITION OF NADPH AND/OR ADP-Fe^{3+} TO LIVER CELLS ISOLATED FROM RATS PRETREATED WITH EITHER PHENOBARBITAL OR PHENOBARBITAL AND ALLYLISOPROPYLACETAMIDE

Animal Pretreatment	Additions to Cell Suspension	Malondialdehyde Formation	
		$\Delta O.D._{532\ nm}/10^8$ cells[b]	
		5 min	30 min
Phenobarbital (6)[a] (Cytochrome P-450 content = 0.05 $O.D._{450\ nm}/10^8$ cells/ml)	NADPH	2.6 ± 0.4	3.4 ± 0.6
	ADP-Fe^{3+}	3.4 ± 0.7	13.9 ± 1.2
	NADPH + ADP-Fe^{3+}	10.3 ± 0.2	37.3 ± 3.5
Phenobarbital and Allylisopropylacetamide (5) (Cytochrome P-450 content = 0.02 $O.D._{450\ nm}/10^8$ cells/ml)	NADPH	2.3 ± 0.2	3.1 ± 0.9
	ADP-Fe^{3+}	5.2 ± 0.9	12.3 ± 2.6
	NADPH + ADP-Fe^{3+}	15.8 ± 2.0	32.5 ± 2.2

The incubation conditions are described in "Methods".

[a] The numbers in parentheses denote the number of separate experiments performed.

[b] These values are calculated as described in Table 11 ± S.E.M.

Treatment of the animals and analyses of cytochrome P-450 are described under "Methods".

B. Studies of Carbon Tetrachloride Stimulation of Lipid Peroxidation of Isolated Rat Liver Parenchymal Cells.

Many studies have demonstrated that *in vitro* lipid peroxidation of rat liver microsomes is stimulated by the metabolism of CCl_4, while only a few studies provided evidence for the stimulation of *in vivo* lipid peroxidation of rat liver microsomes after the administration of CCl_4. Although lipid peroxidation appears to be stimulated *in vivo* during the metabolism of CCl_4, there exists a controversy concerning the ability of some known antioxidants to inhibit CCl_4-mediated lipid deterioration *in vivo*. Furthermore, it has not been substantiated that there is a concomitant loss of polyunsaturated fatty acids of liver microsomes *in vivo* with the production of malondialdehyde from CCl_4-poisoned rats. In addition, a primary concern of many investigators has been the site of interaction of CCl_4 with the drug metabolizing system of rat liver. It is generally accepted that CCl_4 is metabolized *in vivo* by the drug metabolizing system of rat liver microsomes to the $\cdot CCl_3$ radical species, but there is disagreement among these researchers as to the actual locus of interaction of CCl_4 with this system, i.e., whether CCl_4 is metabolized by the NADPH-cytochrome c reductase or the NADPH-cytochrome P-450 reductase enzyme. Because of the previously mentioned gaps of information that exist for the *in vivo* effects of CCl_4 on rat liver, our laboratory attempted to provide additional "*in vivo*" information by using isolated rat liver parenchymal cells.

The concentration of CCl_4 that would yield maximum production of malondialdehyde by cells isolated from untreated rats was determined from

the graph on the left side of Figure 13. When hepatocytes from untreated rats are incubated in a aqueous suspension, they undergo increasing magnitudes of lipid peroxidation with the addition of increasing concentrations of CCl_4. The maximum amount of malondialdehyde is produced with a final concentration of 10 µl of CCl_4 per milliter of incubated cell suspension. The additions of NADPH and/or ADP-Fe^{3+} to these CCl_4-preincubated cells only magnify the magnitude of lipid peroxidation as compared to cells incubated with only CCl_4. The maximal lipid peroxidation under these latter conditions is still produced with a final concentration of 10 µl of CCl_4 per milliter of incubated cell suspension. The addition of these nucleotides enhance lipid peroxidation of hepatocytes by CCl_4, and the relative relationship of this activity is:

NADPH + ADP-Fe^{3+} > NADPH > ADP-Fe^{3+}.

The graph drawn on the right side of Figure 13 illustrates the response of hepatocytes isolated from rats pretreated with phenobarbital to increasing concentrations of CCl_4 and to the effects of the additions of NADPH and/or ADP-Fe^{3+} to the cells suspensions containing CCl_4. Phenobarbital pretreatment of rats did not change the amount of CCl_4 necessary to produce a one-half maximal response, but the amounts of malondialdehyde formed for each concentration of CCl_4 are larger than those of cells isolated from untreated rats. The maximal lipid peroxidation is obtained with a final concentration of 10 µl of CCl_4 per milliter of incubated cell suspension. The relationships of NADPH and/or ADP-Fe^{3+} on lipid peroxidation of liver cells isolated from phenobarbital pretreated rats incubated with CCl_4 are:

NADPH + ADP-Fe^{3+} > NADPH > ADP-Fe^{3+}.

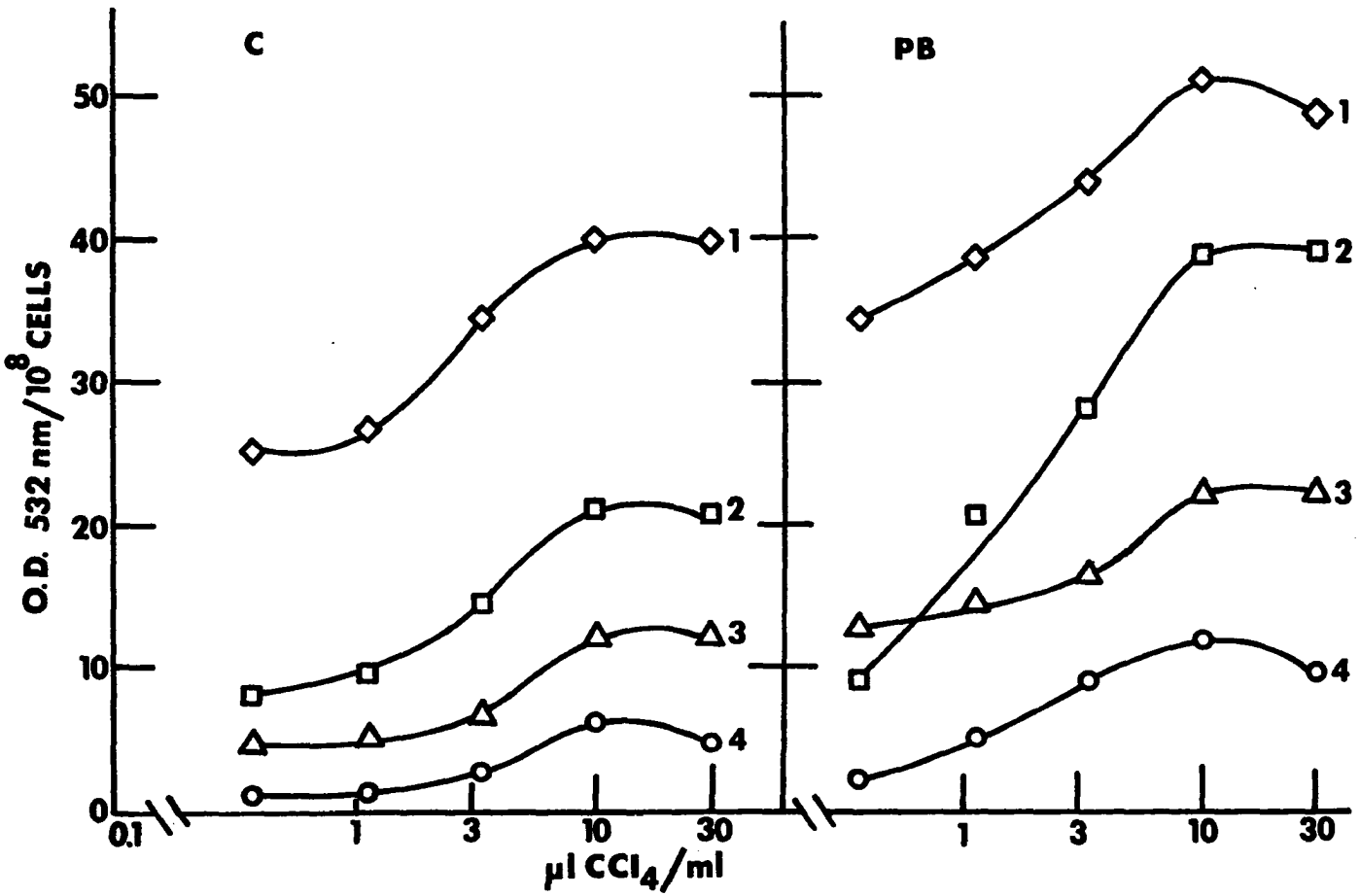


Figure 13. Malondialdehyde production by liver cells isolated from control (C) and phenobarbital-treated (PB) rats incubated with different concentrations of CCl_4 and NADPH and/or ADP-Fe^{3+}. The pretreatment of rats with phenobarbital is described in "Methods". The incubation procedure is also described in "Methods". A description of the "zero" time point is given in Figure 9.

1. cells + NADPH + ADP-Fe^{3+}
2. cells + NADPH
3. cells + ADP-Fe^{3+}
4. cells alone

In view of the potentiating effect of phenobarbital on CCl_4 toxicity in intact animals, liver cells isolated from phenobarbital treated rats should have been more sensitive to lipid peroxidation stimulated by CCl_4 than cells isolated from untreated animals, i.e., the dose response curves for liver cells isolated from phenobarbital treated rats should have shifted to the left. For example, hepatocytes isolated from phenobarbital treated rats might have been expected to undergo maximal lipid peroxidation at 3 µl/ml of CCl_4 rather than 10 µl/ml of CCl_4 as shown for hepatocytes isolated from untreated rats, but this did not occur. Instead, the magnitude of lipid peroxidation of liver cells isolated from phenobarbital treated rats was greater than that of hepatocytes isolated from untreated rats at corresponding concentrations of CCl_4. All subsequent studies of CCl_4 were performed by adding CCl_4 directly to cells to a final concentration of 10 µl/ml.

The previous study demonstrated that liver cells isolated from either untreated rats or phenobarbital pretreated rats produced malondialdehyde in the presence of CCl_4. The additions of NADPH and/or ADP-Fe^{3+} further stimulated the lipid peroxidative process. Figure 14 is a time course study of lipid peroxidation of liver cells isolated from untreated rats stimulated by the <u>in vitro</u> additions of NADPH and/or ADP-Fe^{3+} to liver cell suspensions containing CCl_4. This graph shows that the <u>in vitro</u> addition of CCl_4 to a suspension of hepatocytes stimulates lipid peroxidation as compared to cells incubated without CCl_4. Additions of NADPH and/or ADP-Fe^{3+} further stimulate lipid peroxidation of cell suspensions containing CCl_4, as described for Figure 13. Hepatocytes isolated from phenobarbital treated rats respond in a manner similar

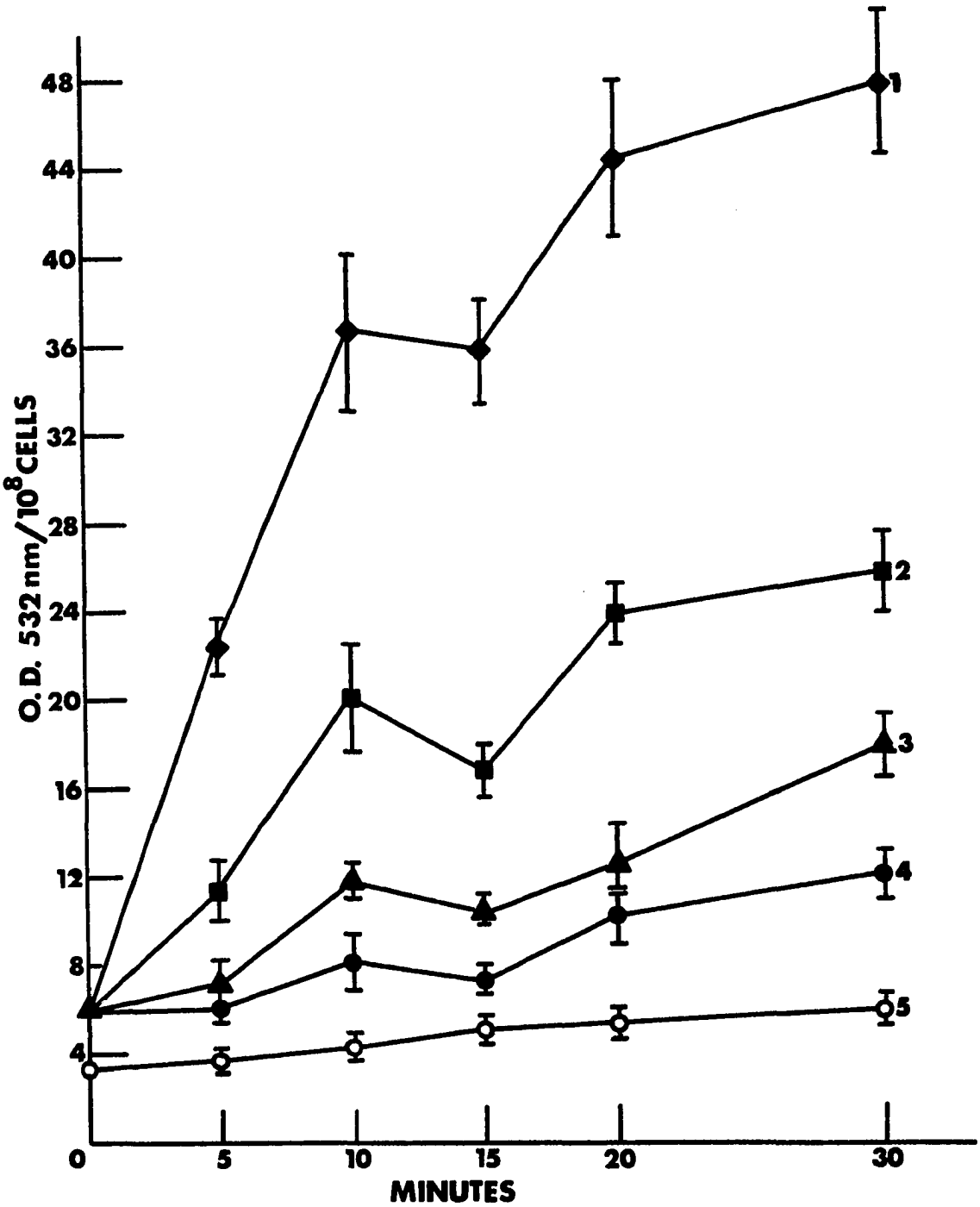


Figure 14. A time course study of malondialdehyde production by liver cells isolated from control rats incubated with CCl_4 and NADPH and/or ADP-Fe^{3+}. The incubation procedure is described in "Methods". A description of the "zero" time point is given in Figure 9.

1. cells + CCl_4 + NADPH + ADP-Fe^{3+}
2. cells + CCl_4 + NADPH
3. cells + CCl_4 + ADP-Fe^{3+}
4. cells + CCl_4
5. cells alone

to liver cells isolated from untreated rats with respect to the addition of either CCl_4 alone or CCl_4 and nucleotides, but the magnitude of lipid peroxidation in each system, except for the one containing NADPH, ADP-Fe^{3+}, and CCl_4, is significantly greater for hepatocytes isolated from phenobarbital treated rats than for cells isolated from untreated rats. Except for the system previously cited, phenobarbital pretreatment of rats enhances the peroxidation of hepatocellular lipids. However, the most important observation is the enhancement of CCl_4-mediated lipid peroxidation of hepatocytes isolated from either untreated or phenobarbital treated rats by the addition of NADPH. Because phenobarbital is known to induce the drug metabolizing system <u>in vivo</u>, the greater stimulatory effect of NADPH on CCl_4-mediated lipid peroxidation of cells isolated from phenobarbital treated rats as compared to hepatocytes from untreated rats indicates that the drug metabolizing system is most likely involved in CCl_4-mediated production of malondialdehyde. Table 11 contains data calculated from Figures 14 and 15. The addition of either CCl_4 or CCl_4 and nucleotides significantly increases lipid peroxidation of hepatocytes isolated from both untreated and phenobarbital treated rats above that produced by their respective control systems after 5 and 30 minutes of incubation. Phenobarbital pretreatment markedly enhances the production of malondialdehyde of cell suspensions containing CCl_4, NADPH and CCl_4, or ADP-Fe^{3+} and CCl_4 as compared to those same systems studied with suspensions of hepatocytes isolated from untreated rats after 5 and 30 minutes of incubation. Carbon tetrachloride-mediated lipid peroxidation of cells incubated with NADPH and ADP-Fe^{3+} appears to be unaffected by phenobarbital pretreatment of rats.

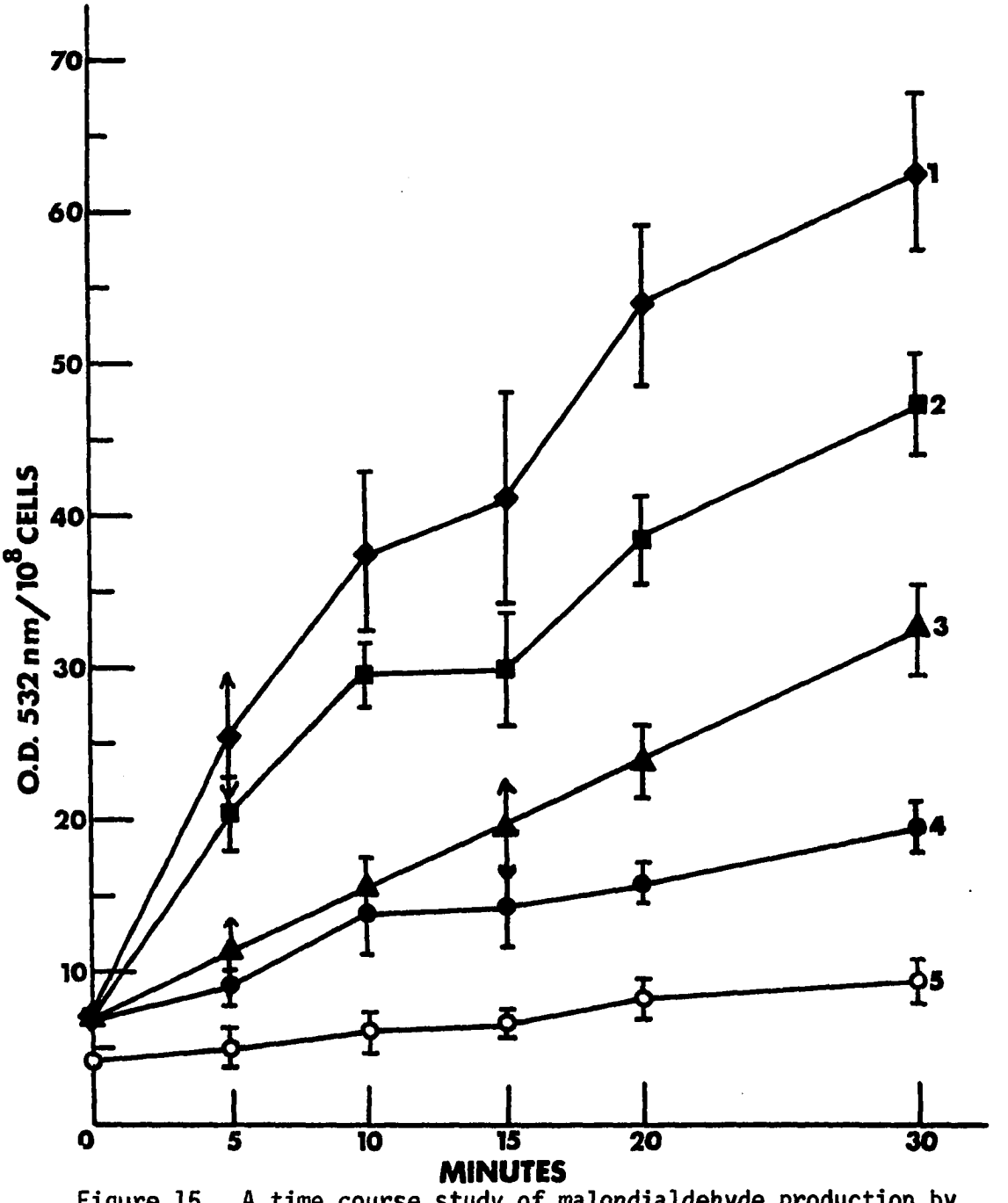


Figure 15. A time course study of malondialdehyde production by liver cells isolated from phenobarbital-treated rats incubated with CCl_4 and NADPH and/or ADP-Fe^{3+}. The rats were pretreated with phenobarbital as described in "Methods". The incubation procedure is also described in "Methods". A description of the "zero" time point is given in Figure 9.

1. cells + CCl_4 + NADPH + ADP-Fe^{3+}
2. cells + CCl_4 + NADPH
3. cells + CCl_4 + ADP-Fe^{3+}
4. cells + CCl_4
5. cells alone

TABLE 11

MALONDIALDEHYDE PRODUCTION OF ISOLATED LIVER CELLS FROM CONTROL AND PHENOBARBITAL-PRETREATED RATS, INCUBATED WITH CCl_4 ALONE OR WITH NADPH AND/OR ADP-Fe^{3+}

Animal Pretreatment	Addition to Cell Suspension	Malondialdehyde Formation	
		Δ O.D.$_{532\ nm}$/10^8 cells	
		5 min	30 min
None (5-6)[a]	CCl_4	2.3 ± 0.5[b]	6.2 ± 0.6[b]
	NADPH + CCl_4	5.3 ± 0.9[c]	13.7 ± 1.7[c]
	ADP-Fe^{3+} + CCl_4	1.1 ± 0.3[c]	5.9 ± 0.7[c]
	NADPH, ADP-Fe^{3+} + CCl_4	16.7 ± 1.0[c]	33.8 ± 1.9[c]
Phenobarbital (6)	CCl_4	4.3 ± 1.1[b]	10.7 ± 1.1[b]
	NADPH + CCl_4	11.2 ± 1.1[c]	27.5 ± 2.3[c]
	ADP-Fe^{3+} + CCl_4	3.5 ± 0.9[c]	14.2 ± 2.8[c]
	NADPH, ADP-Fe^{3+} + CCl_4	17.7 ± 4.1[c]	42.9 ± 3.7[c]

The incubation conditions are described in "Methods".

[a] The number in parentheses denotes the number of separate experiments performed.

[b] These data are calculated by subtracting the value O.D.$_{532\ nm}$/10^8 cells of cells incubated alone for those periods from those obtained for cells incubated with only CCl_4 for 5 and 30 minutes respectively within each group of cells ± S.E.M.

[c] These values are calculated by subtracting the absolute values of O.D.$_{532\ nm}$/10^8 cells of cells incubated with CCl_4 alone for those time periods from those values obtained for cells incubated with the nucleotides for 5 and 30 minutes within each group of cells ± S.E.M.

Absolute values (O.D.$_{532\ nm}$/10^8 cells):	5 min	30 min
Untreated rats (5-6)	3.4 ± 0.4	6.0 ± 0.6
Phenobarbital Pretreated Rats (6)	4.3 ± 0.5	9.2 ± 1.4

Because CCl_4 is a lipid solvent, it is possible that the enhanced lipid peroxidation is related in some way to its ability to alter the structure of membrane lipids. Therefore, another haloalkane, dichloromethane was studied. Dichloromethane has lipid solvent properties similar to CCl_4 but has not been found to be hepatotoxic. Dichloromethane was incubated with rat liver cells under identical conditions as those used for the CCl_4 experiments. Table 12 gives a comparative analysis of the effects of dichloromethane and CCl_4 on lipid peroxidation of hepatocytes incubated with or without NADPH and/or ADP-Fe^{3+}. Dichloromethane does stimulate lipid peroxidation after the addition of NADPH as compared to non-halomethane treated cells, but the stimulatory effect of CCl_4 in all systems investigated is significantly greater. Thus, the stimulatory effect of CCl_4 on lipid peroxidation is primarily due to its metabolism, and not its ability to solubilize membrane lipids. Another lipid solvent, cyclohexane, was also studied, and this compound produced an effect on lipid peroxidation similar to that produced by dichloromethane.

Figures 16 and 17 are electron micrographs of hepatocytes isolated from untreated rats incubated with CCl_4 and CCl_4 and NADPH respectively. Carbon tetrachloride alone has very little effect on the structural morphology of hepatocytes incubated for 30 minutes with the haloalkane. In contrast, liver cells incubated with NADPH and CCl_4 for 15 minutes exhibit gross morphological changes of subcellular organelles as shown in Figure 17. These cells contain many cytoplasmic vacuoles, and some of these have been identified as mitochondria containing no matrices. Electron micrographs of hepatocytes incubated with NADPH alone show no

TABLE 12

MALONDIALDEHYDE PRODUCTION BY ISOLATED RAT LIVER CELLS INCUBATED WITH EITHER DICHLOROMETHANE OR CCl_4

Additions to Cell Suspension	Malondialdehyde Formation		
	O.D.$_{532\ nm}$/10^8 cells/30 min[a]		
	Haloalkane Added		
	None	CH_2Cl_2	CCl_4
None	7.1 ± 0.4	10.6 ± 0.5	13.5 ± 1.0
NADPH	12.0 ± 0.5	15.0 ± 1.0	27.0 ± 1.2
ADP-Fe^{3+}	11.5 ± 1.1	11.2 ± 1.2	19.1 ± 0.3
NADPH + ADP-Fe^{3+}	33.1 ± 5.6	39.4 ± 5.3	50.8 ± 1.8

The incubation conditions are described in "Methods".

[a] The values are averages ± S.E.M. of 3 separate experiments. Malondialdehyde was determined as described under "Methods".

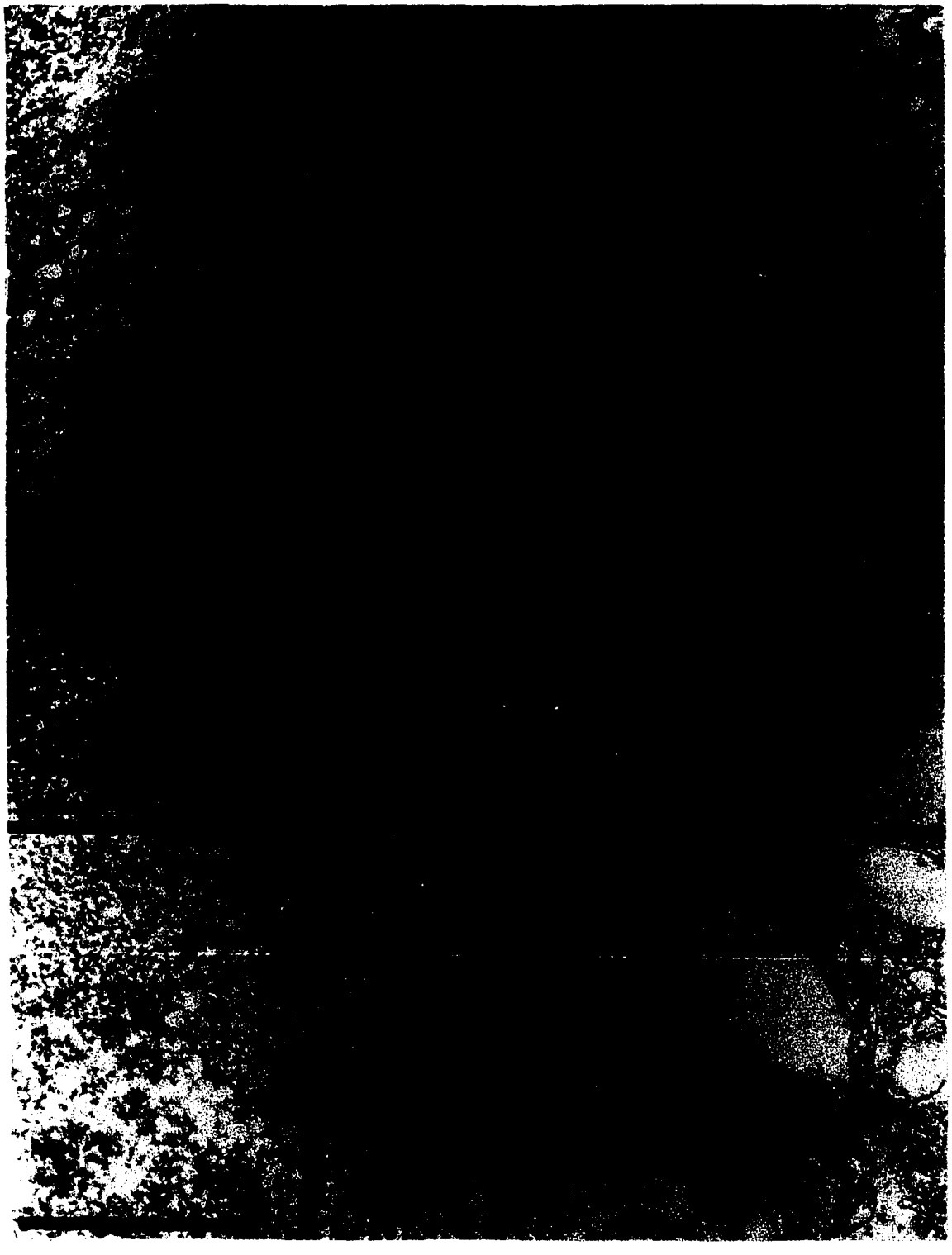


Figure 16. An electron micrograph of a liver cell isolated from a control rat and incubated with CCl_4. The hepatocytes were incubated at 37°C for 30 minutes with CCl_4 (10 µl/ml). Bar denotes one micron.

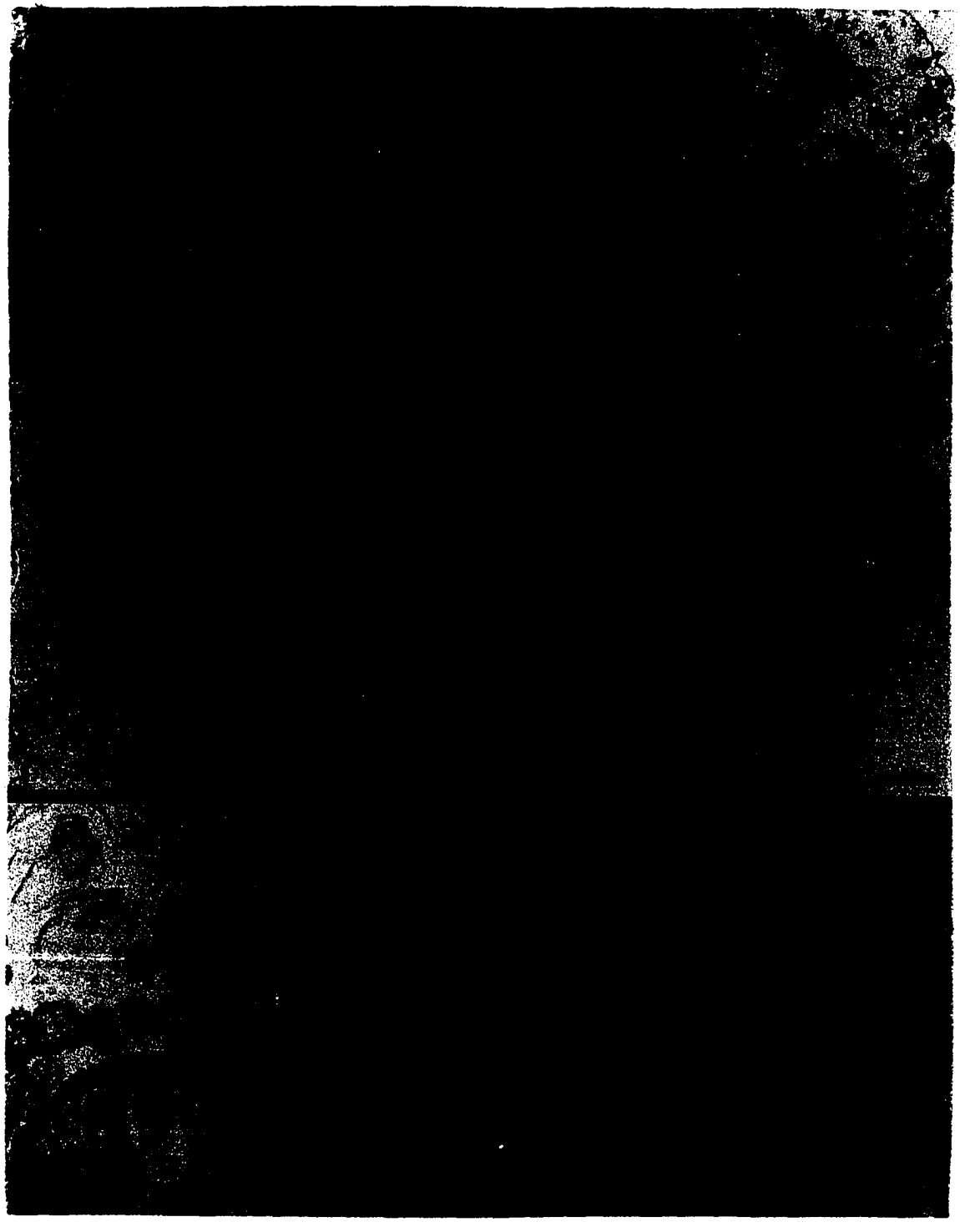


Figure 17. An electron micrograph of a liver cell isolated from a control rat and incubated with NADPH and CCl_4. The hepatocytes were incubated at 37°C for 15 minutes with CCl_4 (10 µl/ml) and an additional 15 minutes after the addition of NADPH (0.6 mM). Bar denotes one micron.

morphological changes of subcellular organelles as compared to Figure 7 (cells incubated alone).

It is generally accepted that CCl_4 is homolytically cleaved during its metabolism by rat liver *in vivo*. The cleavage products are thought to be free radicals which are capable of binding to the lipid and protein portions of rat liver endoplasmic reticulum. These free radicals are also thought to initiate lipid peroxidation of polyunsaturated fatty acids linked to membrane phospholipids of rat liver ER. Neither d-α-tocopherol nor ethoxyquin pretreatment of rats has been reported to inhibit *in vivo* production of diene conjugates initiated after dosing with CCl_4. Also, controversy exists concerning the effectiveness of N,N'-diphenyl-p-phenylenediamine against *in vivo* CCl_4-mediated diene conjugate formation. If lipid peroxidation initiated by the metabolism of CCl_4 is a free-radical mediated process, then it is likely that free-radical scavenging agents would inhibit CCl_4-mediated lipid deterioration. Because the detection of lipid peroxidation *in vivo* has proven to be a difficult task and because the inhibitory effects of d-α-tocopherol, ethoxyquin, and N,N'-diphenyl-p-phenylenediamine against *in vivo* destruction of polyunsaturated fatty acids are not well documented, I studied the effects of these antioxidants on the production of malondialdehyde by isolated rat liver parenchymal cells incubated with CCl_4 and NADPH and/or ADP-Fe^{3+}. The antioxidants were administered to the rats as described in Chapter II, and then the liver cells were isolated from these pretreated animals. Table 13 shows the effects of these antioxidants (administered using Wesson oil as a vehicle for ethoxyquin and N,N'-diphenyl-p-phenylenediamine) as well as the appropriate control

TABLE 13

MALONDIALDEHYDE PRODUCTION BY ISOLATED LIVER CELLS FROM RATS PRETREATED WITH DIFFERENT ANTIOXIDANTS AT TWO DIFFERENT TIME PERIODS OF INCUBATION

Additions to Cell Suspension	Malondialdehyde Formation			
	Δ O.D.$_{532\ nm}$/10^8 cells/5 min[a]			
	Antioxidant Administered			
	None (5-6)[b]	d-α-toc(3)	Ethoxyquin(3)	DPPD(3)
CCl_4	2.3 ± 0.5	1.0 ± 0.3	0.9 ± 0.3	0.2 ± 0.5
NADPH + CCH	5.3 ± 0.9	3.0 ± 0.5	1.3 ± 0.7	0.8 ± 0.2
ADP-Fe^{3+} + CCl_4	1.1 ± 0.3	1.9 ± 0.5	0.7 ± 0.6	0.3 ± 0.3
NADPH, ADP-Fe^{3+}, +CCl_4	16.7 ± 1.0	11.8 ± 2.1	3.6 ± 0.5	0.5 ± 0.3
	Δ O.D.$_{532\ nm}$/10^8 cells/30 min[a]			
	Antioxidant Administered			
	None(5-6)	d-α-toc(3)	Ethoxyquin(3)	DPPD(3)
CCl_4	6.2 ± 0.6	2.6 ± 0.5	-0.5 ± 0.4	-0.6 ± 0.4
NADPH + CCl_4	13.7 ± 1.7	12.0 ± 1.5	1.4 ± 0.3	0.6 ± 0.2
ADP-Fe^{3+} + CCl_4	5.9 ± 0.7	9.3 ± 1.0	3.6 ± 1.8	-0.6 ± 0.4
NADPH, ADP-Fe^{3+}, +CCl_4	33.8 ± 1.9	32.1 ± 2.6	3.2 ± 0.7	0.2 ± 0.2

The incubation conditions are described in "Methods".

[a] The data is calculated as described in Table 12, ± S.E.M.

[b] The numbers in parentheses denote the number of separate experiments performed.

Lipid peroxidation data for liver cells isolated from Wesson oil-treated rats is not given.

systems (liver cells isolated from Wesson oil-treated rats and from untreated rats) on CCl_4-mediated lipid peroxidation. At the injected amount tested, pretreatment of rats with N,N'-diphenyl-p-phenylenediamine yields the most effective inhibition of CCl_4-stimulated lipid peroxidation of the antioxidants which were studied. This was observed at both the 5 and 30 minute intervals of cellular exposure and was true for all of the various conditions which were tested. Ethoxyquin was the next most effective inhibitor, but it did not inhibit the lipid peroxidation produced by the addition of ADP-Fe^{3+} with CCl_4. D-α-tocopherol was the least effective inhibitor of lipid peroxidation by the incubation systems investigated. The only reaction systems that showed depressed lipid peroxidation of hepatocytes by d-α-tocopherol were 1) cells incubated with either CCl_4 or NADPH and CCl_4 for 5 minutes and 2) cells incubated with CCl_4 alone for 30 minutes. In general, pretreatment of rats by Wesson oil injection did not affect lipid peroxidation of hepatocytes except for possibly the systems mentioned previously as being unaffected by ethoxyquin after 5 minutes of incubation. The protective effect of Wesson oil for those systems is probably due to the presence of antioxidants in this commercial preparation. In conclusion, the effectiveness of antioxidant pretreatment against CCl_4-mediated lipid peroxidation of rat hepatocytes as compared to liver cells isolated from untreated rats can be represented as N,N'-diphenyl-p-phenylenediamine > ethoxyquin > d-α-tocopherol > Wesson oil. The formation of malondialdehyde by cells isolated from d-α-tocopherol-deficient rats treated with CCl_4 appears to be similar to that described for hepatocytes isolated from untreated rats incubated with CCl_4. The data is not presented because

this study is still in a preliminary stage of development.

A complete time course study of CCl_4-mediated lipid peroxidation of rat liver cells isolated from animals pretreated with ethoxyquin is given in Figure 18. This graph illustrates that the lipids of these cells are more resistant to lipid peroxidation than are liver cells isolated from untreated, normal animals incubated under the same conditions (Figure 12). The hepatocytes isolated from ethoxyquin-pretreated animals, when incubated with NADPH, ADP-Fe^{3+}, and CCl_4, appear to reach a maximal peroxidative lipid degradation by 15 minutes of incubation after which there follows a sharp decline in the production of malondialdehyde after 5 more minutes of incubation.

The replacement of NADPH by either an NADPH generating system (GS) or NADH on CCl_4-mediated lipid peroxidation of isolated liver cells is given in Table 14. The relationship of the relative stimulatory effect on CCl_4-related lipid peroxidation by the nucleotides is as follows:

GS > NADPH > NADH.

The procedure for direct addition of an emulsion of CCl_4 to the aqueous suspension of hepatocytes required consideration of several reasons, 1) the limited solubility of CCl_4 in aqueous buffer media, 2) the possible limited exposure of the hepatocytes to CCl_4, and 3) the solubilizing effect of CCl_4 on membrane lipids of the cells which may come into contact with the CCl_4 phase during the incubation period. To provide a more uniform exposure of the cells to CCl_4, the latter was diffused in a closed system via the gaseous phase into the incubation medium containing the hepatocytes. The results of this study are given in Table 15. The addition of 10 µl of CCl_4 to the sidearm of Warburg flasks containing 2.0 ml of

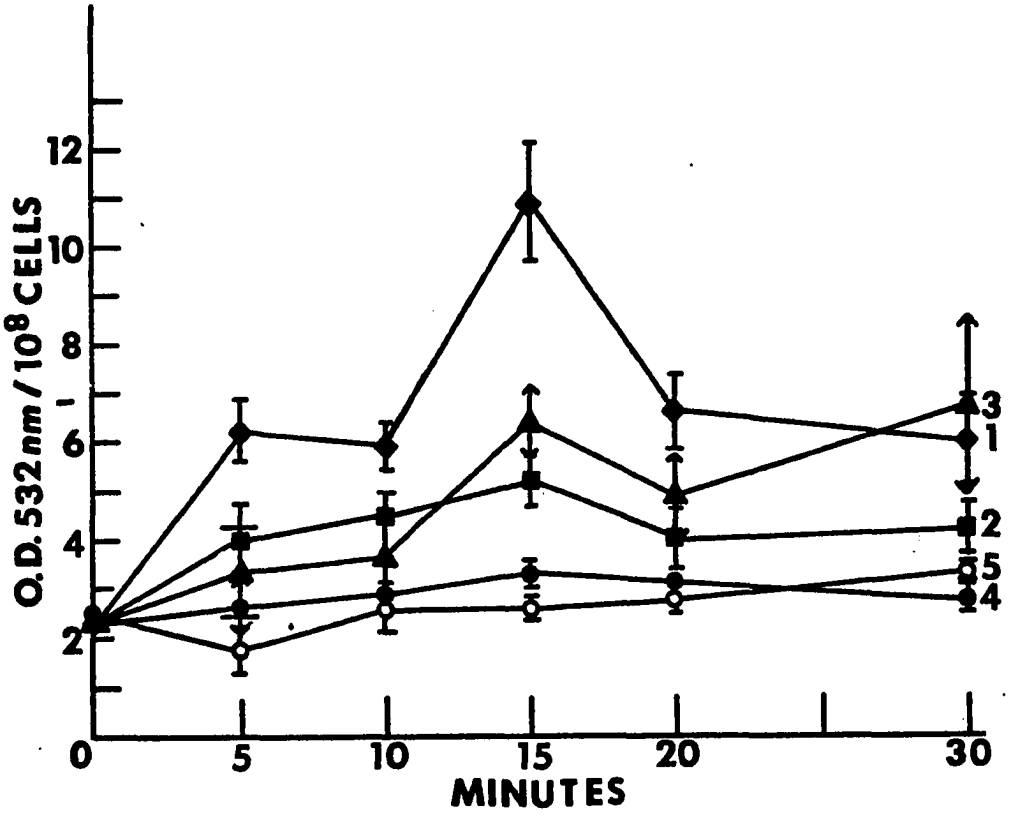


Figure 18. A time course study of malondialdehyde production by liver cells isolated from ethoxyquin-treated rats incubated with CCl_4 and NADPH and/or ADP-Fe^{3+}. The rats were pretreated with ethoxyquin as described in "Methods" prior to the isolation of the liver cells. The incubation procedure is also described in "Methods". A description of the "zero" time point is given in Figure 9.

1. cells + CCl_4 + NADPH + ADP-Fe^{3+}
2. cells + CCl_4 + NADPH
3. cells + CCl_4 + ADP-Fe^{3+}
4. cells + CCl_4
5. cells alone

TABLE 14

MALONDIALDEHYDE PRODUCTION BY ISOLATED CONTROL RAT LIVER CELLS INCUBATED WITH CCl_4 AND AN NADPH GENERATING SYSTEM, NADPH, OR NADH

Additions to Cell Suspension	Malondialdehyde Formation	
	Δ O.D.$_{532\ nm}$/10^8 cells/30 min[a]	
	Experiment 1	Experiment 2
NADPH Generating System	15.5	15.7
NADPH Generating System + CCl_4	40.1	28.5
NADPH	3.1	5.5
NADPH + CCl_4	16.0	18.6
NADH	1.9	0.1
NADH + CCl_4	12.5	9.5

The incubation conditions are described in "Methods".

[a] The values of O.D.$_{532\ nm}$/10^8 cells of cells incubated alone for 30 minutes is subtracted from the values obtained from cells incubated with either nucleotides or nucleotides and CCl_4 for each experiment.

TABLE 15

MALONDIALDEHYDE PRODUCTION BY ISOLATED RAT LIVER CELLS EXPOSED TO A GAS PHASE CONTAINING DIFFERENT AMOUNTS OF CCl_4 OR DICHLOROMETHANE

Additions to Cell Suspension	Malondialdehyde Formation With Different Amounts of Halomethane			
	$\underline{O.D._{532\ nm}/10^8\ cells/30\ min}$[a]			
	Volume of CCl_4 (µl) Added to Side Arm			
	None (4)[a]		5	10	15
None	14.5 ± 2.6	#1	9.0	23.3	18.2
		#2	-	-	12.7
NADPH	19.4 ± 3.4	#1	15.9	48.7	44.5
		#2	-	-	30.3
NADPH + ADP-Fe^{3+}	37.7 ± 6.0	#1	34.7	92.9	88.9
		#2	-	-	61.6
ADP-Fe^{3+}	17.0 ± 1.8	#1	-	-	25.6
		#2	-	-	-
	$\underline{O.D._{532\ nm}/10^8\ cells/30\ min}$[a]			
	Volume of CH_2Cl_2 (µl) Added to Side Arm			
	None (4)[a]		5	10	15
None	14.5 ± 2.6		10.1	18.8	8.3
NADPH	19.4 ± 3.4		13.0	30.3	14.9
NADPH + ADP-Fe^{3+}	37.7 ± 6.0		30.9	-	30.7
ADP-Fe^{3+}	17.0 ± 1.8		-	-	-

[a] The values are calculated from an average ± S.E.M. of 4 cell preparations from which the other data was obtained.

Liver cells were pre-incubated 5 min in Warburg flasks attached to a Gilson Respirometer. Then either CCl_4 or CH_2Cl_2 was added to one side arm and NADPH and/or ADP-Fe^{3+} was added to the other. After 15 min of incubation, NADPH and/or ADP-Fe^{3+} was transferred to the cell suspension. The cells were incubated 30 min, and 1.0 ml was removed for TBA analysis.

buffer and 10^8 hepatocytes stimulated maximum lipid peroxidation as compared to those flasks with side arms containing 5 µl of CCl_4. The stimulation of lipid peroxidation by the addition of 10 µl of CCl_4 to the side-arms is also significantly greater than that produced by corresponding cell systems incubated without CCl_4. Dichloromethane does not stimulate lipid peroxidation of hepatocytes above control values at any concentrations investigated including those concentrations used in the CCl_4 studies. Because the diffusion of CCl_4 into the 2.0 ml of suspended hepatocytes stimulated lipid peroxidation approximately 2 fold greater than that stimulated by CCl_4 added directly to cell suspensions (Table 11), the diffusion method for CCl_4 dispersion was used in subsequent experiments.

It has been assumed that the thiobarbituric acid-reacting chromagen produced after the addition of CCl_4 to the cell systems was malondialdehyde. However, as previously discussed in the studies dealing with the NADPH-dependent enzyme system of rat hepatocytes, it was necessary to demonstrate that the thiobarbituric acid-reacting substance was associated with the loss of polyunsaturated fatty acids from the membrane lipids. The loss of polyunsaturated fatty acids from lipids from one or more subcellular membrane fractions would indicate that the thiobarbituric acid-reacting species is malondialdehyde. For the reasons mentioned in the NADPH-dependent studies of membrane fatty acid content, the liver cells used for this study were obtained from rats pretreated with phenobarbital. Table 16 shows the effect of micro-diffused CCl_4 on lipid peroxidation of hepatocytes isolated from phenobarbital pretreated rats. Carbon tetrachloride also stimulated lipid peroxidation of hepatocytes from phenobarbital treated rats, a phenomenon which is augmented by

TABLE 16

MALONDIALDEHYDE PRODUCTION BY ISOLATED LIVER CELLS FROM PHENOBARBITAL-TREATED RATS

Additions to Cell Suspension	Malondialdehyde Formation
	$\underline{O.D._{532\ nm}/10^8\ cells/30\ min}$[a]
None[b]	3.3 ± 0.2
None	16.3 ± 0.2
NADPH	16.9 ± 1.1
CCl_4[c]	30.5 ± 2.0
NADPH + CCl_4[c]	42.0 ± 2.0

The incubation conditions are described in "Methods".

[a] The values are averages ± S.E.M. for 4 cell preparations.

[b] These cells were incubated at 0°C.

[c] CCl_4 was added to the center wells of modified Warburg flasks.

the addition of NADPH. After the incubation, the liver cells were centrifuged, resuspended in fresh buffer, homogenized, and fractionated into three parts; 1) microsomes, 2) mitochondria, and 3) a fraction composed primarily of nuclei and plasma membranes. The total lipids were extracted from these cellular subfractions and the fatty acid compositions of these extracts were determined by gas-liquid chromatography.

Table 17 shows the fatty acid content of microsomal lipid extracts after the incubation of hepatocytes isolated from phenobarbital pretreated rats either in the presence or absence of CCl_4. These hepatocytes were observed to lose 40-50 percent of each type of fatty acids present in the microsomal membrane when incubated in systems containing CCl_4 in the gas phase. Inclusion of NADPH in these CCl_4-containing systems resulted in no additional loss of fatty acids over that obtained by incubating hepatocytes with CCl_4 alone. The fatty acid content of mitochondria, however, was totally unaffected by any addition to any of the various systems to which the hepatocytes were exposed in these studies is shown in Table 18. Table [19] gives the fatty acid content of the total lipid extract obtained from the nuclei-plasma membrane fraction isolated from hepatocytes isolated from phenobarbital pretreated rats incubated under the conditions specified. The only system that produced a loss of fatty acids was that containing liver cells and CCl_4. The loss consisted of a decrease in arachidonic acid and docosahexenoic acid, but no other fatty acids were diminished. In summation, the effects of either CCl_4 or NADPH and CCl_4 on the fatty acid content of membrane lipids of subcellular membrane fractions isolated after incubation of hepatocytes are 1) 40-50 percent of all fatty acids is lost from the microsomal fatty acids

TABLE 17

FATTY ACID CONTENT OF MICROSOMAL LIPIDS FROM INCUBATED LIVER CELLS ISOLATED FROM PHENOBARBITAL-TREATED RATS

Additions	Fatty Acid Content						Protein Content
	16:0	18:0	18:1	18:2	20:4	22:6	
	mg/10^8 cells[a]						mg/10^8 cells
None-0°	0.67	1.07	0.33	0.60	1.11	0.19	11.58
None-37°	0.69	1.11	0.34	0.60	1.08	0.20	11.72
NADPH	0.77	1.24	0.38	0.70	1.24	0.22	12.74
CCl_4	0.44[b]	0.71	0.25	0.39	0.58	0.12	5.48
NADPH + CCl_4	0.40	0.59	0.22	0.33	0.51	0.10	5.26

The incubation conditions are described in "Methods".

[a] Average value for cells from 4 different rats.

[b] Underlined values are significantly lower than those for cells incubated at 37° without additions, $p < 0.05$.

TABLE 18

FATTY ACID CONTENT OF MITOCHONDRIAL LIPIDS FROM INCUBATED LIVER CELLS ISOLATED FROM PHENOBARBITAL-TREATED RATS

Additions	Fatty Acid Content						Protein Content
	16:0	18:0	18:1	18:2	20:4	22:6	
	mg/10^8 cells[a]						mg/10^8 cells
None-0°	1.01	1.53	0.49	0.97	1.53	0.31	16.90
None-37°	0.99	1.49	0.54	1.09	1.57	0.30	15.85
NADPH	1.06	1.55	0.60	1.19	1.64	0.32	15.39
CCl_4	1.35[b]	2.02	0.80	1.33	1.81	0.38	15.68
NADPH + CCl_4	1.16	1.74	0.67	1.28	1.73	0.32	14.13

The incubation conditions are described in "Methods".

[a] Average value for cells from 4 different rats.

[b] Underlined values are significantly higher than those for cells incubated at 37° without additions, $p < 0.05$.

TABLE 19

FATTY ACID CONTENT OF NUCLEI AND PLASMA MEMBRANES FROM INCUBATED LIVER CELLS ISOLATED FROM PHENOBARBITAL-TREATED RATS

Additions	Fatty Acid Content						Protein Content
	16:0	18:0	18:1	18:2	20:4	22:6	
None-0°	2.65	3.31	1.70	2.89	2.99	0.58	54.05
None-37°	2.42	3.11	1.53	2.91	2.78	0.57	48.15
NADPH	2.55	3.22	1.66	3.12	2.85	0.55	49.17
CCl_4	2.32	2.63	1.64	2.61	1.81[b]	0.37	36.78
NADPH + CCl_4	2.66	3.20	1.82	3.01	2.28	0.46	35.69

The incubation conditions are described in "Methods".

[a] Average value for cells from 4 different rats.

[b] Underlined values are significantly lower than those for cells incubated at 37° without additions, $p < 0.05$

when liver cells are incubated with either CCl_4 or NADPH and CCl_4, 2) the mitochondrial fatty acid content is unaffected when cells are incubated with CCl_4 or NADPH and CCl_4, and 3) CCl_4 alone stimulates the loss of arachidonic acid and docosahexenoic acid from the nuclei-plasma membrane fraction of hepatocytes incubated with this halomethane.

Dichloromethane was incubated in a similar manner with hepatocytes isolated from phenobarbital pretreated rats as previously described for CCl_4 in the fatty acid analysis studies in order to determine whether a similar lipid solvent would be able to produce fatty acid losses analogous to those previously described for hepatocytes incubated with either CCl_4 and/or NADPH and CCl_4. The lipid peroxidation data of hepatocytes incubated with either CCl_4 or dichloromethane is presented in Table 20. Dichloromethane does not significantly stimulate lipid peroxidation of hepatocytes above control values. Fatty acids of membrane lipids were analyzed after the cells were incubated with dichloromethane. Because the data is inadequate to provide clear-cut evidence for the effect of either dichloromethane or NADPH and dichloromethane on fatty acids of subcellular organelles from these cells, only a brief description of the fatty acid data will be presented. Dichloromethane decreased the fatty acid content of microsomal lipids about 40-50 percent as compared to cells incubated without dichloromethane. This is a result similar to that demonstrated for the effect of CCl_4 on microsomal fatty acids. The addition of NADPH to cells incubated with dichloromethane produced a protective effect of the microsomal fatty acids, i.e., there was no loss of any fatty acids as compared to cells incubated either alone or with NADPH alone. The effect of dichloromethane on the fatty acids

TABLE 20

MALONDIALDEHYDE PRODUCTION BY ISOLATED LIVER CELLS FROM PHENOBARBITAL-TREATED RATS INCUBATED WITH EITHER CCl_4 OR DICHLOROMETHANE BY MICRODIFFUSION

Other Additions to Cell Suspension	Malondialdehyde Formation		
	$\underline{O.D._{532\ nm}/10^8\ cells/30\ min}$		
	Halomethane Added		
	None (4)[a]	CCl_4 (4)[a]	CH_2Cl_2
None	16.3 ± 0.2	30.5 ± 2.0	#1 21.1 #2 14.7
NADPH	16.9 ± 1.1	42.0 ± 2.0	#1 23.4 #2 15.4

Liver cells incubated at 0°C produced an $O.D._{532\ nm}/10^8$ cells/30 min equal to 3.3 ± 0.2.

The incubation conditions are described in "Methods".

[a] These values are calculated from an average ± S.E.M. of 4 cell preparations.

of the mitochondrial lipids is uncertain at this time. The nuclei-plasma membrane fraction fatty acids appear to be unaffected by exposure of the liver cells to either dichloromethane or NADPH and dichloromethane.

A preliminary study was carried out on the protein content of the supernatant fractions which were collected after centrifuging down the incubated intact liver cells. Cells incubated with either NADPH and CCl_4 or CCl_4 alone released two times the amount of protein to the incubation medium as compared to the amount released either by cells incubated alone or by cells incubated with NADPH. Dichloromethane did not stimulate the release of protein from hepatocytes.

The role of cytochrome P-450 in the metabolism of CCl_4 and in lipid peroxidation produced by CCl_4 is uncertain. Therefore, we investigated the possible role of cytochrome P-450 in CCl_4-mediated lipid peroxidation by examining the effect of lowering the cytochrome P-450 content of rat liver prior to cell isolation and subsequent incubation of these hepatocytes with CCl_4. Allylisopropylacetamide (AIA) was injected into rats that had been pretreated with phenobarbital, a procedure mentioned previously. This treatment was shown to depress the cytochrome P-450 content of hepatocytes isolated from rats pretreated with phenobarbital 60 percent (Table 9). Figure 19 illustrates 5 time course studies of CCl_4-mediated lipid peroxidation of hepatocytes isolated from rats pretreated with phenobarbital and allylisopropylacetamide. These values are the average of 5 separate studies. Table 21 provides a comparison of the data calculated from the time course studies of CCl_4-mediated lipid peroxidation of hepatocytes isolated from phenobarbital treated rats (Table 10) and cells isolated from rats treated with phenobarbital and

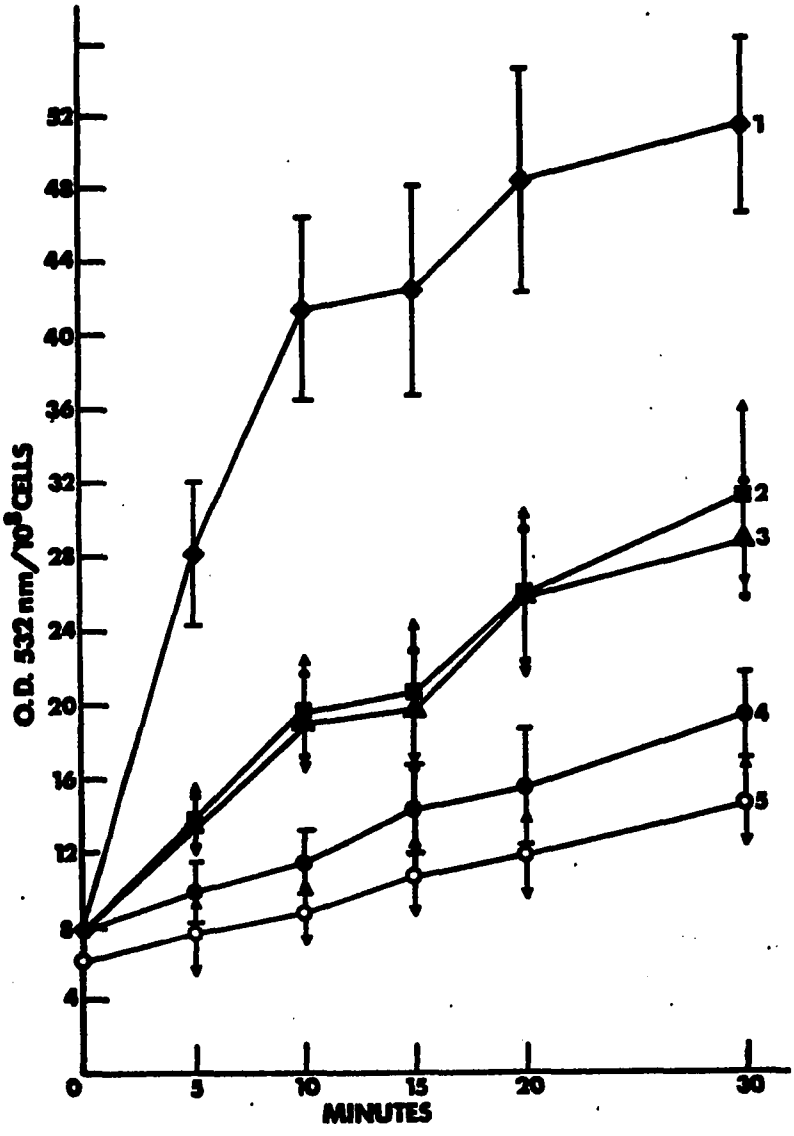


Figure 19. A time course study of malondialdehyde production by liver cells isolated from rats pretreated with phenobarbital and allylisopropylacetamide incubated with CCl_4 and NADPH and/or ADP-Fe^{3+}. The rats were pretreated with phenobarbital and allylisopropylacetamide as described in "Methods" prior to the isolation of the liver cells. The incubation procedure is described in "Methods". A description of the "zero" time point is given in Figure 9.

1. cells + CCl_4 + NADPH + ADP-Fe^{3+}
2. cells + CCl_4 + NADPH
3. cells + CCl_4 + ADP-Fe^{3+}
4. cells + CCl_4
5. cells alone

TABLE 21

MALONDIALDEHYDE PRODUCTION BY ISOLATED LIVER CELLS FROM RATS PRETREATED WITH EITHER PHENOBARBITAL OR PHENOBARBITAL AND ALLYLISOPROPYLACETAMIDE

Animal Pretreatment	Additions to Cell Suspension	Malondialdehyde Formation	
		$\Delta O.D._{532\ nm}/10^8$ cells[a]	
		5 min	30 min
Phenobarbital (6)[b] (Cytochrome P-450 content = 0.05 $\Delta O.D._{450\ nm}/10^8$cells/ml)	CCl_4	4.3 ± 1.1	10.7 ± 1.1
	NADPH + CCl_4	11.2 ± 1.1	27.5 ± 2.3
	ADP-Fe^{3+} + CCl_4	3.5 ± 0.9	14.2 ± 2.8
	NADPH, ADP-Fe^{3+}, + CCl_4	17.7 ± 4.1	42.9 ± 3.7
Phenobarbital and (5) Allylisopropylacetamide (Cytochrome P-450 content = 0.02 $\Delta O.D._{450\ nm}/10^8$cells/ml)	CCl_4	2.1 ± 0.6	4.7 ± 1.3
	NADPH + CCl_4	4.0 ± 0.9	11.9 ± 3.2
	ADP-Fe^{3+} + CCl_4	3.5 ± 0.9	9.4 ± 2.6
	NADPH, ADP-Fe^{3+}, + CCl_4	18.4 ± 2.3	32.0 ± 3.9

The incubation conditions are described in "Methods".

[a] The values are calculated as described in Table 11. These values represent an average ± S.E.M. of 5 or 6 cell preparations.

[b] The numbers in parentheses denote the number of cell preparations used for these experiments.

allylisopropylacetamide (Figure 19). After 5 minutes of incubation, the hepatocytes isolated from rats pretreated with phenobarbital and allylisopropylacetamide and incubated with either CCl_4 or NADPH and CCl_4 produced only 50 and 36 percent of the malondialdehyde respectively than hepatocytes isolated from rats pretreated with phenobarbital alone. The same effect is also produced by liver cells isolated from rats pretreated with phenobarbital and allylisopropylacetamide after 30 minutes of incubation with either CCl_4 or NADPH and CCl_4. Pretreatment of rats with phenobarbital and allylisopropylacetamide has no inhibitory effect on lipid peroxidation when either ADP-Fe^{3+} and CCl_4 or NADPH, ADP-Fe^{3+}, and CCl_4 are added to liver cells isolated from those animals. It appears that the reduction of the hepatic content of cytochrome P-450 significantly depresses the amount of CCl_4-mediated lipid peroxidation of isolated hepatocytes after 5 minutes of incubation with either CCl_4 or NADPH and CCl_4.

CHAPTER IV

DISCUSSION

Lipid peroxidation of different subcellular organelles of rat liver *in vitro* is known to be stimulated by the additions of NADPH and ADP-Fe^{3+} to incubation systems containing either microsomes, mitochondria, or lysosomes. The involvement of an NADPH oxidase system in those studies has been studied in detail by McCay *et al.* (10-17). Until recently, lipid peroxidation mediated by an NADPH oxidase system had not been demonstrated to function *in vivo*, possibly because malondialdehyde, the product most used to estimate lipid peroxidation, was metabolized either by liver mitochondria or by unknown processes during preparation of the tissue for assay. Högberg *et al.* showed that isolated liver cells can undergo lipid peroxidation in the presence of ADP-Fe^{3+} (102). Our study of lipid peroxidation of freshly isolated rat liver parenchymal cells is the first successful attempt to detect the effects of lipid peroxidation on membranes of intact, functional liver cells.

Liver cells were isolated from rats that were fed a commercial ration *ad libitum* and fasted 24 hours prior to liver perfusion. Some of these rats were pretreated with phenobarbital, a compound known to stimulate the synthesis of smooth endoplasmic reticulum, increase the content of the cytochrome P-450, and increase the activity of the drug

metabolizing system of rat liver. When control cells were incubated in suspension buffer at 37°C in a water bath for 30 minutes, they produced malondialdehyde at a slow, but constant rate. Hepatocytes isolated from rats pretreated with phenobarbital also produced malondialdehyde, but at a greater rate and magnitude than that of normal rat hepatocytes incubated under similar conditions (Table 2). This indicates that liver cells are capable of undergoing lipid peroxidation, a process which may be related to a normal cellular process involved in the turnover of membrane lipids. This in turn may be involved in a cellular aging process. The stimulating effect of phenobarbital pretreatment on lipid peroxidation of hepatocytes is probably due to the increased content of membrane-bound lipid as a result of the proliferation of smooth endoplasmic reticulum.

The addition of NADPH to liver cells had little effect on lipid peroxidation of either normal or phenobarbital-treated hepatocytes. The lack of an effect of NADPH is probably due to its inability to penetrate the plasma membrane and initiate endogenous lipid peroxidation. This is postulated due to the effects of either NADPH and ADP-Fe^{3+} or ADP-Fe^{3+} alone on the cellular production of malondialdehyde. As demonstrated by the loss of polyunsaturated fatty acids of the nuclei-plasma membrane fraction of cells incubated with NADPH and ADP-Fe^{3+}, this type of lipid peroxidation most likely occurs at the plasma membrane, and the requirement for exogenous NADPH is evidently due to the presence of an NADPH-cytochrome c reductase enzyme in plasma membranes of rat liver (103). The addition of ADP-Fe^{3+} to liver cells isolated from phenobarbital-treated rats significantly stimulated the production of malondialdehyde

as compared to cells incubated with NADPH alone. Because phenobarbital is known to stimulate the synthesis of rat liver endoplasmic reticulum, the effect of ADP-Fe^{3+} on the production of malondialdehyde in these cells probably occurs at the endoplasmic reticulum in the presence of endogenous NADPH. Although it was not possible to analyze the fatty acids of hepatocytes incubated with ADP-Fe^{3+} alone, a recent publication by Hogberg supports my hypothesis that ADP-Fe^{3+}-mediated lipid peroxidation is an intracellular process. Högberg demonstrated that ADP-Fe^{3+} stimulates endogenous peroxidation of lipids of isolated rat hepatocytes. In addition, phenobarbital-pretreatment of rats causes an increased production of malondialdehyde during 30 minutes of incubation with either NADPH and ADP-Fe^{3+} or ADP-Fe^{3+} alone as compared to that of normal hepatocytes (Table 12). Because I showed that phenobarbital pretreatment of rats produces hypertrophy of liver cells, the stimulatory effect of phenobarbital on either NADPH-ADP-Fe^{3+} or ADP-Fe^{3+}-induced lipid peroxidation is most likely a result of an increase of the surface area of the plasma membrane, which in turn increases the number of potential reaction sites of lipid peroxidation.

Liver cells isolated from phenobarbital-treated rats produced different magnitudes of malondialdehyde when they were incubated in either pure oxygen or room air. For example, hepatocytes incubated for 30 minutes in the experiment designed for the analysis of membrane fatty acids produced significantly larger amounts of malondialdehyde (Table 3) than did hepatocytes incubated with similar additions to test tubes (Figure 10). The liver cells cited in the former experiment were gassed for 10 seconds with pure oxygen prior to the addition of NADPH while

while liver cells in the latter experiment were incubated in room air. The addition of pure oxygen to the modified Warburg flasks produced greater amounts of malondialdehyde probably because more oxygen was available for the peroxidation of membrane lipids. The same reasoning can also be used to explain the differences in the amount of malondialdehyde produced by liver cells incubated with CCl_4 (Table 16 and Figure 15).

The fact that NADPH alone or NADPH + ADP-Fe^{3+} caused negligible loss of fatty acids from the lipids of either the endoplasmic reticula or mitochondria of rat hepatocytes can be explained in a similar manner as previously discussed for their effects on the nuclei-plasma membrane fraction. If the nucleotides are unable to penetrate the plasma membrane barrier, the peroxidation of polyunsaturated fatty acids of these fractions would not be expected.

Several antioxidants were administered either intraperitoneally or orally to rats in an attempt to protect the hepatocytes from lipid peroxidation stimulated *in vitro*. As mentioned in Chapter II,administration of N,N'-diphenyl-p-phenylenediamine, d-α-tocopherol, or ethoxyquin to the rat caused significant inhibition in the formation of malondialdehyde as compared to liver cells of untreated rats. N,N'-diphenyl-p-phenylenediamine was the most effective of the antioxidants tested, a result that could be due to its 1) decreased clearance by the liver and 2) higher affinity for sites in cellular membranes where the potential for peroxidation of polyunsaturated fatty acids is marked. Cawthorne *et al.* showed that orally administered ethoxyquin was rapidly metabolized by female rat livers, while intraperitoneal injections of N,N'-diphenyl-p-phenylene-

diamine and oral administrations of d-α-tocopherol were not substantially metabolized by the livers of female rats (65). The metabolism of ethoxyquin might be different if it is administered intraperitoneally. From Cawthorne's study d-α-tocopherol was not as effective as ethoxyquin in preventing CCl_4-induced liver necrosis, suggesting that the site of localization of these antioxidants in the cellular membranes may have an important influence in determining whether or not lipid peroxidation of membrane-bound polyunsaturated fatty acids will occur under different conditions.

The involvement of cytochrome P-450 on NADPH + ADP-Fe^{3+}-mediated lipid peroxidation of hepatocytes was shown to be negligible. Pretreatment of rats with phenobarbital and allylisopropylacetamide produced a 60% decrease in the hepatocellular content of cytochrome P-450, but lipid peroxidation stimulated by NADPH and/or ADP-Fe^{3+} was unaffected as compared to that of hepatocytes from animals treated with phenobarbital only. This suggests that NADPH-cytochrome c reductase is probably involved in NADPH-ADP-Fe^{3+}-mediated lipid peroxidation. This is supported by the presence of an NADPH-cytochrome c reductase enzyme in plasma membranes (103) and by the studies of Levin et al. which indicated that microsomal cytochrome P-450 is probably not involved in microsomal lipid peroxidation. Therefore, cytochrome P-450 would not necessarily be an ingredient of the peroxidative mechanism of the isolated whole cells, especially because NADPH-ADP-Fe^{3+}-mediated lipid peroxidation of hepatocytes appears to occur at the plasma membranes.

The electron micrographs of hepatocytes isolated from either untreated or phenobarbital-treated rats indicate that incubation per se

has essentially no morphological effect on the cells. Additions of NADPH and/or ADP-Fe^{3+} also have little discernable effect on the morphology of the hepatocytes (micrographs not included). Liver cells incubated with NADPH and ADP-Fe^{3+} have been shown to produce substantial amounts of malondialdehyde, although there is no evidence for structural failure in the plasma membranes as one might expect from the fatty acid analyses of this membrane (micrographs not shown). This could be due to 1) the sensitivity of the thiobarbituric acid analysis, 2) the rapid repair of the lipid components of the plasma membrane after lipid peroxidation, 3) lack of substantial damage to both inner and outer layers of the plasma membrane that would produce substantial extrusion of cytoplasm, and 4) the limitations of electron microscopy to detect the structural aberrations of the plasma membrane.

Carbon tetrachloride was also found to stimulate lipid peroxidation of isolated rat liver cells (Table 11). Figure 13 shows that liver cells isolated from either control or phenobarbital-treated rats produce increasing amounts of malondialdehyde with increasing concentrations of CCl_4 until maximum production of malondialdehyde is reached. Because phenobarbital is known to increase the hepatotoxic effects of CCl_4 administered in vivo, it was expected that phenobarbital pretreatment of rats would make their isolated hepatocytes more sensitive to lower concentrations of CCl_4 than that exhibited by hepatocytes of untreated rats. However, Figure 13 shows that pretreatment of animals with phenobarbital caused no significant change in the half-maximal response of hepatocytes to CCl_4 as compared to hepatocytes from control animals. The maximal response of cells in the phenobarbital graph is substantially larger for

all incubation systems except possibly the one containing NADPH and ADP-Fe^{3+}. A possible explanation for the lack of phenobarbital sensitization of the cells to CCl_4 and the presence of a marked increase in the maximal response is that phenobarbital causes proportionate increases of cytochrome P-450 and membrane-bound polyunsaturated lipids that are available for peroxidation. Assuming that cytochrome P-450 is involved in the metabolism of CCl_4, the increase of both parameters could account for the increase in the maximal response with the cellular sensitization remaining unchanged during CCl_4-stimulated lipid peroxidation. Tables 8 and 11 show that lipid peroxidation is stimulated by either NADPH and ADP-Fe^{3+} or CCl_4. This suggests that the stimulation of lipid peroxidation by the production of radicals by either the NADPH-ADP-Fe^{3+} system or the CCl_4 occurs simultaneously in the cell,but the location of the peroxidative activity in the liver cell also suggests that the two systems are situated at different subcellular sites.

Phenobarbital pretreatment of rats produced an increased lipid peroxidation response by hepatocytes incubated with CCl_4 and NADPH and/or ADP-Fe^{3+} as compared to hepatocytes of control rats (Table 11). The stimulatory effect of phenobarbital on CCl_4-induced lipid peroxidation is most likely due to 1) an increased content of microsomal cytochrome P-450, 2) an increased content of membrane-bound polyunsaturated lipids of the endoplasmic reticulum, 3) an increased surface area of the plasma membrane, or 4) any combination of these three factors. These first two possibilities involve the loss of fatty acids from the membrane lipids of the endoplasmic reticulum promoted by either CCl_4 or NADPH and CCl_4. The third possibility involves the loss of polyunsaturated fatty acids from the nuclei-plasma membrane fraction promoted by CCl_4 alone. The possible

involvement of increased cytochrome P-450 content on the stimulatory effects of CCl_4-mediated lipid peroxidation and the losses of membrane fatty acids will be discussed in more detail later.

Dichloromethane is known not to be hepatotoxic; and therefore, any effect it would have on the production of malondialdehyde of liver cells could probably be attributed to its lipid solvent property. Therefore, the effects of CH_2Cl_2 on lipid peroxidation of untreated hepatocytes is most likely due to its solvent quality (Table 12). It is quite apparent that CCl_4 and NADPH and/or ADP-Fe^{3+} stimulate larger quantities of malondialdehyde than similar incubation systems containing CH_2Cl_2 presumably due to the metabolism of CCl_4 rather than its solvent action on membrane-bound lipids. It is believed that CCl_4 is homolytically cleaved to $\cdot CCl_3$ and $\cdot Cl$, the former radical being the initiator of lipid peroxidation.

An indirect approach was used to determine if CCl_4 was actually being metabolized as previously described. Different antioxidants were administered to rats prior to the isolation of the hepatocytes in an attempt to inhibit CCl_4-mediated lipid peroxidation. The antioxidants used for the study are well-known radical scavenging agents. The results indicate that N,N'-diphenyl-p-phenylenediamine and ethoxyquin protect hepatocytes against CCl_4-stimulated lipid peroxidation better than d-α-tocopherol. The relative effectiveness of these antioxidants against CCl_4-mediated lipid peroxidation can be explained partially by the relative rates of their metabolism by the liver as previously discussed for the NADPH-oxidase enzyme system. The distribution and precise location of the different antioxidants in the different subcellular membranes may play an important role in their relative protective effects on lipid

peroxidation. This would suggest that N,N'-diphenyl-p-phenylenediamine is more closely associated with membrane sites which are susceptible to CCl_4-promoted lipid peroxidation than are the other antioxidants studied. Another possibility is that there may be more of the unbound antioxidant available in the cell for binding $\cdot CCl_3$ thus making it unavailable for attack on unsaturated lipids, i.e., N,N'-diphenyl-p-phenylenediamine is more universally distributed in hepatocytes than either ethoxyquin or d-α-tocopherol. D-α-tocopherol only inhibits lipid peroxidation of hepatocytes stimulated by CCl_4 alone. As previously discussed, the primary attack on membrane lipids by the NADPH, ADP-Fe^{3+} enzyme system is at the nuclei-plasma membrane level. Thus, any of the nucleotides that have been shown to peroxidize the lipids of isolated cells in the nuclei-plasma membrane fraction would still peroxidize those lipids if the antioxidants investigated were not incorporated into that membrane fraction. I believe that d-α-tocopherol is not incorporated in sufficient amounts in those membrane fractions isolated to inhibit lipid peroxidation promoted by either ADP-Fe^{3+} and CCl_4 or NADPH, ADP-Fe^{3+}, and CCl_4 under the conditions studied. This is conceivable for the ADP-Fe^{3+} system if it is assumed that endogenous NADPH is utilized in conjunction with exogenous ADP-Fe^{3+} to promote the peroxidative process. On the otherhand, d-α-tocopherol is known to be incorporated into the endoplasmic reticulum and this correlates with the inhibition of malondialdehyde production by hepatocytes incubated with CCl_4 alone (Table 13). Without d-α-tocopherol pretreatment of rats, there is a substantial loss of total fatty acids from the lipids of the endoplasmic reticulum of these hepatocytes (Table 17), i.e., lipid peroxidation occurs at a rate exceeding homeostatic

mechanisms. Unfortunately, this does not explain the fact that while NADPH and CCl_4 promote the same fatty acid losses from the endoplasmic reticulum, d-α-tocopherol does not inhibit the production of malondialdehyde by the liver cells. The CCl_4 may cause sufficient peroxidation in the plasma membrane prior to the addition of NADPH, so that the exogenous NADPH is now allowed direct access to the cytoplasm and, in conjunction with the CCl_4, overwhelms the limited protective effect of the microsomal membrane-bound d-α-tocopherol. The same reasoning can be applied to the fact that d-α-tocopherol did not inhibit lipid peroxidation stimulated by NADPH, ADP-Fe^{3+}, and CCl_4. In this instance CCl_4, as already suggested, may cause perturbations of the plasma membrane components allowing for the passage of exogenous NADPH into the cytoplasm. Then the NADPH + ADP-Fe^{3+} and the NADPH + CCl_4 radical-producing systems could operate to the extent that the limited amount of d-α-tocopherol was quickly consumed. After the content d-α-tocopherol diminishes, the peroxidation of lipids approaches the maximum rate, similar to that found in hepatocytes of rats not supplemented with tocopherol.

When hepatocytes were incubated with CCl_4 which was diffused into the reaction system, the amount of malondialdehyde was doubled as compared to that of liver cells incubated alone (Table 16). In conjunction with the increased production of malondialdehyde, there was a significant loss of protein and of all fatty acids from the endoplasmic reticulum and in addition, there was a decrease in the polyunsaturated fatty acids of the nuclei-plasma membrane fraction. An unexpected result of exposing hepatocytes to CCl_4 was a significant increase in the short-chained saturated and unsaturated fatty acids of the mitochondria, with no significant loss

of protein from these particles. This suggests that CCl_4 is possibly metabolized both at the plasma membrane, (possibly the nuclear membrane also), as well as at the endoplasmic reticulum. This is evident from 1) the susceptibility of these membranes to CCl_4-mediated lipid peroxidation and 2) the presence of NADPH-cytochrome c reductase in the plasma membranes of rat liver which can be activated by the administration of CCl_4 *in vivo* (103). The magnitude of the fatty acid loss from the plasma membrane-nuclei fraction indicates the loss occurs in the plasma membrane since the nuclear membrane contributes a relatively small percent of the total lipid of this fraction (104, 105). Slater proposed that NADPH-cytochrome c reductase was probably responsible for the homolytic cleavage of CCl_4 by rat liver microsomes. Because there is an NADPH cytochrome c reductase enzyme in plasma membranes of rat liver, our results tend to support Slater's theory concerning the action of CCl_4 on the plasma membrane fatty acid content. However, the independent function of microsomal NADPH-cytochrome c reductase in the metabolism of CCl_4 is still questionable because of the results of lipid peroxidation obtained from the hepatocytes containing lowered cytochrome P-450 levels. This will be discussed in more detail later.

The observation that CCl_4 increases the content of all but the highly unsaturated fatty acids of the mitochondria suggests that some of the saturated and less saturated fatty acids lost from the microsomal membranes may have become adsorbed or incorporated into the mitochondrial fraction. The lack of structural damage to the mitochondria as indicated by the fatty acid data is compatible with morphological studies of CCl_4 on mitochondria *in vivo* (49).

The addition of NADPH to incubation systems containing CCl_4 introduced by microdiffusion also shows 1) a significant increase of malondialdehyde production, 2) a significant loss of protein from the microsomal and the nuclei-plasma membrane fractions of the cells, and 3) a significant decrease of all fatty acids from the microsomal fraction only as compared to control hepatocytes incubated at 37°C. The mitochondrial protein and fatty acid contents remain unchanged. The stimulatory effect of NADPH on CCl_4-mediated lipid peroxidation has already been discussed. It is important to note the specificity of CCl_4-mediated lipid peroxidation for microsomal fatty acids and not those of the nuclei-plasma membranes as well. The stimulating effect of NADPH on the microsome-specific lipid peroxidation caused by CCl_4 also supports the proposal that CCl_4 is metabolized by an NADPH-dependent electron transport system of microsomes. The observation that the fatty acid content of the nuclei-plasma membrane fraction remained unchanged does not necessarily mean that lipid peroxidation did not occur there. On the contrary, lipid peroxidation of the plasma membranes could have occurred, but homeostatic replacement of fatty acids lost by the peroxidative process could mask the process so that no change would be observed during the period of observation. The presence of exogenous NADPH could be involved in maintaining the homeostasis of the fatty acid complement of the plasma membrane. The loss of nuclei-plasma membrane protein, even though the nuclei-plasma membranes fatty acids remained unchanged, suggests that the fatty acid synthesizing and incorporating systems may have been functional but that the protein synthesizing system was unable to maintain the protein content of this mixed membrane fraction. (Because the lipid content of plasma membranes

is sufficiently larger than that of nuclear membranes (104, 105), any significant loss of fatty acids is probably from the plasma membranes.) The replacement of the fatty acids of these membranes probably requires a concomitant replenishment of plasma membrane proteins. Without the replenishment of the protein, it is unlikely that the additional incorporation of membrane lipid would result in a lipo-protein membrane complex stable to homogenization. Therefore, any loss of protein noted in the nuclei-plasma membrane fraction would probably be attributed to the nuclear portion of this fraction. Because NADPH and CCl_4 produced a loss of fatty acids from the microsomal fraction of the isolated liver cells, it is conceivable that the nuclear membrane of these cells could also be damaged as illustrated in Figure 17. This could cause a destabilization of the nulcear membrane that would predispose it to lysis during either the incubation period or the mechanical disruption of the cells for the subcellular fractionation procedures.

The role of cytochrome P-450 in the metabolism of CCl_4 by rat liver microsomes is currently controversial. The evidence that cytochrome P-450 is involved in the metabolism of CCl_4 out weighs the evidence that it involves only the NADPH-cytochrome c reductase pathway. When the cytochrome P-450 content of isolated rat liver parenchymal cells is decreased by 60 percent, the amount of malondialdehyde formed by hepatocytes in the presence of either CCl_4 or NADPH and CCl_4 is decreased 50-60 percent as compared to control cells (Table 21). There is a definite correlation between the loss of cytochrome P-450 and the degree of inhibition of malondialdehyde production by added CCl_4. This suggests that cytochrome P-450 is involved in CCl_4-mediated lipid peroxidation,

and that the cytochrome could be the site at which CCl_4 is homolytically cleaved. However, NADPH-cytochrome c reductase may be involved in the metabolism of CCl_4 at the plasma membrane. Fleischer et al. (106) and Kaspar (107) found little if any cytochrome P-450 in the plasma membranes of rat liver, and Kuchii et al. detected an NADPH-cytochrome c reductase enzyme in that membrane. That NADPH-cytochrome c reductase alone is responsible for CCl_4 metabolism appears unlikely in view of the results in this study with isolated liver cells from animals treated with allylisopropylacetamide and with microsomes of rats pretreated with CCl_4 in which the NADPH-cytochrome c reductase content remained unchanged but the cytochrome P-450 levels fall (101). The results of these experiments suggest that cytochrome P-450 is most likely involved in the metabolism of CCl_4 and probably participates in a rate limiting reaction which produces $\cdot CCl_3$ radicals in the endoplasmic reticulum of rat liver cells. This proposal is in contrast to that suggested for the metabolism of CCl4 by the plasma membranes of isolated hepatocytes. There I proposed that the NADPH-cytochrome c reductase enzyme was involved in the metabolism of CCl_4. There may be different mechanisms for the metabolism of CCl_4 depending on which membrane is involved in the homolytic cleavage of CCl_4.

In addition to exogenous NADPH, an NADPH-generating system as well as NADH were investigated for their effect on CCl_4-mediated production of malondialdehyde (Table 14). The results indicate that in the presence of an NADPH-generating system CCl_4-promoted lipid peroxidation was significantly increased above that which is observed by simple addition of exogenous NADPH to cells exposed to CCl4. The presence of

NADH also enhanced CCl_4-mediated lipid peroxidation, but to a lesser degree than NADPH. This suggests that the cell is probably 1) trans-hydrogenating NADH to available endogenous $NADP^+$ and/or 2) utilizing either the NADH-cytochrome b_5 reductase enzyme system of microsomes to shunt electrons over the NADPH-cytochrome P-450 enzyme system or the NADH-cytochrome c reductase enzyme system of liver plasma membrane for the homolytic cleavage of CCl_4 (103).

CHAPTER V

SUMMARY

Studies of liver cells incubated alone demonstrated that lipid peroxidation is possibly a natural phenomenon. The production of malondialdehyde by isolated rat liver cells is stimulated by CCl_4 or by NADPH plus Fe^{3+}, and the studies indicate that the two processes are not only different, but are additive when occurring at the same time. Both types of lipid peroxidation apparently involve the enzymic generation of free radicals and are enhanced by pretreatment of animals with phenobarbital before the cells are prepared. These studies suggest that phenobarbital induction produces more sites for interaction of CCl_4 with its metabolizing system rather than increasing the affinity of existing sites for the halogenated hydrocarbon. Administration of allylisopropylacetamide to rats before isolation of the cells results in a marked reduction of lipid peroxidation caused by incubation of the cells with CCl_4. The degree to which lipid peroxidation was decreased was observed to be proportional to the decrease in cytochrome P-450 content in the cells caused by the allylisopropylacetamide treatment. The results suggest that cytochrome P-450 may be involved in the metabolism of CCl_4. Allylisopropylacetamide pretreatment enhances lipid peroxidation for endogenous substrates but had little or no effect on lipid peroxidation caused by NADPH and Fe^{3+}.

Several antioxidants are known to inhibit lipid peroxidation stimulated by either CCl_4 or NADPH and ADP-Fe^{3+}. N,N'-diphenyl-p-phenylenediamine, ethoxyquin, or d-α-tocopherol administered to rats significantly depressed the production of malondialdehyde stimulated by either free radical-producing system of liver cells in vitro. N,N'-diphenyl-p-phenylenediamine was the most effective antioxidant. This is attributed to its decreased clearance by the liver and its diffuse distribution throughout the liver cell. The inhibitory effects of these antioxidants on CCl_4-mediated and NADPH-ADP-Fe^{3+}-mediated lipid peroxidation strongly suggest that isolated liver cells of rats are capable of producing the free radicals necessary for the peroxidation of lipids of liver cells. In addition, these antioxidants inhibited the production of malondialdehyde by liver cells incubated alone as compared to that of liver cells isolated from control rats. This suggests that lipid peroxidation is an on-going, free radical-mediated process in liver cells.

The fatty acid content of microsomes, mitochondria, and nuclei and plasma membranes was analyzed in order to locate the source of malondialdehyde produced during lipid peroxidation of hepatocytes. The hepatotoxin, CCl_4, causes major losses of fatty acids and protein from the endoplasmic reticulum and is accompanied by malondialdehyde formation. Loss of polyunsaturated fatty acids in the nuclear-plasma membrane fraction also occurs. On the other hand, treatment of isolated liver cells with NADPH in the presence of a low concentration of Fe^{3+} causes a large loss of polyunsaturated fatty acids only from the plasma membrane. This suggests that NADPH-ADP-Fe^{3+}-mediated lipid peroxidation occurs at the plasma membrane. This is in contrast to the known

peroxidative attack on microsomal lipids promoted by Fe^{3+} and NADPH when the latter is oxidized by microsomal NADPH oxidase. The studies confirm the hypothesis that lipid peroxidation is an on-going phenomenon in liver cells under normal conditions and appears to be in equilibrium with homeostatic mechanisms which stabilize membrane composition. Some exogenous compounds, i.e., CCl_4, overwhelm homeostatic mechanisms and produce marked changes in fatty acid content of membranes. The interaction of carbon tetrachloride with the endoplasmic reticulum of intact liver cells causes a massive augmentation of peroxidative attack on the microsomes resulting in marked alteration of the lipid composition of this membrane. The data indicates that significant peroxidative damage to the plasma membrane also occurs. Destruction of mitochondrial lipids was not observed under any of the conditions described.

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