HEPATIC MICROSOMAL LIPID PEROXIDATION

HEPATIC MICROSOMAL LIPID PEROXIDATION AND ITS EFFECTS ON DRUG METABOLISM

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ABSTRACT

During development of rats, hepatic microsomal NADPH dependent lipid peroxidation increased to reach a maximum activity at 25 days of age and then declined to adult activity by six weeks of age. Increased activity in 25 day old rats resulted in a greater decrease in the ability of microsomes to N-demethylate aminopyrine after preincubation under conditions which promoted lipid peroxidation. Although total NADPH oxidase activity was similar in 25 day old and adult rats, NADPH oxidase activity, which was specific for lipid peroxidation, was increased in 25 day old rats. Microsomal NADPH dependent lipid peroxidation reduced the magnitude of type I and type II spectral binding without affecting the qualitative aspects of the spectra or the absolute amount of cytochrome P-450. It is proposed that decreased drug oxidation following lipid peroxidation in microsomes is due to decreased binding of substrate to cytochrome P-450. NADPH dependent lipid peroxidation was demonstrated for the first time in human hepatic microsomes. Compared to the rat, human microsomes were resistant to decreases in drug oxidation which result from lipid peroxidation. Evidence is presented which supports the concept that NADPH dependent lipid peroxidation can occur *in vivo*. The iron involved in NADPH dependent lipid peroxidation reaction, participates in a bound form even when added from an exogenous source. The problem of iron contamination in studies of microsomal lipid peroxidation is discussed.

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La peroxidation des lipides par les microsomes hépatiques et ses effets sur le métabolisme des drogues

CONDENSE

Dans le cours du développement des rats, l'activité maximale de peroxidation des lipides (dépendante du NADPH) par les microsomes hépatiques est atteinte à l'âge de 25 jours, et elle décline ensuite au niveau adulte d'activité à six semaines d'âge. L'activité accrûe chez les rats âgés de 25 jours résulte eu une plus grande diminution de l'habileté des microsomes à N-déméthyler l'aminopyrine, après une préincubation dans des conditions favorisant la peroxidation des lipides. Bien que l'activité totale de la NADPH oxidase soit similaire dans les rats de 25 jours et les rats adultes, l'activité de la NADPH oxidase spécifique pour la peroxidation des lipides est accrûe dans les rats de 25 jours. La peroxidation des lipides réduit l'amplitude des spectres d'attachement des types I et II sans affecter l'aspect qualitatif des spectres ou la quantité absolue de cytochrome P-450. Il est proposé que la diminution dans l'oxidation des drogues est due à un attachement moindre du substrat au cytochrome P-450. La peroxidation des lipides dépendante du NADPH a été démontrée pour la première fois dans des microsomes d'origine humaine. En comparaison avec le rat, les microsomes humains sont résistants à la diminution de l'oxidation des drogues résultant de la peroxidation des lipides. On présente des resultats qui supportent le concept que la peroxidation des lipides dépendante du NADPH peut se produire "*in vivo*". Le fer, impliqué dans la réaction de peroxidation

des lipides dépendante du NADPH, y participe sous la forme d'un complexe, même s'il est ajouté de source exogène. Le problème de la contamination avec le fer dans les études de peroxidation des lipides par les microsomes est discuté.

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ABBREVIATIONS AND SYMBOLS USED IN THIS THESIS

ADP	Adenosine diphosphate
5'AMP	Adenosine 5'-monophosphate
EDTA	Ethylenediaminetetra-acetic acid
g	Gram
x g	Acceleration of gravity
I_{50}	Concentration of inhibitor reducing response 50%
Km	Michaelis-Menten constant
Ks	Spectral binding constant
M	Molar
nm	Nanometers
NADH	Diphosphopyridine nucleotide, reduced form
NADPH	Triphosphopyridine nucleotide, reduced form
NADP	Triphosphopyridine nucleotide
ΔOD	Change in absorbance
rpm	Revolutions per minute
λ	Wavelength

Prefixes for units of measurement:

n	nano (10^{-9})
μ	micro (10^{-6})
m	milli (10^{-3})
k	kilo (10^{3})

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INTRODUCTION

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The importance of the drug metabolizing oxidative enzyme system, which is located in the hepatic endoplasmic reticulum, is now widely recognized. Many factors alter the activities of hepatic drug metabolizing enzymes and have the potential to play a role in the overall elimination of drugs.

Section I of this Introduction is a general review of hepatic drug oxidizing enzymes and their associated electron transport chain. Included is a review of the major factors which can alter the activity of these enzymes with an emphasis placed on the particular factors which are relevant to the relationship between drug oxidizing enzymes and microsomal NADPH dependent lipid peroxidation.

Section II reviews NADPH dependent lipid peroxidation of hepatic microsomes and its effects on microsomal drug oxidizing enzymes.

Section III is the formulation of the present problem.

SECTION I

A. GENERAL REVIEW OF HEPATIC DRUG OXIDATIVE ENZYMES

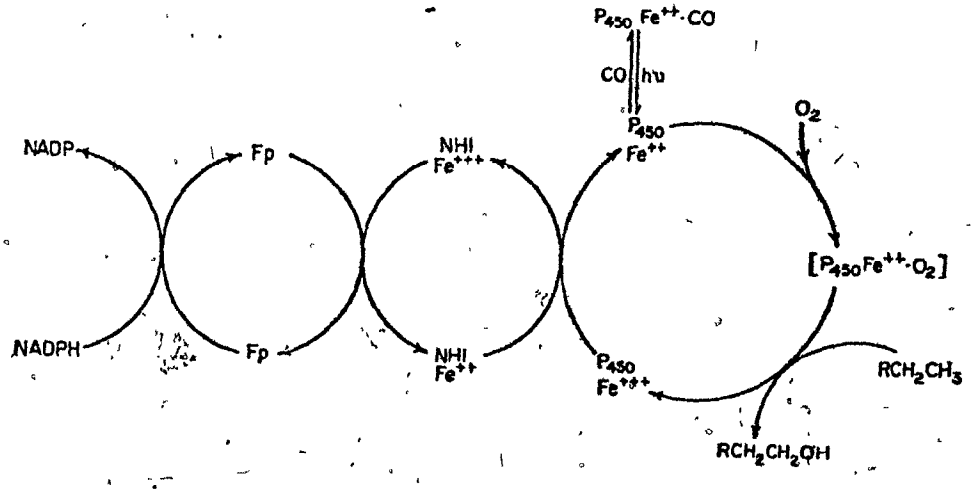
The duration of the pharmacological effect of a drug is controlled by several factors including absorption, distribution, biotransformation and excretion. Drug metabolism is one of the central factors in drug elimination as it is responsible for the formation of polar metabolites from most drugs which terminate pharmacological activity in most cases and results in excretion by the kidney (Williams 1949, 1959). The critical importance of drug metabolism in the excretion of drugs was exemplified by Brodie (1964) who stated that pentobarbital, a lipid soluble drug, would not be eliminated for 100 years if it were not metabolized to polar metabolites.

Many drugs are metabolized by specific enzymes responsible for other biochemical functions in the organism but the vast majority of drugs are metabolized by non-specific enzymes located in the endoplasmic reticulum of the hepatocyte which can be isolated as microsomes by centrifugation techniques. Microsomal metabolism of exogenous chemicals in the liver was first described by Mueller and Miller (1948, 1949, 1953) who demonstrated that azo dyes were reduced and amino-azo dyes were N-demethylated by microsomal preparations in the presence of oxygen and NADPH. Drug metabolism (several different substrates) was first demonstrated in microsomes and shown to require oxygen, NADPH and magnesium ions (Brodie *et al* 1955). The microsomal drug oxidizing system of endoplasmic reticulum of hepatic cells named hepatic mixed function oxidase (Mason 1957) was subsequently shown to oxidize a wide variety of different substrates by several diverse

reactions including aromatic hydroxylation, aliphatic hydroxylation, N-dealkylation, O-dealkylation, deamination, sulphoxidation and N-oxidation (Brodie *et al* 1958, Gillette 1966). This apparently non-specific enzyme oxidation required the transfer of electrons from NADPH via an electron transport chain with the actual oxidation carried out by an intermediate with the overall reaction being:

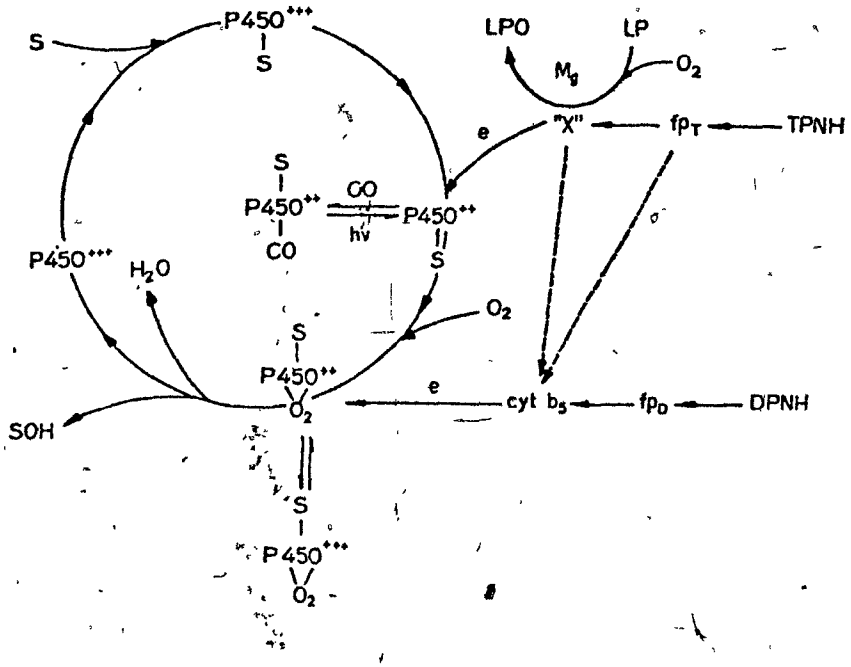
NADPH + Oxygen + Drug = $NADP^+$ + H_2O + Oxidized drug (Gillette 1963).

Cytochrome b_5 (Pappenheimer and Williams 1954), cytochrome P-450 (Klingenberg 1958) and cytochrome c reductase (Phillips and Langdon 1962) were demonstrated in microsomes and proposed as possible participants in the electron transport chain leading to the oxidation of drugs. After determining the involvement of cytochrome P-450 in drug oxidation, Omura *et al* (1965, 1966) proposed the following scheme to represent the oxidation of drugs.



In this scheme the oxidized form of cytochrome P-450 is reduced by electrons originating from NADPH via the flavoprotein cytochrome c reductase and an unidentified non-heme iron component. The reduced cytochrome P-450 reacts

with substrate and activated oxygen to provide the oxidized product (Omura *et al* 1965, 1966). Several other schemes have been proposed by other authors in attempts to achieve a stoichiometric reaction and many now include the involvement of cytochrome b_5 and NADH (Estabrook and Cohen 1969). The most generally accepted scheme for the hepatic mixed function oxidase system is illustrated as follows (Estabrook 1971).



In this scheme the substrate combines with oxidized cytochrome P-450 to form a complex which is then reduced by electrons from NADPH via the electron transport chain which includes cytochrome c reductase and a non-heme iron component. The reduced substrate-cytochrome P-450 complex reacts with molecular oxygen to form a substrate-cytochrome P-450-oxygen complex. Though the exact nature of the next step is obscure it is most likely that this complex is reduced by another electron to form a complex with activated oxygen which then splits to yield oxidized drug, oxidized cytochrome

P-450 and water. Electrons from NADH via cytochrome b_5 may contribute to the second electron input on the cytochrome P-450 complex. The exact role of cytochrome b_5 is, however, highly controversial and though it is likely to play some role (Hildebrandt and Estabrook 1971) in drug oxidation, some authors dispute this claim (Jansson and Schenkman 1973).

An absolute requirement of drug oxidation is that the enzyme system is part of a membrane and disruption of the relationships of the protein components in the membrane leads to a loss of activity (Estabrook *et al* 1971). Several reports (Lu and Coon 1968, Strobel *et al* 1970, Chaplin and Mannering 1970, Liebman and Estabrook 1971) have demonstrated a prominent role of phospholipids in drug oxidation and recently Vore *et al* (1974a, 1974b) has demonstrated an absolute requirement for microsomal lipid in hydroxylation reactions in microsomes.

Cytochrome P-450, which is central to all the proposed models for mixed function oxidase, demonstrates characteristic difference spectra when substrates are added to microsomal suspensions (Narasimhulu *et al* 1965, Remmer *et al* 1966, Imai and Sato 1966). In general, drugs can be divided into two separate spectral groups which have been termed type I (amino-pyrine, hexobarbital) with a λmax at 390 nm and an λmin at 425 nm and type II (aniline) with λmax 430 nm and λmin at 400 nm (Schenkman *et al* 1967a). In addition to these two main classes of difference spectra, several other spectral patterns have been reported for specific chemicals (Schenkman *et al* 1973). Characteristic difference spectra have also been demonstrated in semi-purified preparations of cytochrome P-450 (Lu *et al* 1969a, 1969b). Spectral maxima and minima increase in magnitude with increasing substrate

concentration following Michaelis kinetics. Linear plots can be obtained by plotting 1/ΔOD *vs* 1/substrate concentration and dissociation constants (Ks) can be calculated (Remmer *et al* 1966, Schenkman *et al* 1967). Several attempts have been made to correlate spectral dissociation constants with Michaelis constants (Km) determined during substrate oxidation. Such a correlation would indicate that the binding of drugs represented by spectral difference is an essential step in the oxidation of substrate. However, despite many reports that the Ks and Km of a substrate are similar, there are many raports that dispute any correlation between the two constants. The various conflicting reports have been reviewed by Mannering (1971). These experiments are carried out using a technique which makes several assumptions about soluble enzyme reactions (Michaelis and Menten 1913) and that its application to membrane bound enzymes systems using the same assumptions, is not entirely valid (Gillette 1971). Despite the fact that its exact meaning in molecular terms is obscure, spectral changes due to substrate-cytochrome P-450 interaction have become an important area of study in drug metabolism research. Schenkman and Sato (1968a) have presented evidence that type I spectral changes are due to binding at a site which is not directly at the heme moiety but at a site which allows an increase in the polarity of the 6th ligand of the heme in cytochrome P-450. This substrate binding results in the formation of more polar ligands which increases the flow of electrons to cytochrome P-450 (Schenkman 1968b) and accounts for the increased cytochrome P-450 reductase associated with type I compounds (Gigon *et al* 1969). Holtzman and Rumack (1971) have, however, suggested that the site of substrate binding which produces a spectral

change differs from the binding site responsible for the increase in cytochrome P-450 reductase activity. Type II binding is most likely due to the binding of the type II compound to the cytochrome P-450 to form a ferrihemochrome (Schenkman *et al* 1967a).

B. FACTORS AFFECTING HEPATIC DRUG OXIDATION

Hepatic mixed function oxidase is sensitive to alteration in a large number of ways, including endogenous and exogenous chemicals and physiological factors. As this enzyme system is responsible for the metabolism of many drugs, alteration of the system has become a major influence in both experimental pharmacology and in the practice of therapeutics. The major influences on hepatic mixed function oxidase activity both *in vivo* and *in vitro* are summarized in the following sections.

1. Enzyme Induction. Several hundred unrelated compounds have now been shown to increase the activity of hepatic mixed function oxidase when administered to both animals and man (Conney 1967). Stimulation of this enzyme system is termed induction and can be evoked by barbiturates and related drugs (*e.g.* phenobarbital) and by compounds related to polycyclic hydrocarbons (*e.g.* 3-methylcholanthrene) as reviewed by Conney (1967).

Induction by phenobarbital and other similar compounds results in a four-fold increase in enzyme activity and a concomitant increase in the cytochrome P-450 content of microsomes (Remmer and Merker 1965), cytochrome c reductase (Kuryama *et al* 1969), cytochrome P-450 reductase (Gram *et al* 1968), cytochrome b_5 (Kuryama *et al* 1969), and δ-aminolevulinic acid synthetase (Tephly *et al* 1971). There is an increase in both type I and type II

spectral changes after induction (Schenkman *et al* 1967a). Phenobarbital also increases liver weight, microsomal protein and specific activity and leads to an altered membrane turnover in endoplasmic reticulum (Orrenius *et al* 1969).

Polycyclic hydrocarbon type induction is different in several respects from that obtained with phenobarbital and in general it appears to be a much more selective type of induction, and affects the metabolism of a more selective group of substrates (Conney 1967). Usually cytochrome P-450 is induced but not cytochrome c reductase (Hernandez *et al* 1967). Treatment of animals with phenobarbital and 3-methyl cholanthrene simultaneously produced additive inductive effects (Bidleman and Mannering 1970, Gram and Gillette 1971) a result confirmed in isolated hepatocyte cultures (Gielen and Nebert 1971). Mannering's laboratory suggested that polycyclic hydrocarbons induced the formation of a modified cytochrome P-450 which he termed cytochrome P_1-450 (Sladek and Mannering 1966, 1969a, 1969b, Shoeman *et al* 1969). This cytochrome P_1-450 induced by polycyclic hydrocarbons was shown to have a different extinction maxima when combined in the reduced form to carbon monoxide at 448 nm instead of the normal 450 nm (Alvares *et al* 1967). (Cytochrome P_1-450 is now often termed cytochrome P-448). It is now generally believed that cytochrome P_1-450 results from the *de novo* synthesis of the cytochrome in response to polycyclic hydrocarbon (Alvares *et al* 1971, Fujita and Mannering 1971), rather than a conversion of cytochrome P-450 already present to the cytochrome P_1-450 (Fischer and Spencer 1972). Another major difference between the two classes of induction is that after induction by polycyclic hydrocarbons, only type II spectra are

enhanced compared to type I and type II spectral enhancement after phenobarbital induction (Schoeman *et al* 1969).

2. Enzyme Inhibition. Inhibition of mixed function oxidase has been reported for a large number of compounds and effects both *in vivo* and *in vitro* have been widely demonstrated. Inhibition has been shown to occur by several different mechanisms at different points in the electron transport chain of the cytochrome P-450 system. Some of the more important mechanisms of inhibition are tabled on the following page. As the action of most drugs is terminated through biotransformation by hepatic mixed function oxidase, inhibition of this system by any of the mechanisms described may be of major importance in therapeutics and result in drug accumulation and eventual toxicity. Several examples of drug interaction and toxicity have resulted from the inhibition of hepatic mixed function oxidase [*e.g.* diphenylhydantoin metabolism inhibited by bishydroxycoumarin (Hansen *et al* 1966), and meperidine metabolism inhibited by MAO inhibitors (Eade and Renton 1970a, 1970b)].

3. Species. Qualitative and quantitative interspecies differences in the ability of animals to metabolize drugs by the mixed function oxidase system of hepatic microsomes has been widely reported and is extensively reviewed by Williams (1967), Parke (1968) and Smith (1968). Recently the interspecies differences in the ability of rats, mice, guinea pigs and rabbits to N-demethylate ethylmorphine have been related to differences in NADPH dependent cytochrome P-450 reductase (Davies *et al* 1969). In some species such as fish, drug oxidation is low in microsomes incubated at 37^{0} (Brodie and Maickel 1962) but when incubation temperatures of 10^{0}

ELECTRON TRANSPORT CHAIN	INHIBITOR	MECHANISM	REFERENCE
I. NADPH	Pyrophosphatase	Destroys NADPH	Jeffery and Mannering 1974
II. NADPH-cytochrome c	Cytochrome c methylene blue	Electrons diverted from normal transport chain	Williams and Kamin 1962 Gillette *et al* 1973
	Lipid peroxidation	Electrons diverted to another enzyme system	Orrenius *et al* 1964
III. Cytochrome P-450	SKF-525A Drug oxidation substrates	Direct competition for enzymic sites	Gillette 1966
	Carbon monoxide	Binds to heme iron	Estabrook *et al* 1963
	Oxygen lack	No oxygen to form complex	Brodie *et al* 1955
	Metyrapone	Binds to heme to prevent substrate binding	Netter *et al* 1969
	SH reagents	Blocks sites required by enzymes	Wills 1969c
	Allyl barbiturates	Destroy cytochrome P-450	Levin *et al* 1972
	Lipid peroxidation	Destroy cytochrome P-450	Levin *et al* 1973
	Inhibitors of heme synthesis	Block of cytochrome P-450	Tephly *et al* 1973
IV. Membrane Effects	Detergents	Breakup membrane structure	Wills 1971
	Lipid peroxidation	Destruction of membrane lipids	Hogberg *et al* 1971

and 25° were used, drug oxidation increased (Buhler and Rasmusson, 1968). This interesting observation suggests that in the case of fish maximum activities are obtained at the usual environmental temperature of the liver.

4. Strain. Interstrain differences in drug oxidation within a species have been reported for rats (Mitoma *et al* 1967, Page and Vesell 1969), and in mice (Vesell 1968). Also interstrain differences in the inducibility of drug oxidizing enzymes by phenobarbital have been reported in rabbits (Gram *et al* 1965) and in rats (Page and Vesell 1969).

5. Age. Age has been determined to be an extremely important factor in the activity of drug oxidizing enzymes. In the rabbit the metabolism of several substrates of hepatic mixed function oxidase was deficient at birth (Fouts and Adamson 1959) and reached adult levels at 4 weeks of age. Since this first demonstration of decreased drug oxidation at birth it has been confirmed for a large number of substrates in different species (Jondorf *et al* 1959, Flint *et al* 1964, Gram *et al* 1969, MacLeod *et al* 1972). The deficiency of drug oxidation was related to deficiencies in the associated electron transport chain of the microsomes in the rat (MacLeod *et al* 1972), the rabbit (Fouts and Devereux 1972, MacLeod *et al* 1972), and the pig (Short and Davis 1972). MacLeod *et al* (1972) suggested that the deficiency of drug oxidation was most closely related to a deficiency of cytochrome P-450 reductase in male rats and to a deficiency of cytochrome c reductase in female rats. A similar deficiency in drug oxidation and related electron transport has been demonstrated in human newborn (Yaffe *et al* 1970, MacLeod 1972). This deficiency in the human newborn has been studied in microsomes prepared from livers obtained from premature

infants aged from 28 weeks gestation to full term (Aranda *et al* 1974). As in animals, the deficiency of drug oxidation in human newborns is related to a deficiency in the components of the electron transport system (Aranda *et al* 1974).

6. Sex. In the male rat, drug oxidation has a much higher rate of activity than in the female. This was first reported by Quinn *et al* (1958) who showed that sleeping times for hexobarbital were longer in female rats compared to males and that the differences were due to an increased half life of the drug in females. This was shown to be the result of higher activity of hepatic mixed function oxidase in the male rat (Quinn *et al* 1958). The Km for N-demethylation of ethylmorphine in male rats is 40% that in the female (Davies *et al* 1968) and type I difference spectra have a magnitude of 3 times higher in male rats than in females (Schenkman *et al* 1967b) suggesting that the sex-linked differences may involve the binding of substrates to microsomes. It has also been shown that cytochrome P-450 content, NADPH cytochrome P-450 reductase, and substrate enhanced NADPH cytochrome P-450 reductase are lower in females compared to the male (Gillette and Gram 1969). Recently the sex difference in drug oxidizing enzymes of microsomes has been extensively studied in Mannering's laboratory (El Defrawy *et al* 1974a, 1974b, Cohen and Mannering 1974, Sladek *et al* 1974). It was concluded that temporal decreases in the female and temporal increases in the developing rat partially accounted for the difference and that there was also a qualitative difference in the cytochrome P-450 found in the two sexes and that the changes may be related to the hormonal status of the sexes.

Sex differences in drug metabolism have also been reported in mice though the differences are of a lower magnitude than those in the rat (Vesell 1968). It would appear from the lack of reports in the literature that sex differences do not occur to any significant degree in other species.

SECTION II

A. GENERAL HISTORY

In 1963 Hochstein and Ernster reported an enzymatic peroxidation of endogenous lipid in isolated rat liver microsomes which was dependent on NADPH. Prior to this, nonenzymatic lipid peroxidation was a well recognised phenomenon and had been described for a number of tissues and conditions. Deutsch *et al* (1941) had noted that ascorbic acid induced the formation of lipid peroxide and then several authors (Elliot and Libet 1944, Simon *et al* 1944) demonstrated the catalytic effects of iron on lipid peroxidation. Tappel (1953, 1955) studied the effects of hematin on the peroxide formation from linoleic acid esters. Wilbur *et al* (1954) and Ottolenghi *et al* (1955) demonstrated that lipid peroxides can be generated in tissues *in vivo* and that they can be formed *in vitro* when tissues are exposed to ultraviolet radiation.

One of the major contributions to this were the experiments which showed for the first time that peroxidation of endogenous lipid occurred in subcellular particles (Ottolenghi *et al* 1955, Ottolenghi 1959). In mitochondria, ascorbic acid enhanced lipid peroxidation and iron salts catalyzed the reaction. Ottolenghi (1959) believed that this reaction was a co-oxidation of ascorbic acid and unsaturated lipid and was similar to the co-oxidation scheme of Elliot and Libet (1944) which required a metal catalyst. Also interesting is the fact that Ottolenghi (1959) mentioned that peroxidation of lipid took place in microsomes to a greater extent than mitochondria, though he never reported these results in detail. Tappel and Zalkin (1960) also showed that hematin could stimulate lipid

peroxidation in subcellular particles, and in vitamin E deficient animals lipid peroxides could be detected in several tissues (Zalkin and Tappel 1960).

The demonstration that an enzyme linked lipid peroxidation existed in rat hepatic microsomes resulted in a completely new area of research into lipid peroxidation and its effects (Hochstein and Ernster 1963). The peroxidation of endogenous membrane lipids of the microsomes required NADPH, ADP and gaseous oxygen and the reaction was heat sensitive. This enzymatic NADPH dependent lipid peroxidation could not be evoked in mitochondria though non-enzymatic ascorbate induced lipid peroxidation occurred in both microsomes and mitochondria. The only other NADPH dependent oxidative enzyme system which also required oxygen known at that time in microsomes was the NADPH dependent drug oxidizing system described by Gillette *et al* (1957). Because of the similarities in the requirements of the two systems (NADPH, oxygen) and the fact that SKF-525-A (β-diethylaminoethyldiphenylpropylacetate) inhibited both reactions, Hochstein and Ernster (1963) proposed a close association or coupling between NADPH dependent lipid peroxidation and drug oxidation and devehowever a scheme which involved the transfer of electrons from NADPH along a common transport pathway which included the flavoprotein cytochrome c reductase which had been described by Phillips and Langdon (1962) and involved the *active oxygen* of drug metabolism (Mason 1957). An ADP-iron complex was also central to the scheme. It was at the active oxygen step where the electron pathway was proposed to diverge for either drug oxidation or lipid peroxidation. In retrospect it is surprising how accurate this proposal was

when one considers how little data the authors had to substantiate their hypothesis. Even after most of the steps of the electron transport chain for drug metabolism had been substantiated and the *active oxygen* was shown to involve cytochrome P-450 (Omura and Sato 1964a, 1964b) the original proposal of a common electron transport chain was maintained and as will be discussed later in this Introduction some researchers still maintain an active involvement of cytochrome P-450 in NADPH dependent lipid peroxidation. At the same time it was reported that increased NADPH oxidation occurred in microsomes of rat liver (Beloff-Chain *et al* 1963) when ADP was added to the reaction mixtures. These authors at that time did not, however, recognise that this increase in NADPH oxidation was due to lipid peroxidation activity stimulated by ADP and the association between the two facts was not made. Initially the exact role of ADP in lipid peroxidation was unknown though Hochstein and Ernster (1963) proposed an ADP-$Fe^{2+}O_2$ complex as the *active oxygen* in their scheme. Later in the same year, these authors added a note to their chapter in the Ciba Foundation on Cellular Injury (Hochstein and Ernster 1964) which stated that the ADP used was contaminated with iron complexes and later they reported that other iron complexes such as a pyrophosphate-iron complex could substitute for the apparent dependence of lipid peroxidation for ADP (Hochstein *et al* 1964).

B. MECHANISM OF LIPID PEROXIDATION

Non-enzymatic lipid peroxidation produced malonaldehyde which reacted with thiobarbituric acid to produce a red chromogen (Bernheim *et al*

1958, Sinnhuber *et al* 1958). As this reaction was utilised to characterise NADPH dependent lipid peroxidation in isolated rat microsomes (Hochstein and Ernster 1963) it was assumed that the product resulting from lipid peroxidation was malonaldehyde derived from the degradation of unsaturated lipid of the microsomal membrane.

Later it was demonstrated that membrane phospholipids were modified during peroxidation reactions by a reduction in their arachidonic acid and docosahexanoic acid content (May *et al* 1965) and that this occurred via chain cleavage at the β position of polyunsaturated fatty acids (May and McCay 1968a). One mole of phospholipid fatty acid was used for each mole of NADPH used along with four moles of oxygen (May and McCay 1968b) and the products formed during the reaction consisted of several aldehydes and phospholipids with carbonyl functions on the β-acyl groups. One of the reaction products was positively identified as malonaldehyde (Niehause and Samuelsson 1968) and utilising (H^3)-arachidonic acid it was shown that the malonaldehyde could be produced from phospholipid arachidonate contained in hepatic microsomal membranes. This was confirmed when May and Reed (1973) found difference spectra in microsomes after peroxidation which were due to malonaldehyde and conjugated dienes. It was also shown that phospholipid peroxides which were always associated with the β positions of polyunsaturated fatty acids were transient intermediates in the NADPH dependent lipid peroxidations of microsomal membranes (Tam and McCay 1970). The number of derivatives obtained was approximately equal to the number of double bonds in the fatty acids.

During the reaction in which phospholipids were degraded, there was

a distinct structural change in the membrane itself as measured by a decrease in the turbidity of microsomal suspensions. In these particular experiments no distinct gross structural changes were determined by electron microscopy of microsomal particles but the microsomes did not aggregate like normal microsomes (Tam and McCay 1970). More recently Arstila *et al* (1972) and Hogberg *et al* (1973) have described distinct changes in liver and kidney microsomes after lipid peroxidation using the electron microscope. Both authors reported aggretates of vesicles which had a typical trilaminar membrane structure, several dense amporphous patches and much unidentified debris. A major difference in the two reports was that Hogberg *et al* (1973) recorded the attachment of ribosomes to the vesicle membrane surfaces but in the electron micrographs of Arstila *et al* (1972) there was a complete absence of ribosomes at the membrane surface after lipid peroxidation had taken place. Hogberg *et al* (1973) also showed a decrease of 20:4 and 22:6 unsaturated fatty acids at low levels of peroxidation and at high levels of peroxidation, a decrease in 18:2 fatty acids.

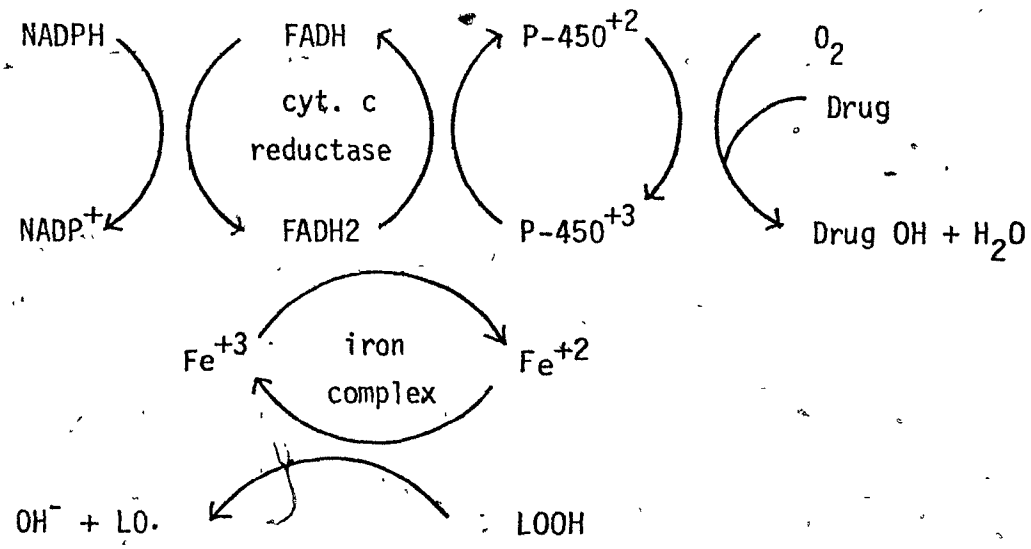
During NADPH oxidation and the generation of lipid peroxides there is evidence that a free radical is generated (McCay *et al* 1971, Pfeifer and McCay 1971). It is therefore possible that NADPH lipid peroxidation involves a free radical intermediate as it is well established that free radicals can cleave unsaturated lipids and form peroxides (Tappel 1972) and free radical trapping agents such as N,N' diphenyl-p-phenylenediamine, inhibit lipid peroxidation (Hochstein and Ernster 1963). Though the precise reaction mechanism and sequence of reactions has still to be decided it is certain that NADPH catalyses the peroxidation of polyunsaturated

fatty acids at the β unsaturated position in the lipid membrane structure of the endoplasmic reticulum and that the process results in marked changes in the structure and function of the membrane.

While other workers concentrated on defining the nature of the lipid structures involved in lipid peroxidation of microsomes, Wills (1969a, 1969b, 1969c) described the basic properties and requirements of the NADPH dependent lipid peroxidation reaction in rat microsomes. Iron chelating agents were effective inhibitors of the reaction and iron added to microsomes could increase peroxidation activity and reverse the effects of chelating agents. The iron involved in the reaction was non-heme in nature but as it was resistant to washing it was assumed to be bound in some way. Chelating agents appeared to act on the bound form of the iron and did not act by removing iron from the reaction mixtures. Cytochrome c which inhibited drug oxidation by diverting electrons from the electron transport chain after the cytochrome c reductase step (Williams and Kamin 1962, Gillette *et al* 1973) also inhibited NADPH dependent lipid peroxidation (Wills 1969a). As cytochrome c reductase is the flavoprotein proposed in the electron transport scheme of Hochstein and Ernster (1963) for lipid peroxidation, the evidence that cytochrome c inhibits both systems is support for the idea that lipid peroxidation and drug oxidation may share a common electron transport pathway. Wills (1969a) proposed that the electron transport chain, which includes cytochrome c reductase, can switch between the oxidation of drugs and the peroxidation of unsaturated lipids of endoplasmic reticulum. Lipid peroxidation activity can be induced by treating animals with phenobarbital (Nilsson *et al* 1964). Barbiturates

are well known for their induction of the components of the electron transport system of hepatic drug oxidation and the resulting increased oxidation of substrates (Gelboin 1971). Direct proof of the involvement of a drug oxidation electron transport component was provided when Pederson and Aust (1972) demonstrated NADPH dependent peroxidation of extracted microsomal lipid by a purified preparation of the flavoprotein NADPH cytochrome c reductase. This isolated enzyme system differed from the system in whole microsomes by having an absolute requirement for an EDTA-Fe complex as well as the usual requirement for iron, added in this experiment as iron-ADP. Antibodies prepared against purified cytochrome c reductase effectively inhibited microsomal N-demethylation, microsomal lipid peroxidation, and the lipid peroxidation catalysed by isolated cytochrome c reductase (Pederson *et al* 1973). It was also shown that by adding EDTA-Fe to microsomal peroxidation reaction mixtures, NADH could substitute for the requirement of NADPH in normal lipid peroxidation mixtures. These authors suggested that microsomes contained a component required for lipid peroxidation which could only be reduced via the NADPH cytochrome c reductase step and that this component could be replaced by an EDTA-Fe complex which could then be reduced via an NADH system. This evidence fits well with the theoretical concept that cytochrome c reductase functions to reduce an iron containing component which promotes lipid peroxidation in intact microsomes (Bidlack and Tappel 1973) as illustrated on the following page.

In intact microsomes, NADH cannot reduce the iron containing component via cytochrome c reductase but can in some unknown way reduce EDTA-Fe complex which can apparently replace this NADPH-cytochrome c reductase



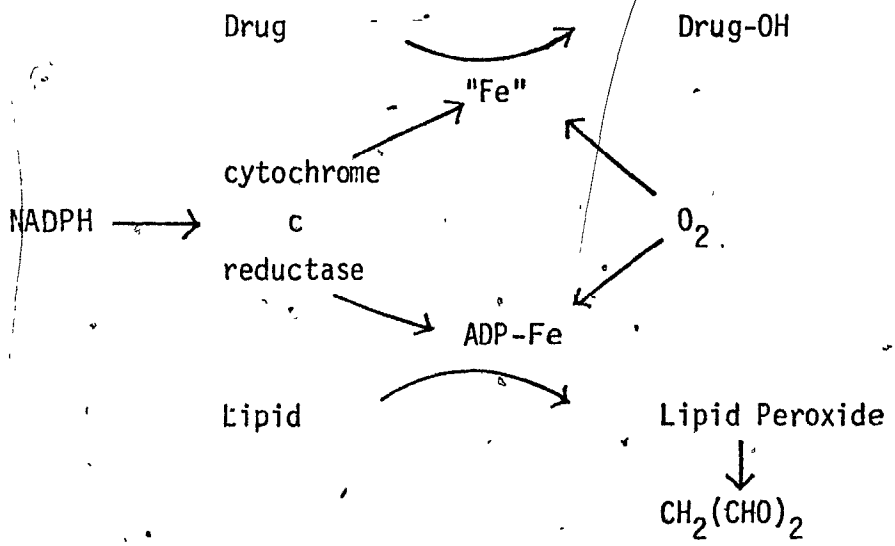
specific iron containing component (Pederson *et al* 1973). More recently (Hogberg *et al* 1974) isolated an NADPH cytochrome c reductase solubilized from microsomes which were subjected to lipid peroxidation. This fraction itself was capable of supporting an NADPH dependent lipid peroxidation of mitochondria when added to mitochondrial suspensions containing iron.

Recently Radtke and Coon (1974) demonstrated that a purified preparation of the hemeprotein cytochrome P-450 promoted lipid peroxidation in the presence of lipid peroxides. NADPH is not apparently required for this system. The exact nature and extent of cytochrome P-450 involvement in lipid peroxidation in microsomes has still to be evaluated.

C. LIPID PEROXIDATION AND DRUG OXIDATION

Similarities in NADPH and oxygen requirements for both microsomal lipid peroxidation and microsomal drug oxidation (Hochstein and Ernster 1963) suggested that the two processes might be closely related and possibly interfere with each other. Two substrates of the drug oxidation

system, codeine and aminopyrine, inhibited lipid peroxidation in rat microsomes (Orrenius *et al* 1964) as measured by the thiobarbituric acid reaction, oxygen uptake, and the oxidation of NADPH. Direct effects of these compounds on lipid peroxidation was ruled out and it was concluded that NADPH dependent lipid peroxidation and NADPH dependent drug oxidation utilized the same electron transport pathway and the following scheme was proposed to explain that data.



Although there was no direct evidence it was suggested that this involved the flavoprotein cytochrome P-450 ("Fe" in the proposed scheme) which was Klingenberg's (1958) carbon monoxide binding pigment, which at that time was just being implicated in the drug oxidation pathway (Estabrook *et al* 1963). The proposed sharing of a common electron transport chain was also supported by the evidence that carbon monoxide which inhibited drug oxidation by binding with cytochrome P-450 (Conney *et al* 1957) reversed the inhibition of lipid peroxidation by drug oxidation substrates (Ernster

and Nordenbrand 1967). Wills (1969c) reported similar results to support the argument for a link between drug oxidation and lipid peroxidation. The evidence indicated that microsomal lipid peroxidation and drug oxidation interfered with each other by competing for reducing equivalents from flux along at least part of a common electron transport chain. These experiments using substrates of drug oxidizing enzymes to inhibit lipid peroxidation led to a greater understanding of lipid peroxidation mechanisms, though recently (Pederson and Aust 1974) it has been suggested that the right conclusion was reached (*i.e.* that lipid peroxidation and drug oxidation share part of a common electron transport pathway) but that the reasoning used to reach the conclusion was in error. It has been suggested that substrates do not block lipid peroxidation by competing for available electrons but by forming anti-oxidant metabolites via cytochrome P-450 and that the anti-oxidant metabolites are responsible for the inhibition of lipid peroxidation. This interpretation may be limited to benzpyrene which was the only substrate studied, although it has been extensively shown that several anti-oxidants are potent inhibitors of lipid peroxidation (Gram and Fouts 1966, Wills 1970, Vainio 1974).

A second type of interference of drug oxidation as a result of lipid peroxidation was reported by Wills (1969c). By increasing lipid peroxidation by incubating microsomes with NADPH or by exposing microsomes to ionizing radiation, the activity of aminopyrine N-demethylation and aniline hydroxylase was reduced. As the reduction in drug oxidation after radiation was also accompanied by increased lipid peroxidation it appeared that the decrease in drug metabolism was due to membrane destruction (Wills

and Wilkinson 1970). This meant that drug oxidation was not only reduced by competition for electrons by lipid peroxidation, but that lipid peroxidation actually destroyed the membrane, its components, or altered the membrane in some way to prevent the drug oxidation system from operating. It was subsequently shown (Wills 1971) that other microsomal enzymes, including glucose-6-phosphatase, aminopyrine N-demethylase, aniline hydroxylase, NADPH oxidase and menadione-dependent NADPH oxidation, were all reduced in activity after lipid peroxidation had been promoted. On the other hand, other microsomal enzymes, including $NAD^+/NADP^+$ glycohydrolase, adenosine triphosphatase, esterase and NADPH cytochrome c reductase, were not inactivated by lipid peroxidation activity (Wills 1971). Detergents, such as deoxycholate, added to microsomes inactivated the same enzymes as lipid peroxidation activity and had no effect on the ones unaffected by lipid peroxidation. As these affects of lipid peroxidation were not due to the formation of hydroperoxides, malonaldehyde or other lipid peroxidation breakdown products, it was concluded that the effects of lipid peroxidation in microsomal membranes was due to a loss of structure or integrity of the membranes (Wills 1971). This effect of lipid peroxidation was confirmed by Hogberg *et al* (1973) who also showed that a number of microsomal parameters which are known to be dependent on membrane integrity, were effected by the process of lipid peroxidation, including a reduction in activity of aminopyrine N-demethylase, 3,4-benzpyrine hydroxylase and glucose-6-phosphatase, and an increase in activity of UDP glucuronyltransferase. Another effect on microsomal enzymes was demonstrated recently by Hogberg *et al* (1974) who isolated solubilized NADPH cytochrome c

reductase from microsomes which had been subjected to lipid peroxidation. This was similar to detergent treatment as it has been shown (Ernster *et al* 1962) that deoxycholate solubilized cytochrome c reductase from microsomal membranes. The release of NADPH cytochrome c reductase and other membrane proteins had already been demonstrated by Bidlack and Tappel (1973) using non-enzymatic lipid peroxidation of microsomal membranes.

Wills (1971) went so far as to suggest that lipid peroxidation might be important in the regulation of microsomal mixed function oxidase and was involved in the turnover of the membranes involved in this system. Wills (1972b) expanded this suggestion to provide a biological function for menadione related quinones. He submitted a hypothesis which allowed the quinones to rapidly oxidize all available NADPH normally used by drug oxidation and lipid peroxidation. The subsequent lack of lipid peroxidation protected the membrane from degradation, thereby regulating the turnover of membranes in the endoplasmic reticulum. The turnover of endoplasmic reticulum, therefore, would depend on lipid peroxidation activity which in turn depends on quinone levels in the hepatocyte. If the suggestion that lipid peroxidation controls microsomal membrane integrity is valid, then it would not be unreasonable to suggest that by inhibiting lipid peroxidation in microsomes the normal membrane turnover would be altered to produce an increase in drug oxidation activity. However, α-tochopherol which inhibits lipid peroxidation by anti-oxidant properties (McCay *et al* 1971) had no effect on the time course of hexobarbital or codeine metabolism (Gram and Fouts 1966). Similarly, Anders (1969) showed that inhibition of lipid peroxidation had no effect on the hydroxylation of aniline in

microsomes and Wills (1969c) did not show stimulation of aminopyrine N-demethylation after inhibition of lipid peroxidation with EDTA. These results would tend to oppose the idea that lipid peroxidation plays a central role in the control of the activities of enzymes located in the membrane of the endoplasmic reticulum. In one special case, the epoxidation of aldrin to dieldrin by microsomes from the pig liver, inhibition of lipid peroxidation led to an increased enzyme activity (Lewis *et al* 1967). These same authors could not produce such a stimulation of activity in microsomes obtained from housefly.

Recently it has been demonstrated by Kamataki and Kitagawa (1973) that N-demethylation of ethyl morphine is increased in microsomes when lipid peroxidation is inhibited by EDTA, o-phenanthroline, a,a' dipyridyl, or cobalt chloride and that N-demethylation of ethyl morphine is decreased when lipid peroxidation is stimulated by iron. These results would fit the pattern predicted if lipid peroxidation was playing a central role in the control of microsomal membrane function. However, as linearity of some other drug oxidation reactions are dependent on the extent of lipid peroxidation (Jacobson *et al* 1973) and Kamataki and Kitagawa (1973) used incubation times of longer duration than that used by most laboratories the stimulation of drug metabolism reported may be a result of an incubation artifact and have little to do with normal membrane turnover in the microsomes. This particular point is investigated in this thesis.

Another mechanism for the effect of lipid peroxidation on the drug oxidizing system of microsomes is the destruction of the heme in cytochrome P-450 as a result of the peroxidation reaction. This was suggested by the

fact that carbon monoxide was released when microsomes were incubated with NADPH and this could be blocked by EDTA (Nishibayashi *et al* 1967). Carbon monoxide formed from a biological source can be the result of heme breakdown (Ludwig *et al* 1957, Sjostrand 1952, Coburn 1970). Hrycay and O'Brien (1971) demonstrated that lipid peroxides could destroy cytochrome P-450 and reduce drug oxidation activity in hepatic microsomal suspensions. Schacter *et al* 1972, 1973) showed that high levels of lipid peroxidation in microsomes resulted in the loss of cytochrome P-450, the degradation of microsomal heme and a resultant evolution of carbon monoxide. The evolution of carbon monoxide was in the ratio of one mole heme lost to one mole of carbon monoxide formed. This was confirmed by Hogberg *et al* (1973) who showed microsomal lipid peroxidation reduced cytochrome P-450 levels and also by Levin *et al* (1973) who showed that inhibition of lipid peroxidation with EDTA could prevent the loss of cytochrome P-450, heme destruction and evolution of carbon monoxide. It was also demonstrated that in rats treated with labelled δ-[3,5-^{3}H]amino levulinic acid, labelled heme breakdown products were detected during lipid peroxidation. This established a direct role for the involvement of lipid peroxidation in the destruction of heme in cytochrome P-450, and the resulting loss of drug oxidation activity. As lipid peroxidation has been shown to occur in a purified preparation of cytochrome c reductase without the presence of cytochrome P-450 (Pederson and Aust 1972), it is likely that cytochrome P-450 does not take part in the lipid peroxidase reaction and that its destruction is not due to its participation in the reaction, but is a consequence of the peroxidation reaction. The destruction of cytochrome P-450 by allyl containing

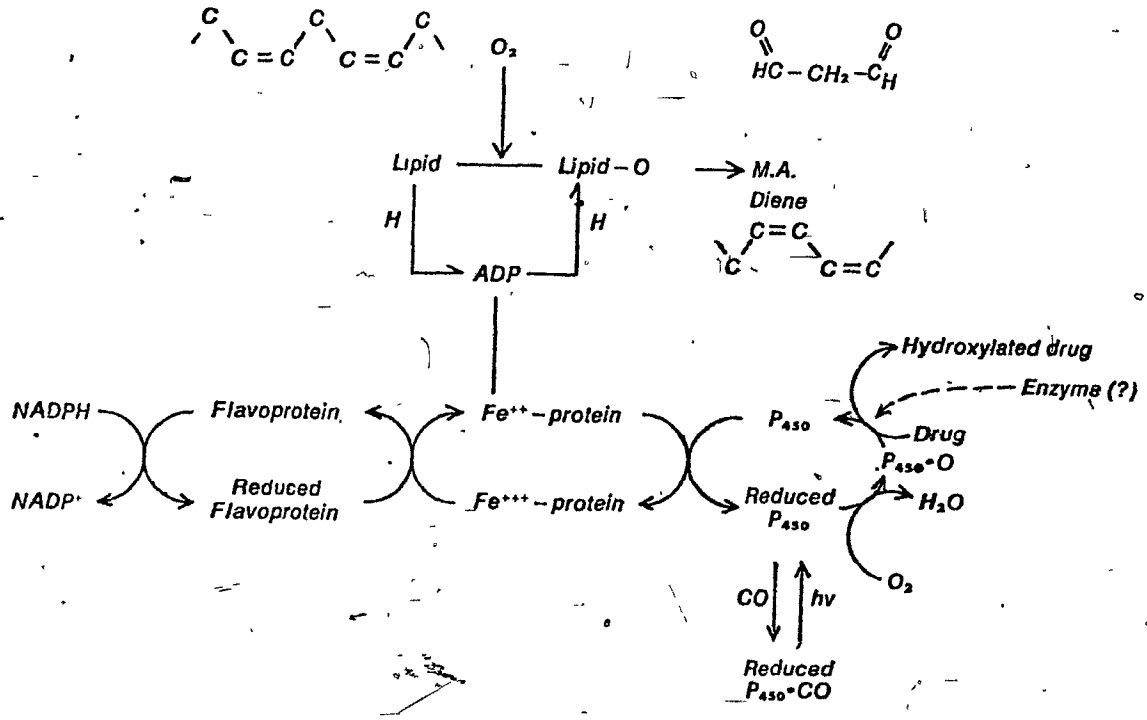
barbiturates (Levin *et al* 1972) occurs by a mechanism unrelated to the destruction of cytochrome P-450 by lipid peroxidation (Levin *et al* 1973). The disappearance of cytochrome P-450 was shown to be directly related to a decrease in pentobarbital and acetanilide oxidation in microsomes of the rat resulting from the effects of lipid peroxidation (Jacobson *et al* 1973). By inhibiting lipid peroxidation with EDTA, linearities for these substrate reactions could be extended because the breakdown of cytochrome P-450 was prevented. In the rabbit, a species with a low level of peroxidation, pentobarbital metabolism was linear for an extended period even without the addition of EDTA, and cytochrome P-450 levels did not decrease.

In a discussion after a report by Schacter *et al* (1973) at the Second International Symposium on Microsomes and Drug Oxidations, Mannering (1973) pointed out that in his laboratory he was able to demonstrate non linearity of many substrate oxidations and the production of carbon monoxide by microsomes, but he had failed to demonstrate a simultaneous reduction in cytochrome P-450 content. In his reaction mixtures, Mannering did not actively promote lipid peroxidation with Fe and ADP as had most other authors (Schacter 1972, 1973, Levin *et al* 1973, Jacobson 1973). In spite of the incubation mixture differences, the experiments of Mannering dissociated the decrease in drug metabolism and the evolution of carbon monoxide during lipid peroxidation from cytochrome P-450 destruction and, therefore, challenges the apparently solid evidence of the other work in this area.

Drug oxidation in microsomes can be enhanced by adding the 100,000 x g soluble fraction to the reaction mixture (Van Dyke and Wineman 1971, Gandolfi and Van Dyke 1973, Nelson *et al* 1973). The addition of soluble

fraction also extends the linearity of the time course of benzpyrine hydroxylase in microsomes (Kuntzman 1969). As inhibition of lipid peroxidation might enhance drug oxidation (Kamataki and Kitagawa 1973) and extend the linearity of drug oxidation reactions (Jacobson *et al* 1973), the enhancement of drug oxidation seen by adding soluble supernatant fraction to microsomes could be due to an inhibitor of lipid peroxidation being present in the soluble fraction. Kamataki *et al* (1974) demonstrated that soluble supernatant fraction inhibited lipid peroxidation and that this inhibition could partially explain the augmentation of drug oxidation caused by the soluble fraction. Warner and Neims (1975) have recently isolated two fractions from soluble supernatant which can augment aminopyrine N-demethylation and aniline hydroxylase, but have not tested these fractions for inhibitory activity on lipid peroxidation. Should the hypothesis that lipid peroxidation inhibition by soluble supernatant fraction leads to increased drug oxidation by microsomes prove correct, then a case can be made for the endogenous control of lipid peroxidation (increased by iron, reduced by soluble fraction) being a possible mechanism for the control of the oxidative drug metabolism in the endoplasmic reticulum. The fact that drug oxidation can be altered in both directions by endogenous means, makes Wills' (1971) proposal that lipid peroxidation may control drug oxidation much more feasible.

As far as can be determined from the evidence presently available, the following diagram summarizes the relationship between the drug oxidizing scheme and the scheme for NADPH dependent lipid peroxidation. Lipid peroxidation can effect electron flux, membrane structure and cytochrome P-450



Scarpelli and Trump 1971

levels. Alteration of any one of these factors could play a vital role in the total capacity of the liver to eliminate drugs.

D. *IN VIVO* LIPID PEROXIDATION

Lipid peroxidation has been shown to take place *in vivo* in animals fed toxic chemicals (Gloshal and Recknagel 1965), vitamin E deficient diets (Zalkin and Tappel 1960), or choline deficient diets (Monserrat *et al* 1969), and in animals exposed to radiation (Wills and Wilkinson 1967). As far as is known, NADPH dependent lipid peroxidation has not been involved in any of these processes. As has been suggested by Wills (1971), NADPH dependent

lipid peroxidation may play a role in the natural turnover of endoplasmic reticulum membrane, and it is important to determine if NADPH dependent lipid peroxidation takes place *in vivo*, and to demonstrate that it is not a phenomenon confined to isolated preparations of microsomes. Mice which were treated with iron-dextran (Imferon), showed an increased NADPH lipid peroxidation activity in subsequently isolated microsomes (Wills 1972a). This increased lipid peroxidation was accompanied by a decrease in microsomal aminopyrine and p-chloro-N-methyl aniline oxidation in microsomes. Wills interpreted his results to indicate that an increased *in vivo* NADPH dependent lipid peroxidation in the endoplasmic reticulum of hepatocytes, led to a decrease in drug metabolism. It has been demonstrated that by treating rats *in vivo* with iron and ascorbic acid, the level of thiobarbituric acid reacting material (indicating lipid peroxidation had taken place), is increased in isolated mitochondria (Fujita 1973) and in isolated microsomes (Fujita 1974). In both mitochondria and microsomes, the unsaturated fatty acid content of the subcellular particle involved was also reduced, a finding which would be expected if lipid peroxidation had taken place. A decrease in lipid peroxidation activities should lead to an increased drug oxidation because of a reduced breakdown of microsomal membrane. Though lipid peroxidation was not measured, the N-demethylations of aminopyrine increases in iron deficient rats (Catz *et al* 1970), and was decreased in vitamin E deficient rats (Carpenter 1972).

Despite the arguments discussed, it is generally believed that the occurrence of *in vivo* NADPH dependent lipid peroxidation has not yet been proved conclusively. Several strong arguments have been proposed to support

the idea of a normal *in vivo* suppression of non-enzymatic lipid peroxidation, including the fact that a) polyunsaturated lipids are protected in membrane structures, b) *in vivo* anti-oxidants inhibit lipid peroxidation, c) iron catalysis is prevented by membrane structures, d) glutathione peroxidase destroys peroxides and, e) oxygen tension is low in membranes (Barber and Bernheim 1967, Tappel 1972). These same arguments may not be entirely valid for enzymatic lipid peroxidation, as it is the *protective membrane* of the microsome which is the substrate for the reaction, and both iron and oxygen are present in high enough concentration to serve the closely related drug oxidizing system. Because of the central role played by the microsomal drug oxidizing enzymes of the hepatic endoplasmic reticulum in the elimination of drugs and its possible ease of alteration by lipid peroxidation of the membranes, the *in vivo* demonstration of lipid peroxidation has become of major importance.

Lipid peroxidation has recently been shown to control the half life of epoxide intermediates and their breakdown by epoxide hydrolase (Watabe and Akamatsu 1974), during the oxidation of olefins and arenes by microsomes. As these epoxides are toxic or carcinogenic, a mechanism such as lipid peroxidation, which might control the *in vivo* half life of the epoxide, becomes extremely important in the determination of the potential carcinogenicity of the epoxide.

Recently, Hogberg *et al* (1974) have reported that in isolated hepatocytes, ADP-Fe^{+3} can initiate an increase in the NADPH-cytochrome c reductase activity in the 100,000 x g supernatant fraction after the cells are homogenized. As the same enzyme can be released from isolated microsomes by

lipid peroxidation, this experiment may be the first to demonstrate lipid peroxidation in the whole cell.

E. FACTORS AFFECTING LIPID PEROXIDATION

As is the case with drug metabolizing enzymes of microsomes, lipid peroxidation of microsomes is influenced both positively and negatively by a number of factors.

1. Enhancement of Lipid Peroxidation. Iron added to microsomes increases the activity of lipid peroxidation (Orrenius *et al* 1964, Wills 1969b). In at least one study (Levin *et al* 1973), iron added to microsomes did not stimulate lipid peroxidation. However, the basal lipid peroxidation in this study was high, indicating that the microsomes utilized contained adequate iron to promote maximal activity. Lipid peroxidation activity can also be increased by treating animals with the classic microsomal enzyme inducer, phenobarbital (Nilsson *et al* 1964).

2. Inhibition of Lipid Peroxidation. Inhibition of lipid peroxidation has been reported for a number of different compounds, acting by several different mechanisms, as outlined in the following diagram.

Inhibitor	Mechanism of Action	Reference
EDTA o-Phenanthroline 8-OH-Quinoline	Chelates iron	Wills 1969b
Aminopyrine Codeine	Competes with substrates of drug oxidation for electrons	Orrenius *et al* 1964

Cytochrome c	Diverts electrons	Wills 1969a
Vitamin E DPPD Butylated hydroxyanisole	Anti-oxidant	Tappel 1972 Levin *et al* 1973 Vianio 1974
Deoxycholate	Destroys membrane	Wills 1969c
Antibodies *vs* cytochrome c reductase	Blocks electron transport via cytochrome c reductase	Pederson & Aust 1973
Iodoacetamide p-Chloromercuribenzoate	Blocks thiol groups	Wills 1969c
GSH Cysteine	Thiol reagent	Wills 1969a

3. Species. Different levels of lipid peroxidation activity have been reported in several different species. Levels of microsomal lipid peroxidation in the mouse and guinea pig (Kamataki and Kitagawa 1974), and in the pig (Lewis *et al* 1967), were in the same range as those widely reported for the rat (Wills 1969a, Kamataki and Kitagawa 1974). Lower levels have been reported for the rabbit (Gram and Fouts 1966, Jacobson *et al* 1973, Kamataki and Kitagawa 1974), and activity could not be detected in the housefly (Lewis *et al* 1967). The differences in the rabbit cannot be accounted for by low iron content in rabbit microsomes, as even when maximally stimulated with iron added to microsomes, lipid peroxidation in the rabbit was less than one third that found in the rat under the same conditions (Kamataki and Kitagawa 1974). Also, it is unlikely that the difference is due to different amounts of the flavoprotein cytochrome c reductase in the two species, as cytochrome c reductase activities in the rat and rabbit are almost identical (MacLeod 1972). The low level of peroxidation in

rabbit microsomes is the most likely explanation for the prolonged linearity of substrate metabolism in the rabbit as compared to the rat (Gram and Fouts 1966, Jacobson *et al* 1973). The occurrence of NADPH dependent lipid peroxidation has not yet been determined in the human.

4. Strain. Several strains of rats have been studied in different laboratories, but as interlab differences are large in Sprague-Dawley rats, no strain difference could be assessed using data from different laboratories.

5. Age. Drug oxidation has been shown to be dependent on the age of the animal, and in most species it is deficient at birth (Fouts and Adamson 1959, Jondorf *et al* 1959, Flint *et al* 1964) compared to adult activities. This deficiency in drug oxidation is related to a deficiency in the components of the electron transport chain associated with drug oxidation (MacLeod *et al* 1972, Fouts and Devereux 1972, Aranda *et al* 1974). As NADPH dependent lipid peroxidation shares part of the same electron transport chain, it is reasonable to assume that lipid peroxidation activity can vary with age. However, El Defrawy *et al* (1974a) showed that lipid peroxidation in the rat is constant between 21 and 56 days of age, when activities were expressed as activity per gm liver. When expressed as activity per mg microsomal protein, lipid peroxidation tended to decrease slightly between 21 and 56 days of age. All other reports of NADPH dependent lipid peroxidation activities in microsomes have been carried out using animals of adult ages.

In mitochondria, Williams (1966) reported that spontaneous lipid peroxidation in the liver was several fold greater in the fetus when compared to the values found in adult liver mitochondria. She has also reported that

mitochondrial lipid peroxidation was similar in newborn rats and adults (Williams 1973), but no determinations were made in rats aged between birth and adulthood. The increased lipid peroxidation in fetal mitochondria may be related to a vitamin E deficiency reported in rats of this age (Rose and Gyorgy 1950).

6. Sex. Male rats have a higher lipid peroxidation activity than female rats (El Defrawy *et al* 1974), which is analagous to the higher drug oxidation activity reported for male rats (Quinn *et al* 1958).

S E C T I O N III

FORMULATION OF THE PROBLEM

The close relationship between the activity of NADPH dependent lipid peroxidation and drug metabolism in hepatic microsomes, suggested these studies on the role of lipid peroxidation in microsomal drug oxidation. The main questions considered in the study were as follows.

1. What is the normal developmental pattern of NADPH dependent lipid peroxidation in microsomes of immature rats aged from birth to adulthood? Do factors which affect lipid peroxidation in adult animals, have similar effects in immature animals?

2. To what extent does the changing activity of lipid peroxidation in the developing rat, contribute to the deficiency of drug oxidation demonstrated in immature rats?

3. Do increased lipid peroxidation activities in immature rats have an intensified effect on the function of the drug metabolizing enzyme system of microsomes compared to the effects found in the adult rat?

4. What mechanisms are involved in the reduction of drug oxidative enzymes following NADPH dependent lipid peroxidation?

5. To what extent does lipid peroxidation control the activity of drug oxidation in microsomes?

6. What is the role of NADPH dependent lipid peroxidation in the drug oxidation differences in the male and female rat?

7. What are the conditions required for lipid peroxidation of endogenous lipid in human microsomes? Are the microsomal factors which are altered by lipid peroxidation in the rat, also altered in human microsomes?

8. Can NADPH dependent lipid peroxidation occur *in vivo* and is it a factor to be considered in the overall eliminations of drugs from both animals and man?

9. What is the nature of the iron which is a required cofactor in microsomal NADPH dependent lipid peroxidation?

To date, NADPH dependent lipid peroxidation has been shown to markedly reduce the ability of microsomes to metabolize drug substrates, but its role in conditions of reduced drug oxidations, such as immaturity or in sex differences in the rat, has not been determined. It is therefore appropriate to determine the role of lipid peroxidation in these deficiencies and to establish the possible mechanism by which lipid peroxidation reduces drug oxidative activity in microsomes.

The question as to whether NADPH dependent lipid peroxidation occurs *in vivo*, was investigated, as this is crucial before lipid peroxidation can be considered a factor which must be taken into account in the overall elimination of drugs in both animals and man. Evidence available to date, has not really proved that NADPH dependent lipid peroxidation is more than a reaction confined to isolated microsomal preparations.

The overall objective of the studies described, has been to determine the role of lipid peroxidation in the alteration of drug oxidation in immature animals and man, and to establish the significance of such an interaction when drugs are used in the human patient.

MATERIALS AND METHODS

MATERIALS

A. CHEMICALS AND REAGENTS

Throughout these studies, standard reagent grade laboratory chemicals were obtained from local suppliers, and were manufactured by Fisher Scientific Co., Fairlawn, New Jersey or, by J.T. Baker Chemical Co., Phillipsburg, New Jersey.

Non-standard reagents, and reagents supplied by other than the two companies mentioned, are listed below.

Acetyl acetone: Fisher Scientific Co.

Albumin: J.T. Baker Chemical Co.

Aminopyrine: Ciba Co. Ltd., Dorval, Quebec. Gift.

Aniline, certified grade: Fisher Scientific Co.

Biuret reagent, Gornall-Bardawill-David formula: Harleco Chemical Co., Philadelphia, Pennsylvania.

Carbon monoxide: Union Carbide (Medigas), St. Laurent, Quebec.

Cytochrome c (horse heart): Sigma Chemical Co., St. Louis, Missouri.

Disodium monohydrogen phosphate: J.T. Baker Chemical Co.

Epon 812: Shell Chemicals, Montreal, Quebec.

Ethylenediaminetetra-acetic acid: Fisher Scientific Co.

Formaldehyde: British Drug Houses (Canada) Ltd., Montreal, Quebec.

Ferrous sulphate: Fisher Scientific Co.

Ferrozine[3(2-pyridyl)-5,6-diphenyl 1,2,4-triazine]: Nutritional Biochemicals Corp., Cleveland, Ohio.

Hydrazine sulphate: Eastman Organic Chemicals, Rochester, New York.

Hexobarbital: Sigma Chemical Co., St. Louis, Missouri.

8-Hydroxyquinoline: Fisher Scientific Co.

Isocitric Acid Dehydrogenase (pig heart): Sigma Chemical Co., St. Louis, Missouri.

Imferon (Iron-Dextran): Fisons Ltd., Don Mills, Ontario.

Malonaldehyde: Eastman Organic Chemicals, Rochester, New York.

NADP (Triphosphopyridine nucleotide): Sigma Chemical Co., St. Louis, Missouri.

NADPH (Triphosphopyridine nucleotide, reduced form): Sigma Chemical Co., St. Louis, Missouri.

NADH (Diphosphopyridine nucleotide, reduced form): Sigma Chemical Co., St. Louis, Missouri.

Potassium dihydrogen phosphate: J.T. Baker Chemical Co.

Sodium dithionite: Fisher Scientific Co.

Sodium Isocitrate (DL-isocitric acid, tri-sodium salt): Sigma Chemical Co., St. Louis, Missouri.

Semicarbazide hydrochloride: Sigma Chemical Co., St. Louis, Missouri.

Sulphamic acid: K and K Laboratories, Plainville, New York.

Thiobarbituric acid: Eastman Organic Chemicals, Rochester, New York.

Trichloracetic acid: Fisher Scientific Co.

Zirconia clad porous silica glass beads: Corning Biological Products Group, Medfield, Massachusetts.

B. TISSUES

1. Rats. Sprague-Dawley rats obtained from Canadian Breeding Farms, St. Constant, Quebec, were used throughout these experiments. All rats were allowed to acclimatize for 3 days before use after receipt from the breeder. Young animals were obtained from litters born in the McIntyre Animal Center, McGill University, and raised to the required age. Unless

otherwise stated, rats were male and were weaned at 21 days of age. Diet consisted of Purina rat chow and water *ad libitum*, for animals over 21 days old. Animals under 21 days old were allowed to suckle freely, and had free access to solid food and water until weaned. In experiments where a mean ± standard error was determined for several animals, the individual animals used to determine that mean were never litter mates.

2. Humans. Liver specimens were obtained from human infants during autopsies performed within a few hours of death. Tissue samples were placed in a polythene bag which was kept on ice inside a thermos jar, until the sample was homogenized and subcellular fractions prepared.

METHODS

A. PREPARATION OF MICROSOMES AND OTHER SUBCELLULAR FRACTIONS

Microsomes and other subcellular fractions were prepared by a modification of the general procedures reviewed by Fouts (1971). Liver samples, obtained from various sources, were rinsed with cold phosphate buffer (0.1 M, pH 7.4) and finely minced using scissors, and then washed and decanted three times with phosphate buffer to remove excess blood. The mince was suspended in four volumes phosphate buffer per unit weight of liver, and homogenized at 16,000 rpm in a Sorvall Omni-mix homogenizer for 20 seconds.

The homogenate was centrifuged at 10,000 x g for 10 minutes in a refrigerated centrifuge, to remove unbroken cells, cell wall fragments, nuclei and mitochondria. The floating fatty layer was removed by suction, and the supernatant which was termed 10,000 P fraction, was removed with a Pasteur pipette. The supernatant was recentrifuged at 100,000 x g for 60 minutes in a Beckman L3-40 refrigerated ultra centrifuge, to obtain a microsomal pellet which was termed microsomes or 100,000 P fraction. The microsomal pellet was resuspended in phosphate buffer, using a glass homogenizer and two strokes with a Teflon pestle, to yield a suspension containing 20-30 mg microsomal protein per ml. The supernatant from the 100,000 x g centrifugation, which was removed with a Pasteur pipette, was used in some experiments and was termed 100,000 S fraction. All enzyme determinations were carried out on freshly prepared microsomes or other subcellular fractions; as it has been our experience that microsomal enzyme activities are reduced during storage.

B. DETERMINATION OF PROTEIN IN SUBCELLULAR FRACTIONS

Protein was determined using the standard biuret method (Kabat and Meyer 1967). Commercially prepared biuret reagent was used. Albumin was used to standardize the reaction, the standard curve being determined for protein concentrations 0.5 to 6 mg albumin per ml.

C. DETERMINATION OF MICROSOMAL AMINOPYRINE N-DEMETHYLATION

The N-demethylation of aminopyrine in microsomes was determined by measuring the amount of formaldehyde, trapped as a semicarbazone, which results from the cleavage of the N-methyl group from aminopyrine (Cochin and Axelrod 1959). The reaction mixture contained 0.1 ml microsomes, 36 µmoles magnesium chloride, 24 µmoles neutralized semicarbazide HCl, aminopyrine in the amount given in the results, and 0.1 ml NADPH generating system consisting of 0.66 µmoles NADP, 16 µmoles sodium isocitrate, and 0.5 units isocitric acid dehydrogenase. Other additions, such as iron or EDTA, were added to the reaction mixture in a volume of 0.5 ml. The reaction mixture was made up to a total volume of 2 ml with phosphate buffer (0.1 M, pH 7.4). Reaction mixtures were incubated for 15 minutes at 37^{0} on a Dubnoff shaking water bath, unless otherwise stated in the Results. The reactions remained linear with respect to time utilizing these reaction conditions.

The reactions were terminated and the mixtures deproteinised by adding 0.5 ml 15% zinc sulphate followed by 0.5 ml saturated barium hydroxide. The mixtures were thoroughly agitated on a vortex mixer after each

addition, and then centrifuged at 1,500 x g for 5 minutes.

Formaldehyde was assayed in the clear supernatant by adding 1 ml Nash reagent, which contained 0.2 ml acetyl acetone + 15 g ammonium acetate in 50 ml water (Nash 1953), to 2 ml clear supernatant, and heating the mixture in a water bath at 60° for 30 minutes. Occasionally, a precipitate which could be removed by centrifugation at 1,500 x g for 5 minutes, occurred during heating. The absorbance of the cooled, clear samples was determined at 415 nm in a spectrophotometer. Formaldehyde concentration was determined by comparison to a linear standard curve, prepared using standard concentrations of formaldehyde (0.5 - 5 mg HCHO/ml). In all experiments, reaction mixtures incubated without substrate, were subtracted from the experimental incubation mixtures to correct for any formaldehyde or other material reacting with Nash reagent which may be formed from a non-substrate source. Absorbance of blank reaction mixtures varied between 0.020 and 0.035. Results were expressed as specific activity (*i.e.* nmoles formaldehyde formed/mg microsomal protein/minute).

D. EXPERIMENTS TO PROMOTE MICROSOMAL LIPID PEROXIDATION BY PREINCUBATION

In some experiments, microsomes were preincubated to promote NADPH dependent lipid peroxidation prior to the addition of substrate for the assessment of drug metabolism in peroxidized microsomes. Reaction mixtures similar to that used for aminopyrine N-demethylation (except that the aminopyrine was omitted), were preincubated for varying time periods in a shaking water bath at 37°. At the end of the preincubation period, a further 0.1 ml NADPH generating system was added, along with 0.5 ml 20 mM

aminopyrine (giving a final concentration of 5 mM), and N-demethylation was determined as previously described.

E. DETERMINATION OF MICROSOMAL CYTOCHROME P-450

Cytochrome P-450 was determined by measuring the absorbance of the carbon monoxide-reduced cytochrome P-450 complex at 450 nm (Omura and Sato 1964a). As this original method does not compensate for carbon monoxide formed in the reaction mixtures during incubation, the modification of the method by Raj and Estabrook (1970) was utilized in the present experiment. This method allows the determination of microsomal cytochrome P-450 in the presence of endogenously produced carbon monoxide, and also has the added advantage in that it eliminates interference by any contaminating hemoglobin.

Microsomes were diluted to a concentration of 2 mg microsomal protein per ml with phosphate buffer (0.1 M, pH 7.4), to give a total volume of 5 ml. The sample was gassed with carbon monoxide for thirty seconds and then divided equally between two spectrophotometer cuvettes, and 0.5 mM NADH was added to both cuvettes. A few crystals of sodium dithionite were added to the experimental cuvette and read against the other reference cuvette (without dithionite), at 490 nm and 450 nm, in a double beam spectrophotometer. Cuvettes were kept tightly closed with rubber stoppers except during additions. The molar concentration of cytochrome P-450 was calculated from the difference in absorption (450 nm - 490 nm) using an extinction coefficient of 91 nm^{-1} cm^{-1} (Omura and Sato 1964b).

F. DETERMINATION OF MICROSOMAL NADPH CYTOCHROME C REDUCTASE

NADPH cytochrome c reductase activity was estimated by determining the appearance of the reduced form of cytochrome c at 550 nm in a spectrophotometer (Phillips and Langdon 1962). Reaction mixtures contained 36 µmoles magnesium chloride, 0.15 µmoles cytochrome c, and 25 µl of microsomes, made up to 2.0 ml with phosphate buffer (0.1 M, pH 7.4), and placed in both the sample and reference cuvettes, in a dual beam spectrophotometer, with temperature control set at 37^{o} . The reaction was initiated by adding 1 µmole NADPH in 0.1 ml buffer to the experimental cuvette, and 0.1 ml buffer to the reference cuvette. The change in absorbance at 550 nm was recorded on a potentiometric recorder over the first minute of the reaction, using a chart speed of 1 inch/minute. The initial rate of change of absorbance was estimated from the initial linear portion of the reaction (usually the first 20 seconds). Using an extinction coefficient of 19.1 nm^{-1} cm^{-1} (Peters and Fouts 1970), the activity of cytochrome c reductase was calculated and expressed as nmoles cytochrome c reduced/mg microsomal protein/minute.

G. DETERMINATION OF MICROSOMAL NADPH OXIDASE

NADPH oxidase was determined by measuring the decrease in absorbance of NADPH at 340 nm, as described by Gillette *et al* (1957). The reaction mixture contained 0.1 ml microsomes and 36 µmoles magnesium chloride made up to 1.5 ml with phosphate buffer (0.1 M, pH 7.4), and 0.5 ml water containing iron or EDTA in the concentration given in Results. Two ml of

mixture was placed in both the experimental and reference cuvettes in a double beam spectrophotometer and allowed to attain 37°. The reaction was initiated by adding 0.4 µmole NADPH in 20 µl buffer to the experimental cuvette, using a Hamilton microliter syringe. The linear decrease in absorbance at 340 nm was followed for 5 minutes using a potentiometric recorder running a chart speed of 1 inch/minute. Utilizing an extinction coefficient of 6.22 mM^{-1} cm^{-1} for NADPH (Ernster 1967), NADPH oxidase activity was calculated and expressed as nmoles NADPH oxidized/mg protein/minute. The fraction of NADPH oxidation which is specific for lipid peroxidation of endogenous microsomal membrane, was determined as explained in the Results section.

H. DETERMINATION OF MICROSOMAL NADPH DEPENDENT LIPID PEROXIDATION

After incubating microsomes with NADPH or an NADPH generating system, lipid peroxidation was determined by measuring the amount of thiobarbituric acid reacting material generated, as described by Ottolenghi (1959). The reaction mixture contained 0.2 ml microsomes, 36 µmoles magnesium chloride, 0.5 ml iron or EDTA solution in water to give the final concentrations described in Results, and 0.1 ml of an NADPH generating system containing 0.66 µmoles NADP, 16 µmoles sodium isocitrate and 0.5 units isocitric acid dehydrogenase. The reaction mixture was made up to a total volume of 2 ml with phosphate buffer (0.1 M, pH 7.4). Incubations were carried out for 15 minutes in a Dubnoff shaking water bath at 37°, unless otherwise stated in the Results. To avoid the danger of iron contamination, care was taken during incubation to ensure that splashes of

water from the water bath did not contaminate the reaction mixtures.

The reaction was terminated by the addition of 0.5 ml 40% trichloroacetic acid, and the mixture cooled in an ice bath. The cooled mixture was then added to 2 ml 0.67% thiobarbituric acid in water, and heated on a water bath at 90° for 20 minutes. The mixture was then cooled and again mixed thoroughly with a further 0.5 ml 40% trichloroacetic acid. After waiting for 5 minutes, the mixture was centrifuged at 1,500 x g for 5 minutes. The absorbance of the clear supernatant was determined at 535 nm in a spectrophotometer. The reaction was quantified by comparing experimental samples to a standard curve prepared from known amounts of malonaldehyde, which is one of the known thiobarbiturate reacting products resulting from lipid peroxidation (Niehaus and Samuelsson 1968). To simplify expression of the results, lipid peroxidation was expressed as nm malonaldehyde produced/mg microsomal protein/15 minutes. Malonaldehyde has been used almost exclusively in other studies published as the standard for expressing lipid peroxidation activity.

In the experiments in which thiobarbituric acid reacting material was measured in microsomes prepared from livers of rats treated with iron to determine if lipid peroxidation products were present, 25 nmoles EDTA was added to the buffer at all steps in the preparation of the microsomes. This was done to minimize thiobarbituric acid reacting materials formed via lipid peroxidation during the process of preparation. In this determination, 0.2 ml microsomes were diluted to 2 ml followed by 0.5 ml 40% trichloroacetic acid. Thiobarbituric acid reacting products were determined as previously described.

Thiobarbituric acid solutions (0.67%) were made by dissolving thiobarbituric acid in distilled water at 60°. This provided a solution which was stable for several hours, and yielded blanks of a much lower value than when thiobarbituric acid was dissolved in dilute sodium hydroxide solution as used by some other investigators. An absorbance of 0.020 - 0.030 was usually obtained when blank incubation mixtures were reacted with thiobarbituric acid.

I. DETERMINATION OF NON-HEME IRON

Non-heme iron was determined by a modification of the method of Wills (1969b). Ferrozine [3(2-pyridyl)-5,6-diphenyl 1,2,4-triazine] was substituted for bathophenanthroline as the color reagent (Stookey 1970). Microsomes (0.2 ml) were diluted to 2 ml with water, and 0.4 ml 40% trichloroacetic acid was added and the mixture heated in a water bath at 90° for 10 minutes. After cooling, the mixtures were centrifuged at 1,500 x g for 5 minutes, and one ml clear supernatant was used for the determination of iron.

One ml of supernatant was added to a tube containing 1 ml 50% ammonium acetate, followed by 0.1 ml of a saturated solution of hydrazine and 0.2 ml ferrozine (1 mg/ml). After thorough mixing, the mixture was allowed to remain at room temperature for 20 minutes before the absorbance of the solutions was determined in a spectrophotometer at 562 nm. Iron concentration was assessed by comparison to a standard curve prepared using ferrous sulphate. Values were expressed as nmoles non-heme iron/mg protein.

J. DETERMINATION OF MICROSOMAL DIFFERENCE SPECTRA AND SPECTRAL DISSOCIATION CONSTANTS

Difference spectra and corresponding spectral dissociation constants, were determined for aniline and hexobarbital, between the wavelengths 350 to 500 nm, in a double beam spectrophotometer, as described by Remmer *et al* (1966) and Schenkman *et al* (1967a). Microsomal suspensions were diluted to a protein concentration of 2 mg/ml using phosphate buffer (0.1 M, pH 7.4). Two ml of the diluted microsomes were placed in each of the two cuvettes in a double beam spectrophotometer. A blank spectrum was then determined, using a potentiometric recorder chart speed of 1 inch/minute and a wavelength scan speed of 50 nm/minute. Substrate (either hexobarbital or aniline) was added to the experimental cuvette in a volume which was always less than 20 µl, to yield the overall concentrations of 0.05 - 1 mM as detailed in the Results. A corresponding volume of buffer was added to the reference cuvette and the spectrum determined. The difference between the experimental and blank spectrum was plotted to yield a corrected difference spectrum between 350 nm and 500 nm.

To determine spectral dissociation constants, the difference in extinction between 500 nm and 425 nm ($\Delta OD_{500-425}$) for hexobarbital, and between 430 nm and 390 nm ($\Delta OD_{430-400}$) for aniline, was determined over the range of substrate concentrations given in Results. Double reciprocal plots of $1/\Delta OD$ *vs* 1/concentration, were constructed. Approximate spectral dissociation constants were determined by extrapolating the line to the x axis, yielding an intercept = Ks (Schenkman *et al* 1967a). Because of limitations in assessing Ks from linear double reciprocal plots, spectral

dissociation constants for each of the substrates were determined by fitting the data to a rectangular hyperbola equation with a computer program as described in the statistics section of Methods.

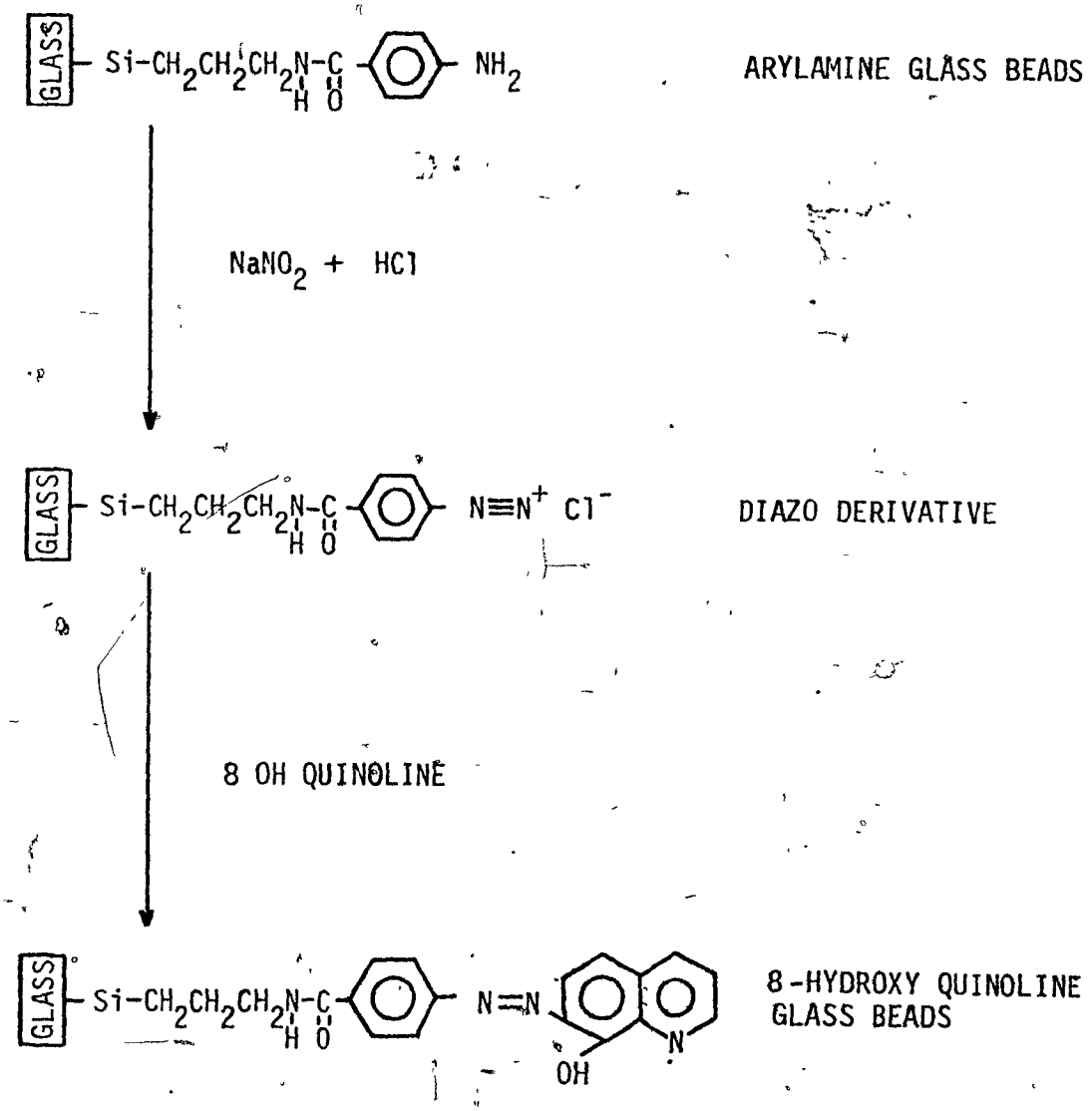
In experiments designed to determine the effects of preincubation on difference spectra, the preincubations were carried out in 2 ml of an incubation mixture containing 0.2 ml microsomes, 36 μmoles magnesium chloride, 0.1 ml NADPH generating system containing 0.66 μmoles NADP, 16 μmoles sodium isocitrate and 0.5 units isocitric acid dehydrogenase and iron in the concentrations described in Results. Immediately prior to the determination of difference spectra by the method just described, microsomal suspensions were diluted to 2 mg microsomal protein/ml.

K. PREPARATION OF 8-HYDROXYQUINOLINE COVALENTLY BOUND TO GLASS BEADS

The arylamine derivative of zirconia clad porous silica glass beads (MAO 3930, Corning) was prepared by Dr. Man Sen Yong, Department of Pharmacology, McGill University, Montreal, by the method of Weetal (1969). These arylamine glass beads were then used in the synthesis of an 8-hydroxyquinoline derivative as outlined in figure 1. Arylamine glass beads (200 mg) were diazotized in 50 ml 0.5 M HCl with 30 mg sodium nitrite and stirred in an ice bath for 30 minutes. The product was washed with 100 ml cold water, 100 ml 1% sulphamic acid solution and then 100 ml cold water. The diazotized glass beads were subsequently coupled to 8-hydroxyquinoline by adding the beads to 50 ml saturated solution of 8-hydroxyquinoline in 0.05 M sodium carbonate. The product was then placed in a Buchner funnel (medium disc size) at room temperature and washed with 5 litres 1 M sodium chloride.

FIGURE 1. The synthesis of 8-OH quinoline covalently linked to zirconia clad porous silica glass beads.

Details of the synthesis of 8-OH quinoline glass beads from arylamine glass beads are outlined in Methods.


GLASS
Si-CH2CH2CH2N-C
H O
NH2
ARYLAMINE GLASS BEADS
NaNO2 + HCl
GLASS
Si-CH2CH2CH2N-C
H O
N≡N+ Cl-
DIAZO DERIVATIVE
8 OH QUINOLINE
GLASS
Si-CH2CH2CH2N-C
H O
N=N
OH
N
8-HYDROXY QUINOLINE
GLASS BEADS

The product was then washed with 25 litres distilled water over a period of two days. The 8-hydroxyquinoline glass beads were stored under 100 ml 0.1 M HCl at 4°C. Immediately prior to use, the beads were washed with 5 x 20 ml aliquots distilled water to remove the HCl.

L. ABSORPTION OF IRON BY 8-HYDROXYQUINOLINE GLASS BEADS

8-Hydroxyquinoline immobilized on glass beads was used to remove free iron from solutions and from microsomal suspensions. In most experiments, a portion of glass beads were blotted on a filter paper and 10 mg of damp beads were weighed. All weights of glass beads referred to in Results signify this wet weight. The glass beads and solutions or suspensions of microsomes were mixed and gently shaken on a water bath at 37°. The mixtures were then centrifuged at 1,500 x g for 5 minutes to sediment the glass beads. Supernatants of microsomes treated in this way were regarded as free iron deficient preparations. The supernatant was then removed and utilized for determination of iron, aminopyrine N-demethylation activity or NADPH dependent lipid peroxidation activity.

M. GLASSWARE - REDUCTION OF IRON CONTAMINATION.

Because of the extreme sensitivity of lipid peroxidation reactions to the concentration of iron in incubation mixtures, it was of importance to minimize iron contamination of the glassware used in these studies. Inconsistent lipid peroxidation activities were found in preliminary studies using glassware washed with detergent, hot water and rinsed with distilled

water and dried in an air circulating oven. To further minimize iron contamination in this study, all test tubes used were chemical free disposable glassware (Corning Cat. No. 99445). The use of these tubes for all incubations and most procedures, greatly reduced the tube-to-tube variation in lipid peroxidation determinations. When other glassware was required, it was washed in 0.1 M HCL and deionized water immediately prior to use, to reduce iron contamination. As far as possible, all pipetting was carried out using automatic pipetting devices with disposable polyethylene tips. When pipettes of a larger size were required, all glass pipettes were used which had been washed in chromic acid and rinsed in deionized water.

N. ELECTRON MICROSCOPY OF MICROSOMAL PELLETS

Microsomes used for electron microscopy were prepared in a manner similar to that already described in section A of Methods. The only difference was the addition of 25 μM EDTA to the phosphate buffer at all stages of the preparation.

Microsomal pellets (100,000 x g pellet) were fixed in 3% glutaraldehyde in phosphate buffer (0.1 M pH 7.4) for 3 hours at room temperature. After the first 30 minutes in the fixative, the pellet was gently lifted from the bottom of the centrifuge tube and cut into 1 mm wide slices and then replaced in the fixative. After the 3 hour fixing period, the tissue was washed 3 times with phosphate buffer and allowed to stand in phosphate buffer overnight at 4^{0}. The tissue was then dehydrated in graded ethyl alcohol solutions (70% up to 100%) and embedded in Epon 812. Sections were cut with a diamond knife and mounted on copper grids. After staining with

uranyl acetate and lead citrate, sections were examined under a Philips 300 electron microscope.

0. STATISTICAL METHODS AND COMPUTER PROGRAMING

1. The students t test for unpaired data was used in this study to determine statistical significance of the difference between two means. Significance throughout the study was defined as being at the 5% level, *i.e.*, $p < 0.05$.

2. Estimation of the spectral dissociation constants (Ks) in substrate difference spectra experiments, was carried out by an iterative fit of the data (ΔOD *vs* substrate concentration) (Wilkinson 1961) to the standard Michaelis and Menten equation (Michaelis and Menten 1913) used to describe enzyme catalyzed reactions. Absorbance change was substituted for velocity in the original equation as follows:

$$\Delta OD = \frac{\Delta OD \text{ max} \times S}{Ks + S}$$

where ΔOD = absorbance change

Ks = spectral dissociation constant

S = substrate concentration

The equation is in the form of a rectangular hyperbola. The iterative fit of experimental data to the equation was carried out using a FORTRAN computer program described by Cleland (1966). The original program was slightly modified to enable its use on a remote MUSIC terminal connected to the main computer center at McGill University. An IBM 360 computer was used in the system. The program estimates Ks, OD max, 1/Ks, 1/OD max, and

Ks/OD max, which were used to fit the line on the double reciprocal plots.

The symbols used in the FORTRAN program, and the program itself arranged for use in the McGill remote terminal MUSIC system, and a typical arrangement for data input, is illustrated on the following pages.

Symbols used in the program:

V(1), V(2), *etc.*	Experimental velocities
A(1), A(2), *etc.*	Corresponding substrate concentrations
W(1), W(2), *etc.*	Weighting factors for velocities
S(1,1), S(2,1) S(1,2), *etc.*	The array in which matrix 9 is set up. After the solution is obtained, this array contains the regression coefficients and the inverse matrix.
Q(10, Q(2), *etc.*	The expressions in equation 6.
SM(1), SM(2), *etc.*	Scale factors used to equalize diagnols of the matrix during solution.
SS(1), SS(2), *etc.*	During solution, the S array is overprinted several times. Information in the first column which is erased by the overprinting but still needed during the calculations is stored in the SS array.
JJ	Number of data sets processed.
NP	Number of data points in a given data set (number of data cards following title card).
M	With M = 1, the matrix solution subroutine uses statements 15 and 16 to make the preliminary fit as discussed in Section II-D. With M = 2, statements 17 and 18 are used to make the iterative fit described in Section II-E.
N	The number of constants to be determined (2 in this program). The matrix solution subroutine and certain other statements are common to all programs and are written in terms of N,N+1, and N+2.
CK	The constant K in equation 1. CK is first preliminary estimate of K, but becomes refined to its final value by the iterative procedure. In statement 18, for instance, CK on the right is the preliminary estimate; on the left, the newly refined estimate.
NT	Number of iterations. This is set at 3 here, but may be set to any number by changing the IF (NT - ?) statement.
D	Denominator; used to simplify arithmetic in calculating Q's for the iterative fit.
S2	Experimental variance (residual least square)
S1	Square root of experimental variance (sigma)
SL, VINT, VK	K/V (slope of the line described by equation 2); 1/V (vertical intercept of this line); V/K.
SEV, SECK, SEVI, SESL, SEVK	Standard errors of the estimates of V (maximum velocity), K (Michaelis constant), 1/V, K/V, V/K.
WCK, WV, WSL, WVI, WVK	Weighting factors for further analysis for K, V, K/V, 1/V, V/K.
I, J, K, L	Counting indices for various DO loops in the program.

PROGRAM:

```
0001 /LOAD FORTG
0002       DIMENSION V(100),A(100),W(100),S(3,4),Q(3),SM(3),SS(3)
0003
0004
0005       PRINT 100
0006 100   FORMAT(35H FIT TO HYPERBOLA - V=    VMAX*A(K+A)///)
0007 11    FORMAT(I3,17X,48H
     )
0008 1     FORMAT (3F10.5)
0009       JJ=0
0010 14    READ 11,NP
0011       IF (NP) 99,99,12
0012 12    M=1
0013       N=2
0014       P=NP-N
0015       N1=N+1
0016       N2=N+2
0017       GO TO 2
0018 15    READ 1, V(I),A(I),W(I)
0019       IF (W(I)) 19,19,20
0020 19    W(I)=1.
0021 20    Q(1)=V(I)**2/A(I)
0022       Q(2)=V(I)**2
0023       Q(3)=V(I)
0024       GO TO 13
0025 16    CK=S(1,1)/S(2,1)
0026       JJ=JJ+1
0027       PRINT 11,JJ
0028       NT=0
0029       M=2
0030       GO TO 2
0031 17    D=CK+A(I)
0032       Q(1)=A(I)/D
0033       Q(2)=Q(1)/D
0034       Q(3)=V(I)
0035       GO TO 13
0036 18    CK=CK-S(2,1)/S(1,1)
0037       NT=NT+1
0038       IF (NT-3) 2,21,21
0039 21    S2=0
0040       DO 22 I=1,NP
0041 22    S2=S2+(V(I)-S(1,1)*A(I)/(CK+A(I)))**2*W(I)
0042       S2=S2/P
0043       S1=SQRT(S2)
0044       SL=CK/S(1,1)
0045       VINT=1./S(1,1)
0046       VK=1./SL
0047       DO 10 J=2,N1
0048       DO 10 K=1,N
0049 10    S(K,J)=S(K,J)*SM(K)*SM(J-1)
0050       SEV=S1*SQRT(S(1,2))
```

```
0051          SECK=S1*SQRT(S(2,3))/S(1,1)
0052          SEVI=SEV/S(1,1)**2
0053          S(1,3)=S1*SQRT(CK**2*S(1,2)+S(2,3)+2*CK*S(1,3))
0054          SESL=S(1,3)/S(1,1)**2
0055          SEVK=S(1,3)/CK**2
0056          WCK=1./SECK**2
0057          WV=1./SEV**2
0058          WSL=1./SESL**2
0059          WVI=1./SEVI**2
0060          WVK=1./SEVK**2
0061          PRINT 30,CK,SECK,WCK
0062          PRINT 31,S(1,1),SEV,WV
0063          PRINT 32,SL,SESL,WSL
0064          PRINT 33,VINI,SEVI,WVI
0065          PRINT 34,VK,SEVK,WVK
0066          PRINT 35,S2,S1
0067 30       FORMAT(7H K   = F12.6,13HS.E.(K)     = F11.6,5H W = E14.5)
0068 31       FORMAT(7H V   = F12.6,13HS.E.(V)     = F11.6,5H W = E14.5)
0069 32       FORMAT(7H K/V = F12.6,13HS.E.(K/V)   = F11.6,5H W = E14.5)
0070 33       FORMAT(7H 1/V = F12.6,13HS.E.(1/V)   = F11.6,5H W = E14.5)
0071 34       FORMAT(7H V/K = F12.6,13HS.E.(V/K)   = F11.6,5H W = E14.5)
0072 35       FORMAT (12H VARIANCE = E14.5,10H  SIGMA = F12.7//)
0073          GO TO 14
0074 C        MATRIX SOLUTION SUBROUTINE
0075 2        DO 3 J=1,N2
0076          DO 3 K = 1,N1
0077 3        S(K,J)=0
0078          DO 4 I=1,NP
0079          GO TO (15,17),M
0080 13       DO 4 J=1,N1
0081          DO 4 K=1,N
0082 4        S(K,J)=S(K,J)+Q(K)*Q(J)*W(I)
0083          DO 5 K=1,N
0084 5        SM(K)=1./SQRT(S(K,K))
0085          SM(N1)=1.
0086          DO 6 J=1,N1
0087          DO 6 K=1,N
0088 6        S(K,J)=S(K,J)*SM(K)*SM(J)
0089          SS(N1)=-1.
0090          S(1,N2)=1.
0091          DO 8 L=1,N
0092          DO 7 K=1,N
0093 7        SS(K)=S(K,1)
0094          DO 8 J=1,N1
0095          DO 8 K=1,N
0096 8        S(K,J)=S(K+1,J+1)-SS(K+1)*S(1,J+1)/SS(1)
0097          DO 9 K=1,N
0098 9        S(K,1)=S(K,1)*SM(K)
0099          GO TO (16,18),M
0100
0101 36       FORMAT(23H PROGRAM COMPLETED FOR I4,6H LINES)
0102 99       PRINT 36,JJ
0103          STOP
0104          END
0105 /DATA
```

DATA MATRIX:

```
0105 /DATA
0106   6
0107     0.004      0.10
0108     0.008      0.20
0109     0.010      0.30
0110     0.013      0.50
0111     0.015      0.70
0112     0.018      1.00
0113
0114 END
0115 /END
```

Note: data deck must end with a blank card which is line 113 in this example.

RESULTS

A. MICROSOMAL NADPH DEPENDENT LIPID PEROXIDATION AND ITS EFFECTS ON AMINOPYRINE N-DEMETHYLATION AND NADPH OXIDASE IN THE DEVELOPING AND ADULT RAT

The activity of NADPH dependent lipid peroxidation in hepatic microsomes prepared from male rats aged from birth to adulthood is shown in figure 2. A low level of 0.34 ± 0.04 nmoles malonaldehyde/mg microsomal protein/15 minutes was noted at birth and maintained close to that level for the first two weeks of life. Between two weeks and three weeks of age, activity increased sharply to reach a maximum level of 3.52 ± 0.35 nmoles malonaldehyde/mg microsomal protein/15 minutes at 25 days of age. Activity decreased gradually over the following three weeks to a value between 0.20 and 0.30 nmoles malonaldehyde/mg microsomal protein/15 minutes, which was maintained into the adult age group. In the experiment illustrated, adult activity in rats weighing 290 gms was 0.20 ± 0.04 nmoles malonaldehyde/mg microsomal protein/15 minutes. EDTA (25 μM) added to the incubation mixtures, inhibited NADPH dependent lipid peroxidation by at least 90% at all ages tested, as indicated by the dotted line in figure 2.

Stimulation of NADPH dependent lipid peroxidation activity by ferrous iron and its inhibition by EDTA in rats aged 21 days, 25 days, and adults, is illustrated in figure 3. In control incubation mixtures, without additions of either iron or EDTA, lipid peroxidation activity was respectively, 1.88 ± 0.17, 3.79 ± 0.11, and 0.15 ± 0.03 nmoles malonaldehyde/mg microsomal protein/15 minutes for 21 day, 25 day, and adult rats. The addition of 25 μM ferrous sulphate to the reaction mixtures, stimulated NADPH dependent lipid peroxidation activity to 13.13 ± 0.58, 19.69 ± 0.69

FIGURE 2. NADPH dependent lipid peroxidation in hepatic microsomes prepared from rats aged from birth to adulthood.

Each value represents the mean ± S.E. of the results obtained from 5 individual rats. ●——● normal lipid peroxidation, o------o lipid peroxidation in the presence of 25 μM EDTA.

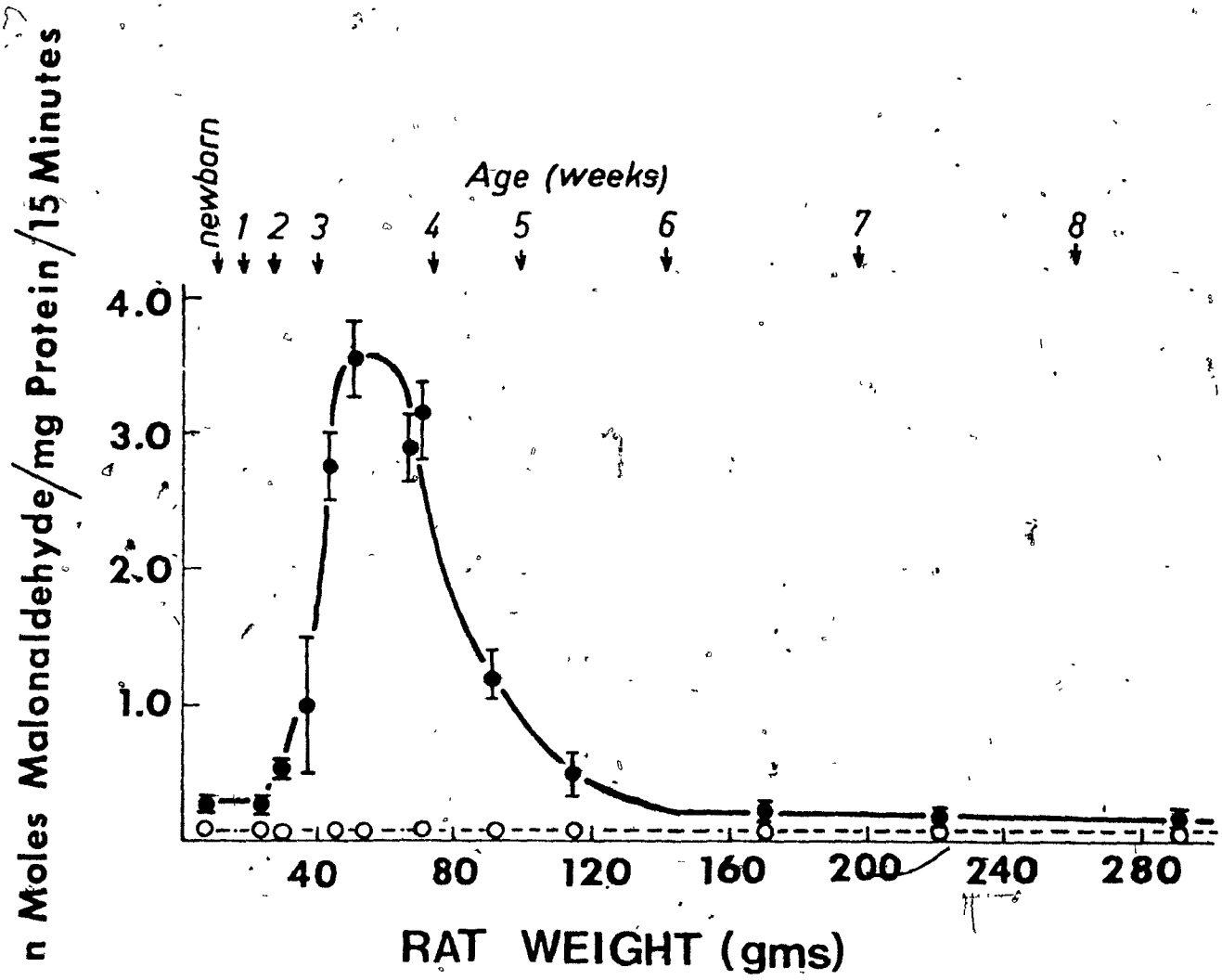
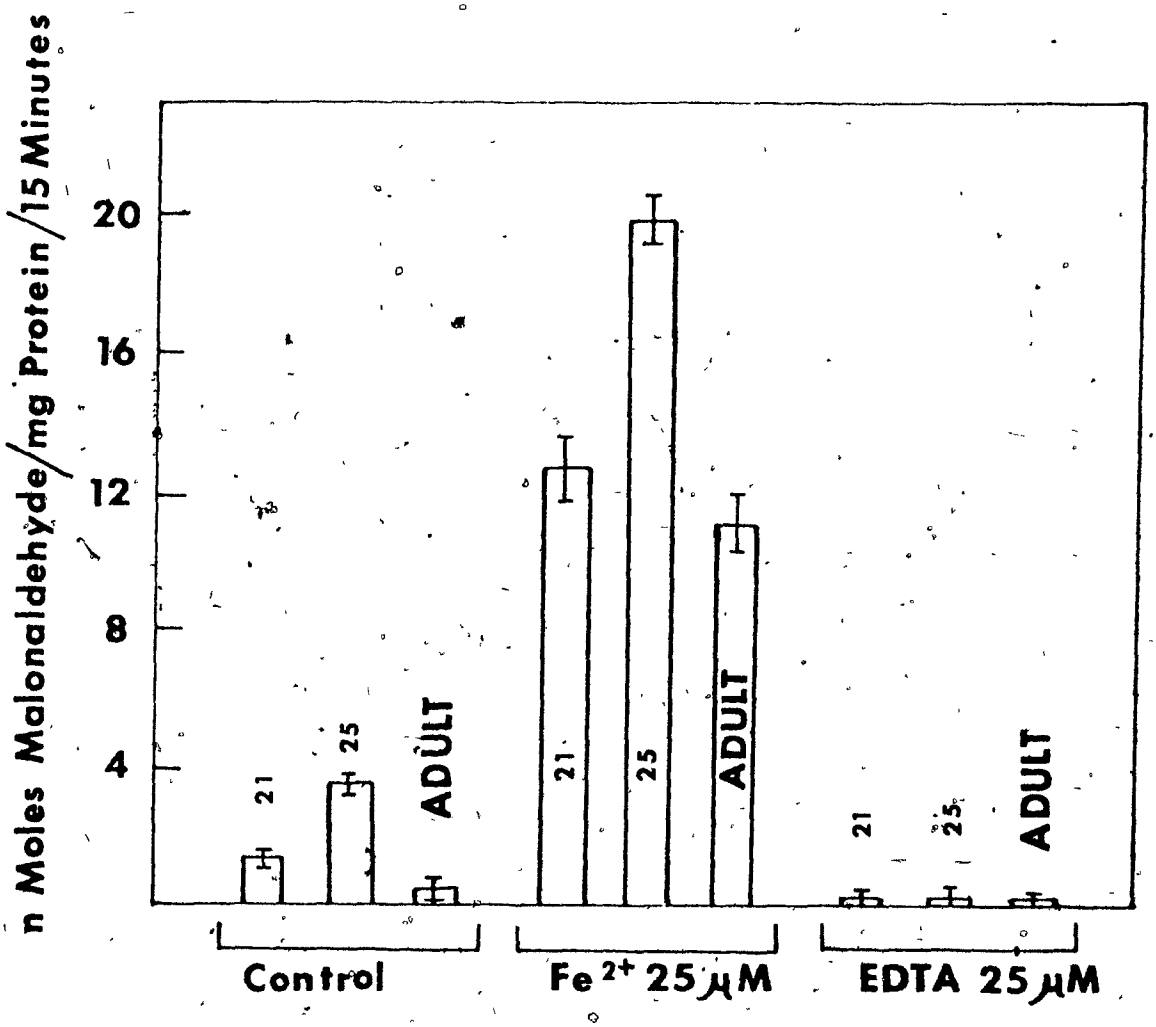

n Moles Malonaldehyde/mg Protein/15 Minutes
Age (weeks)
newborn
1
2
3
4
5
6
7
8
4.0
3.0
2.0
1.0
40
80
120
160
200
240
280
RAT WEIGHT (gms)

FIGURE 3. The effect of iron and EDTA on NADPH dependent lipid peroxidation in hepatic microsomes prepared from 21 day, 25 day and adult rats.

Incubation mixtures containing 25 μM ferrous sulphate or 25 μM EDTA where indicated. Each Bar represents the mean ± S.E. obtained from 5 individual incubation mixtures containing microsomes pooled from 3 rats.


n Moles Malonaldehyde/mg Protein/15 Minutes
20
16
12
8
4
21
25
ADULT
21
25
ADULT
21
25
ADULT
Control
Fe 2+ 25 µM
EDTA 25 µM

and 11.47 ± 0.41 nmoles malonaldehyde/mg microsomal protein/15 minutes for 21 day, 25 day and adult aged rats, respectively. This represents an 11-fold stimulation at 21 days, 5.3-fold stimulation at 25 days and 77-fold stimulation in adults, for NADPH dependent lipid peroxidation activity in microsomes. In all age groups, NADPH dependent lipid peroxidation was almost totally inhibited by 25 μM EDTA added to the reaction mixtures.

As illustrated in figure 4, NADPH dependent lipid peroxidation activity in the presence of 25 μM ferrous iron was stimulated to a maximum in both 25 day and adult rat microsomes. Maximum stimulation of NADPH dependent lipid peroxidation was attained at an iron concentration of 5 μM in 25 day old rats and was attained at an iron concentration of 15 μM in adult rats. EDTA inhibited NADPH dependent lipid peroxidation at concentrations ranging from 1 to 25 μM when added to reaction mixtures containing microsomes prepared from 25 day and adult rats (figure 5). Because the basal levels of lipid peroxidation differ in 25 day and adult rats, the data has been replotted in the inset of the diagram using % inhibition of control activity for each particular age group. The % inhibition for the two ages studied was identical, and the I_{50} as calculated from the graph for both 25 day and adult rats, was approximately 2 μM EDTA.

The time course of NADPH dependent lipid peroxidation in 25 day and adult rats is shown in figure 6. Lipid peroxidation continued to increase for at least 20 minutes for both control and iron stimulated microsomes in both age groups. The reactions are, however, not linear, particularly in the case of the reaction mixtures containing 25 μM iron. Because of this deviation from linearity, lipid peroxidation was expressed as malonaldehyde

FIGURE 4. The stimulation of NADPH dependent lipid peroxidation by different concentrations of ferrous iron in hepatic microsomes prepared from 25 day old and adult rats.

Incubation mixtures contained ferrous sulphate in concentrations ranging from 1 μM to 25 μM. Each point represents an individual incubation mixture containing microsomes obtained from a pool of 3 rats. ●——● 25 day old rats, o——o adult rats.

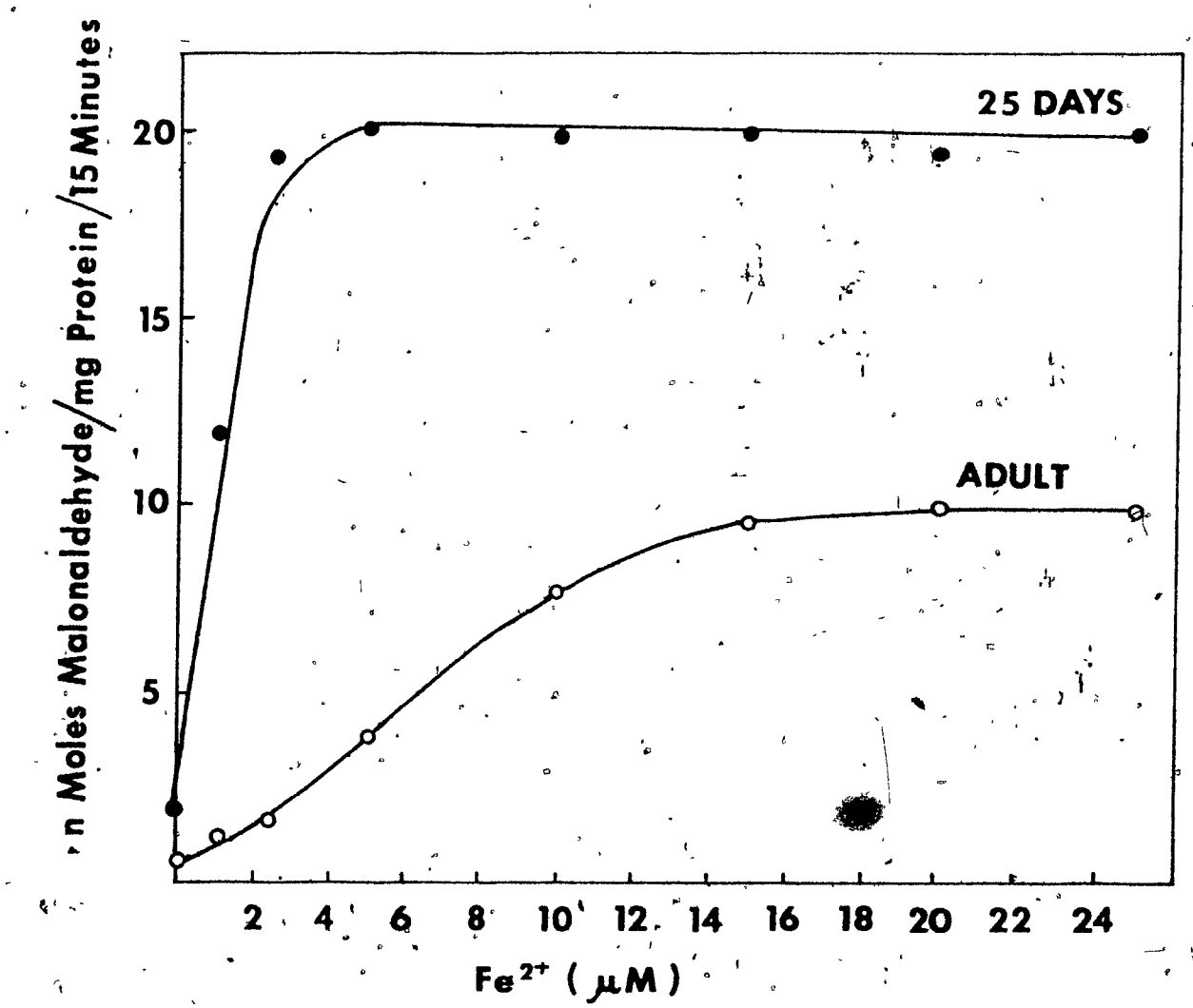

25 DAYS
ADULT
n Moles Malonaldehyde/mg Protein/15 Minutes
20
15
10
5
2
4
6
8
10
12
14
16
18
20
22
24
Fe2+ (μM)

FIGURE 5. The inhibition of NADPH dependent lipid peroxidation by different concentrations of EDTA in hepatic microsomes prepared from 25 day old and adult rats.

Incubation mixtures contained EDTA in concentrations ranging from 1 μM to 25 μM. Each point represents an individual incubation mixture containing microsomes obtained from a pool of 3 rats. In the main diagram, inhibition is illustrated by a decrease in the actual amount of malonaldehyde produced. This data is replotted in the inset using % inhibition of control activity for each age group. ●——● 25 day old rats, o——o adult rats.

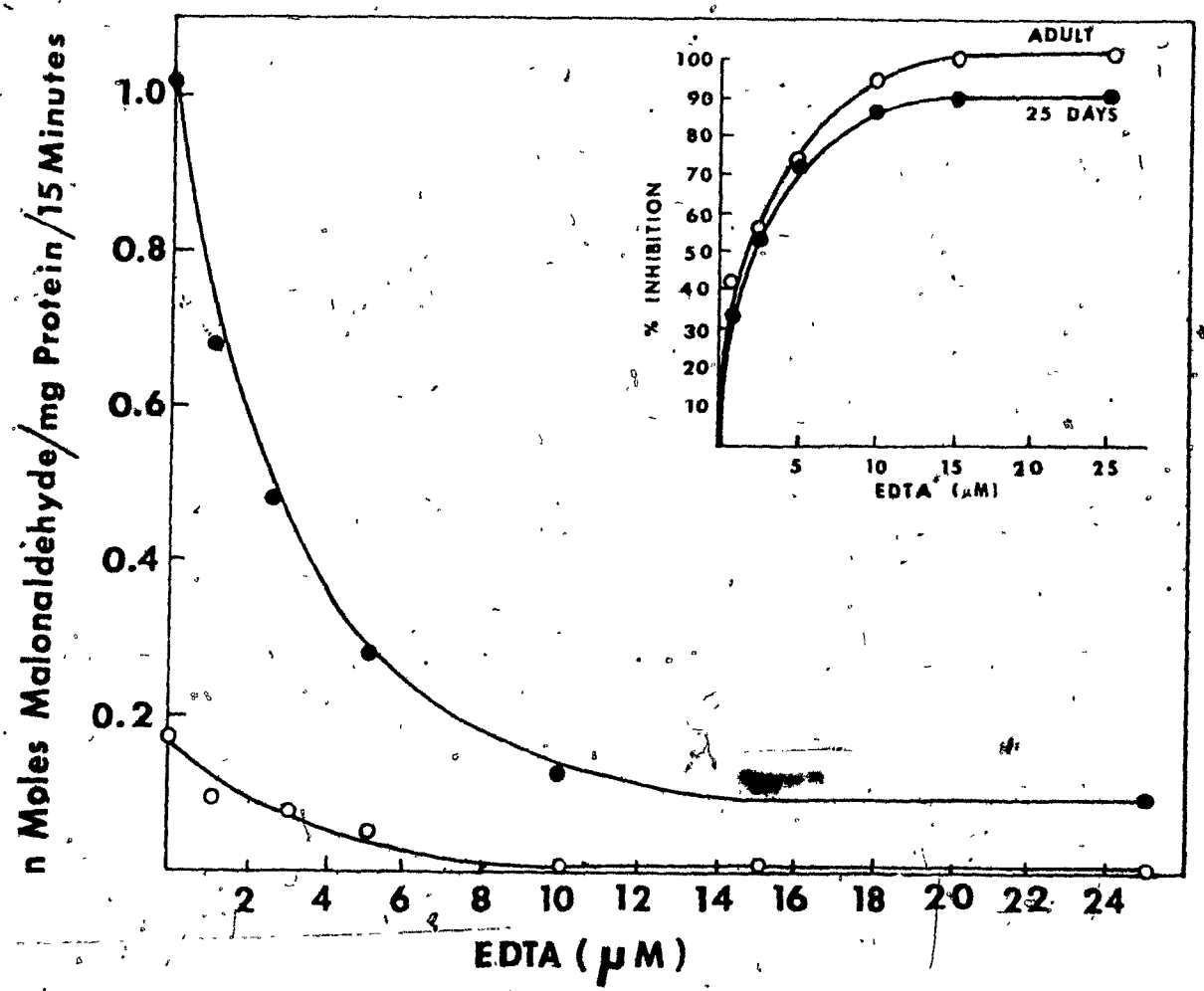
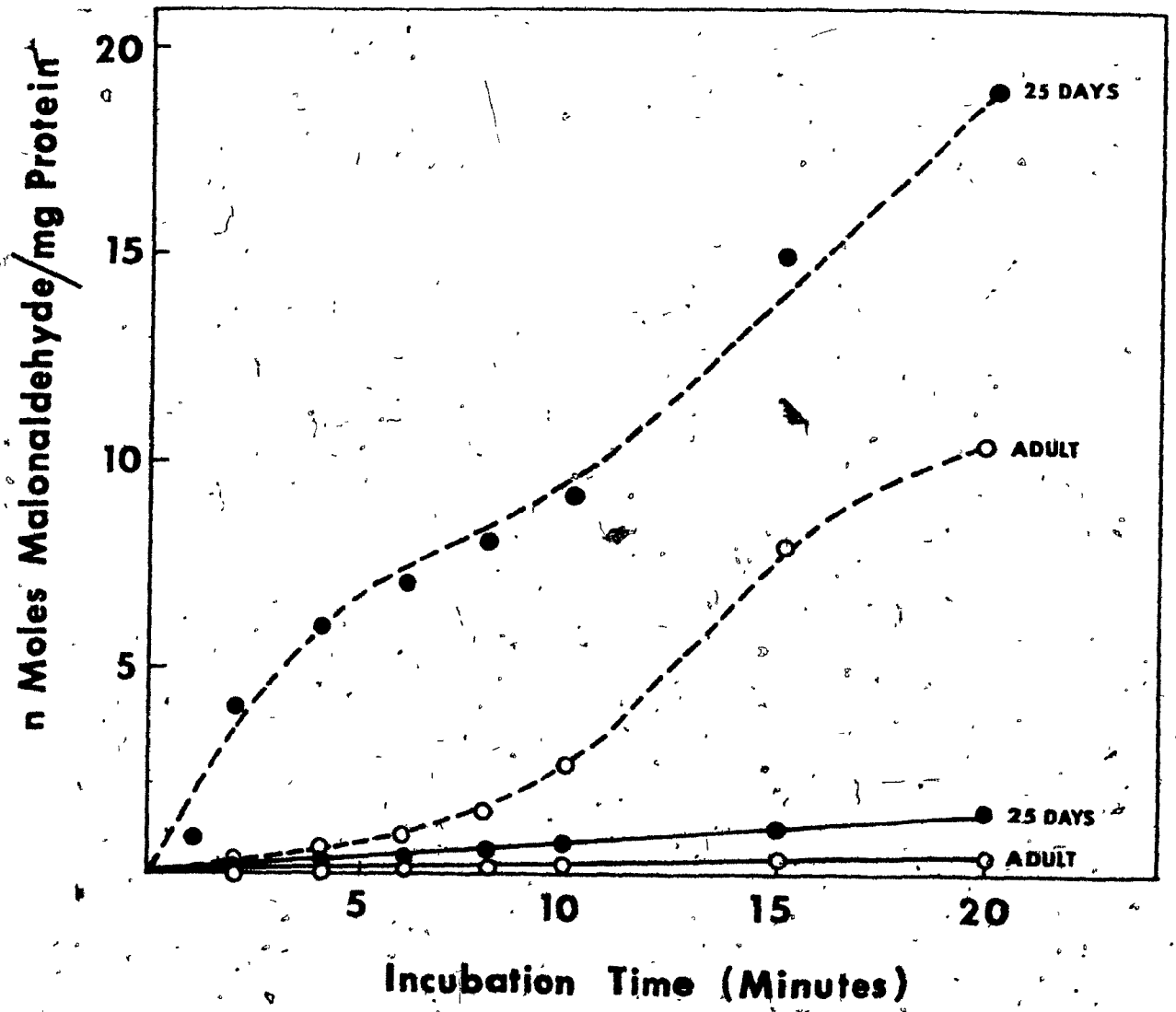

n Moles Malonaldehyde/mg Protein/15 Minutes
1.0
0.8
0.6
0.4
0.2
2
4
6
8
10
12
14
16
18
20
22
24
EDTA (μM)
ADULT
25 DAYS
% INHIBITION
100
90
80
70
60
50
40
30
20
10
5
10
15
20
25
EDTA (μM)

FIGURE 6. The time course of NADPH dependent lipid peroxidation in control and iron stimulated hepatic microsomes prepared from 25 day old and adult rats.

Incubation mixtures with and without 25 μM iron added, were incubated for periods ranging from 0 to 20 minutes. Each point represents an individual incubation mixture containing microsomes obtained from a pool of 3 rats. ●———● 25 day old rats (control), ●------● 25 day old rats (25 μM Fe^{2+}), o———o adult rats (control), o------o adult rats (25 μM Fe^{2+}).


n Moles Malonaldehyde/mg Protein
20
15
10
5
25 DAYS
ADULT
25 DAYS
ADULT
5
10
15
20
Incubation Time (Minutes)

formed/15 minutes incubation time, which was the incubation time utilized throughout the experiments.

Non-heme iron levels determined in rat microsomes prepared from rats aged 25 days and adults, are shown in table 1. The level of 7.06 ± 0.31 nmoles non-heme iron/mg microsomal protein in 25 day old rats was not significantly different from the level of 7.44 ± 0.26 nmoles non-heme iron/mg protein in adult rats.

Aminopyrine, which was the substrate for drug oxidation used throughout this study, inhibited NADPH dependent lipid peroxidation at concentrations ranging from 0.1 to 10 mM when added to reaction mixtures containing microsomes prepared from 25 day and adult rats (figure 7). Because the basal levels of lipid peroxidation differ in 25 day and adult rats, the data has been plotted in the inset of the diagram using % inhibition of control activity for each particular age group. The % inhibition for the two ages studied is identical, and the I_{50}, as calculated from the graph for both 25 day and adult rats, is approximately 1 mM aminopyrine.

Microsomes were preincubated without substrates for 10 and 30 minutes with an NADPH generating system to promote lipid peroxidation. Aminopyrine was then added and the rate of aminopyrine N-demethylation determined and compared to aminopyrine N-demethylation activity in microsomes which had not been preincubated (figures 8 and 9). To minimize lipid peroxidation activity after the preincubation period, a concentration of 5 mM aminopyrine was used for the determination of N-demethylation activity. As already shown in figure 7, 5 mM aminopyrine inhibits NADPH dependent lipid peroxidation by 90%. In 25 day old rats (figure 8), preincubation of control

Table 1

Non-Heme Iron Content of Microsomes Prepared From 25 Day Old And Adult Rats

Age	Non-Heme Iron (nmoles/mg protein)
25 days	7.06 ± 0.31
Adult	7.44 ± 0.26

Values represent the mean ± standard error of 5 individual rats which were not litter mates.

$p > 0.05$

FIGURE 7. The inhibition of NADPH dependent lipid peroxidation by different concentrations of aminopyrine in hepatic microsomes prepared from 25 day old and adult rats.

Incubation mixtures contained aminopyrine in concentrations ranging from 0.1 to 10 mM. Each point represents an individual incubation mixture containing microsomes obtained from a pool of 3 rats. In the main diagram, inhibition is illustrated by a decrease in the actual amount of malonaldehyde produced. This data is replotted in the inset using % inhibition of control activity for each age group. ●——● 25 day old rats, o——o adult rats.

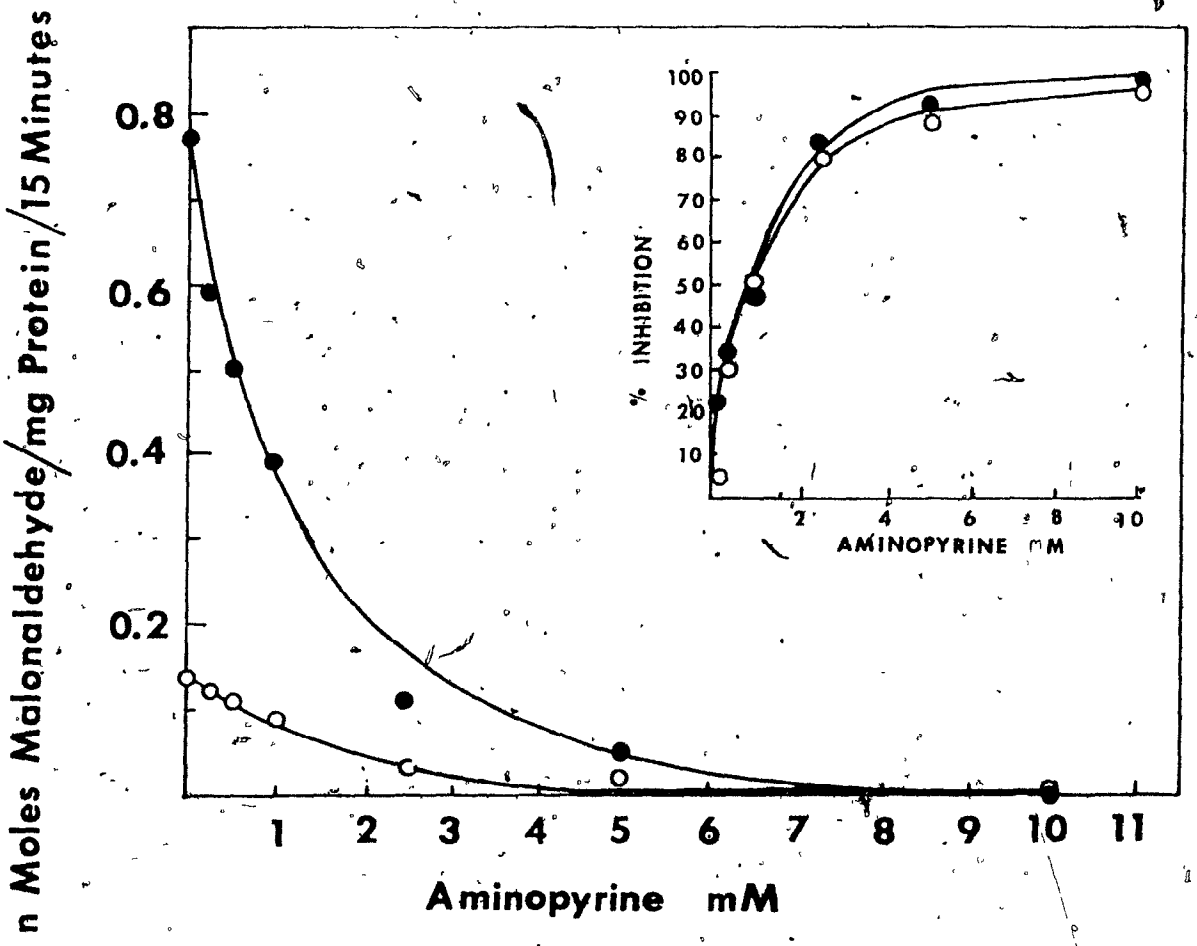

n Moles Malonaldehyde/mg Protein/15 Minutes
0.8
0.6
0.4
0.2
1
2
3
4
5
6
7
8
9
10
11
Aminopyrine mM
% INHIBITION
100
90
80
70
60
50
40
30
20
10
2
4
6
8
10
AMINOPYRINE mM

FIGURE 8. The effect of preincubation of hepatic microsomes with an NADPH generating system and iron or EDTA on the subsequent N-demethylation of 5 mM aminopyrine in 25 day old rats.

Preincubations were carried out for the times indicated under the conditions detailed in Results before N-demethylation of aminopyrine was determined. Each value represents the mean ± S.E. obtained from 5 individual incubation mixtures which contained microsomes pooled from 6 rats aged 25 days.

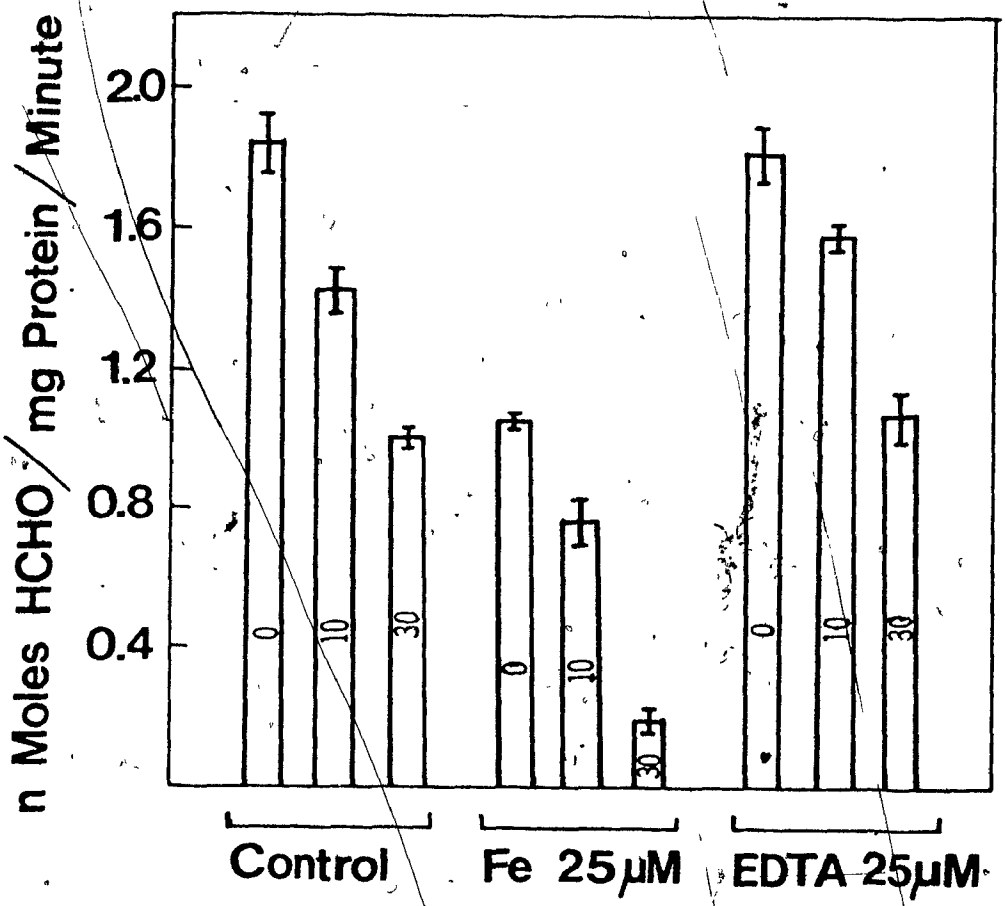
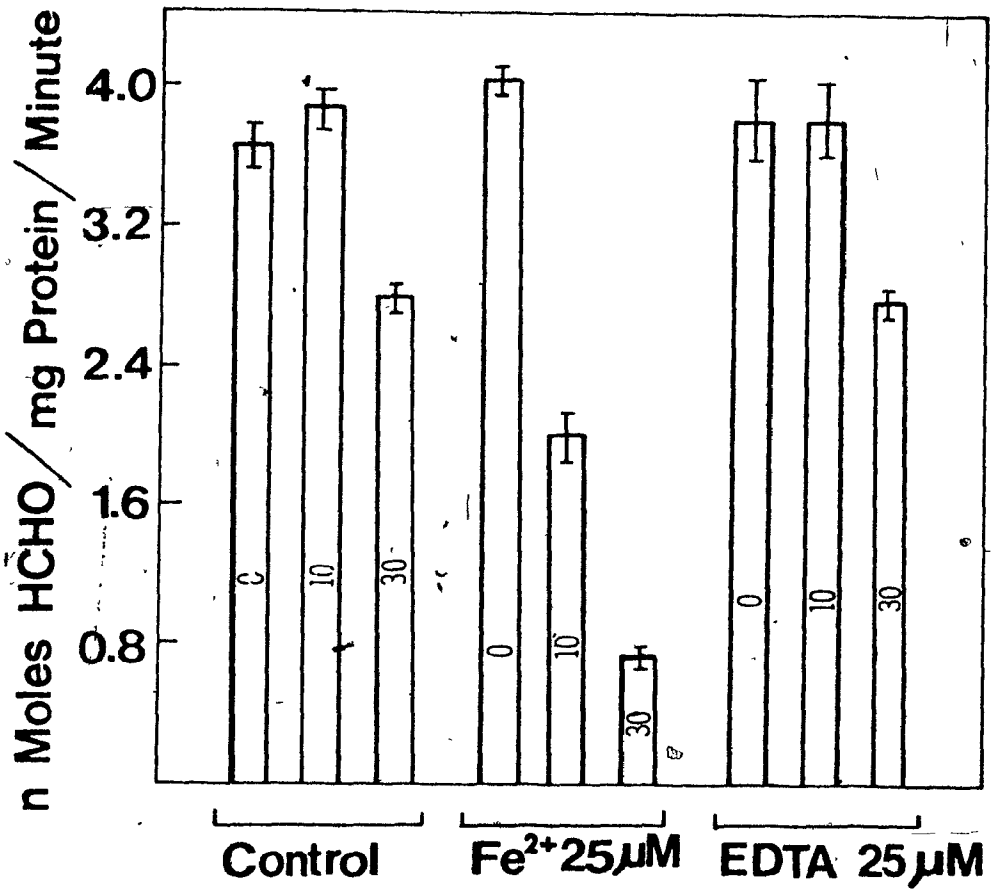

n Moles HCHO / mg Protein / Minute
2.0
1.6
1.2
0.8
0.4
0
10
30
0
10
30
0
10
30
Control
Fe 25µM
EDTA 25µM

FIGURE 9. The effect of preincubation of hepatic microsomes with an NADPH generating system and iron or EDTA on subsequent N-demethylation of 5 mM aminopyrine in adult rats.

Preincubations were carried out for the times indicated under the conditions detailed in Results and the N-demethylation of aminopyrine was determined. Each value represents the mean ± S.E. obtained from 5 individual incubation mixtures which contained microsomes pooled from 6 adult rats.


n Moles HCHO / mg Protein / Minute
4.0
3.2
2.4
1.6
0.8
0
10
30
0
10
30
0
10
30
Control
Fe2+25μM
EDTA 25μM

microsomes with an NADPH generating system had a marked effect in reducing the activity of aminopyrine N-demethylation. After 10 minutes preincubation, aminopyrine N-demethylation was reduced by 20% and after 30 minutes preincubation, was reduced by 50%. The addition of 25 μM ferrous iron to the preincubation reaction mixture, produced a further decrease in the ability of microsomes to N-demethylate aminopyrine. In these incubation mixtures, N-demethylation was reduced by 50%, 60% and 90%, after preincubation times of 0, 10 and 30 minutes, respectively. In contrast to iron, EDTA (25 μM) added to the preincubation reaction mixtures had little effect in preserving the ability of the microsomes to N-demethylate aminopyrine when compared to preincubation without any additions.

In contrast to the results obtained with 25 day old rats, in microsomes prepared from adult rats (figure 9), preincubation with an NADPH generating system for 10 minutes had no effect on the ability of the microsomes to N-demethylate aminopyrine compared to microsomes which were not subjected to preincubation. Preincubation of adult microsomes for 30 minutes, reduced aminopyrine N-demethylation activity by 20%. When preincubations were carried out in the presence of 25 μM ferrous iron, aminopyrine N-demethylation was reduced by 50% after 10 minutes and by 80% after 30 minutes preincubation. Addition of EDTA to the preincubation reaction mixtures had no effect in preventing the slight decrease in N-demethylation seen after preincubating control microsomes for similar times. In figures 8 and 9, the difference in the basal level of aminopyrine N-demethylation without preincubations (1.866 ± 0.070 nmoles HCHO/mg microsomal protein/minute in the 25 day old rat compared to 3.672 ± 0.114 nmoles HCHO/mg microsomal

protein/minute in adults) is accounted for by the normal age differential in the activity of this substrate oxidation in the developing rat.

Total NADPH oxidase activity, in microsomes prepared from rats aged 25 days and adult, is shown in figure 10. In control microsomes, NADPH oxidase activity was 10.472 ± 0.422 nmoles NADPH oxidized/mg microsomal protein/minute at 25 days, compared to 12.264 ± 1.278 nmoles NADPH oxidized/mg microsomal protein/minute in the adult. Ferrous iron (25 μM) added to the incubation mixture, stimulated NADPH oxidation 2.5 times in the 25 day old rats and by 1.4 times in the adult rats. When EDTA (25 μM) was added, the incubation mixture's NADPH oxidation activity was reduced 14% in 25 day old rats and 4% in adult rats, when compared to the control incubations for the corresponding ages. NADPH oxidation determined in this experiment, represents the total NADPH utilized by the incubation mixtures, and includes in the total, the NADPH used specifically for NADPH dependent lipid peroxidation of microsomes.

NADPH oxidation utilized specifically for the peroxidation of the endogenous lipid of the microsomal membrane is shown in figure 11. These values were calculated by determining the activity of NADPH oxidase, which was sensitive to inhibition by the 25 μM EDTA added to the incubation mixtures. The following formula, used in the calculation of this value, was: (NADPH oxidase activity used specifically for lipid peroxidation) = (Total NADPH oxidase activity) - (NADPH oxidase activity in the presence of 25 μM EDTA). In control microsomes, NADPH oxidase activity for lipid peroxidation was 3.8 times greater in 25 day old rats as compared to adult rats. When 25 μM ferrous iron was added to the reaction mixtures, NADPH oxidase

FIGURE 10. The effect of 25 μM iron and 25 μM EDTA on total NADPH oxidation in hepatic microsomes prepared from 25 day old and adult rats.

Incubation mixtures contained 25 μM iron or 25 μM EDTA as indicated. Each value represents the mean ± S.E. of the results obtained from 5 individual rats at each age.

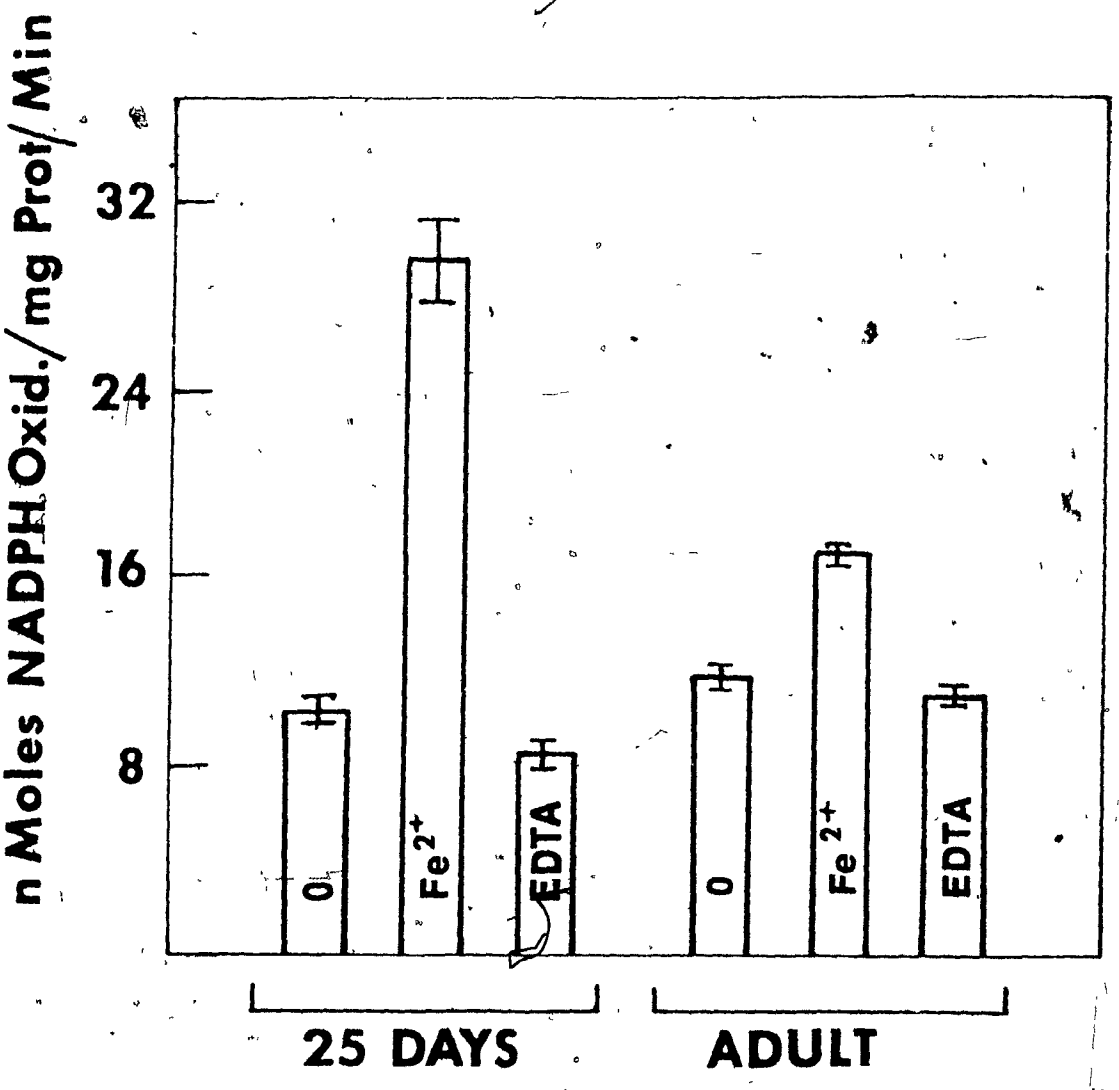
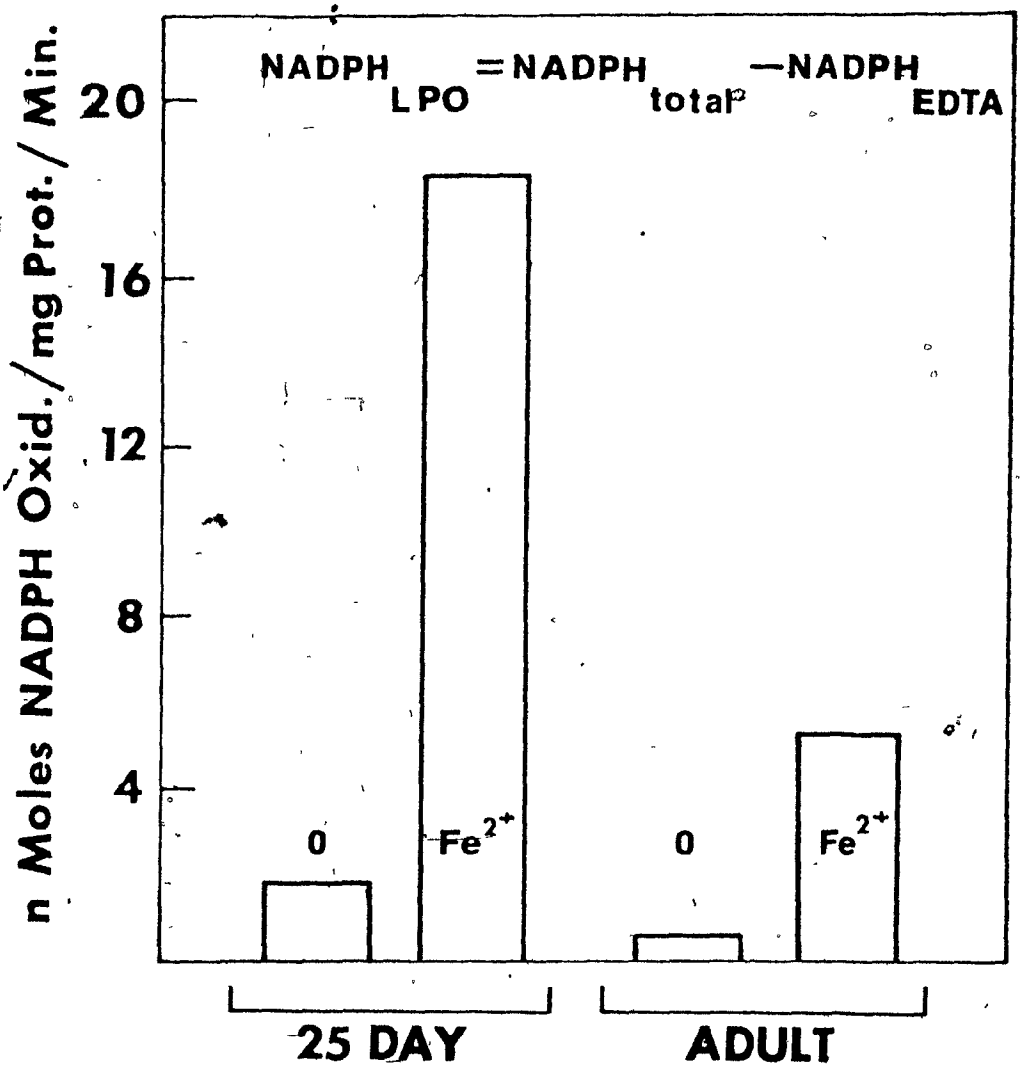

n Moles NADPH Oxid./mg Prot/Min
32
24
16
8
0
Fe^{2+}
EDTA
0
Fe^{2+}
EDTA
25 DAYS
ADULT

FIGURE 11. The effect of 25 μM iron on NADPH oxidation specifically utilized for lipid peroxidation in hepatic microsomes prepared from 25 day old and adult rats.

The values shown are the amount of NADPH oxidation which is sensitive to EDTA inhibition, which is calculated using the formula (NADPH used for lipid peroxidation) = (total NADPH oxidized) - (NADPH oxidation in the presence of EDTA). Each bar represents the mean of the values calculated in this way for 5 individual rats.


NADPH LPO = NADPH total − NADPH EDTA
n Moles NADPH Oxid./mg Prot./Min.
20
16
12
8
4
0
Fe2+
0
Fe2+
25 DAY
ADULT

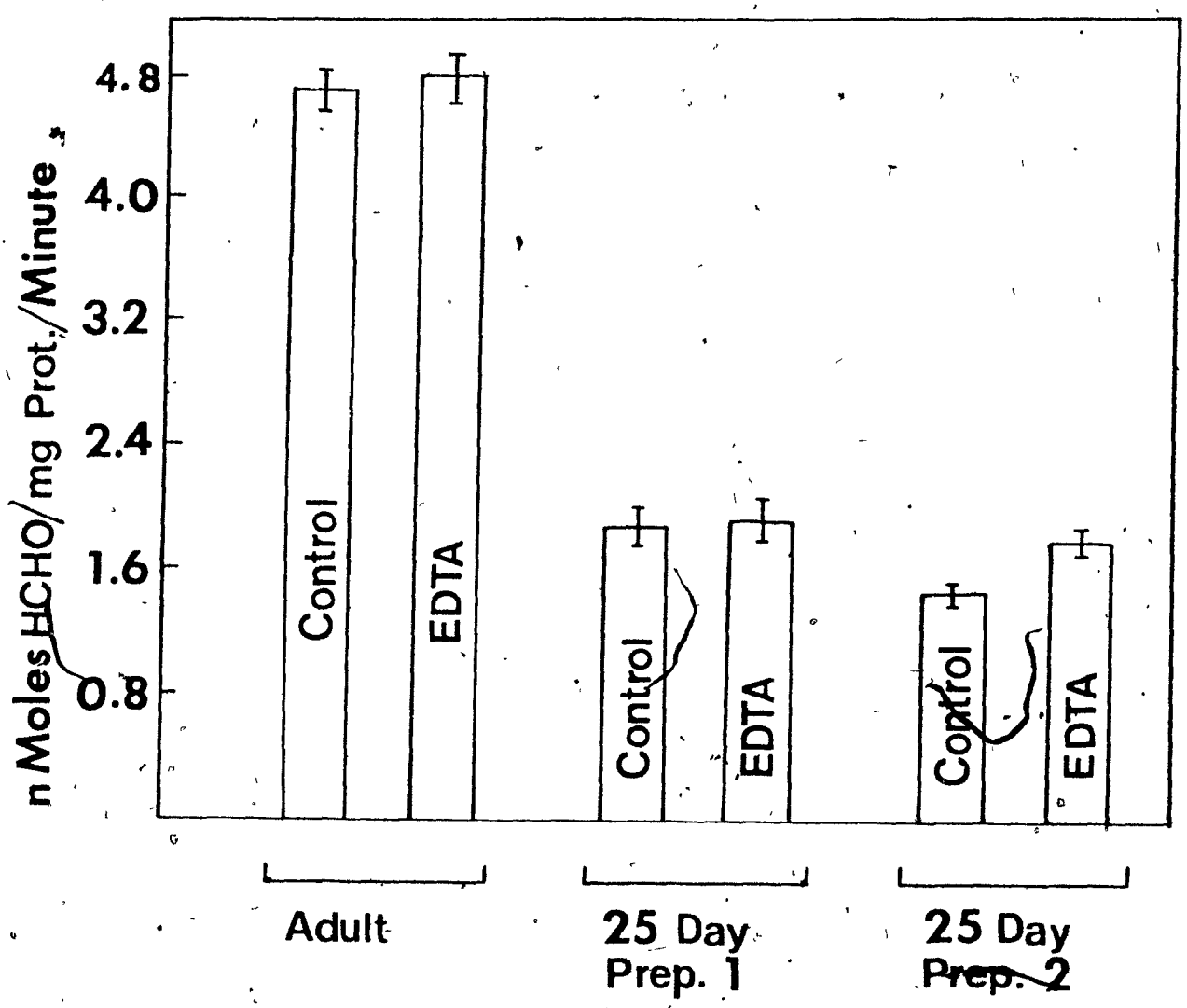
activity for lipid peroxidation was stimulated by 10 fold in 25 day old rats and by 12 fold in adult rats. Iron stimulated NADPH oxidase for lipid peroxidation is 3.3 fold greater in 25 day old rats compared to adult rats. The general pattern for NADPH oxidation, specific for lipid peroxidation, is qualitatively very similar to the pattern for NADPH dependent lipid peroxidation, as determined by the thiobarbituric acid reaction in control and iron stimulated microsomal incubation mixtures, as illustrated in figure 3.

B. THE POTENTIATION OF DRUG OXIDATION BY INHIBITION OF NADPH DEPENDENT LIPID PEROXIDATION IN HEPATIC MICROSOMES IN 25 DAY OLD AND ADULT RATS

The effect of EDTA on N-demethylation of aminopyrine is shown in figure 12 for several separate microsomal preparations in 25 day old and adult rats. In about 50% of microsomes prepared from 25 day old rats, 25 μM EDTA added to reaction mixtures potentiated aminopyrine N-demethylation, as represented by preparations 1 and 2 in figure 12. In preparation 1 from 25 day old rats, 25 μM EDTA had no effect on N-demethylation of aminopyrine and, in preparation 2, 25 μM EDTA increased the activity of aminopyrine N-demethylation. In most preparations which are represented by preparation 2, an increase of about 10% was observed for aminopyrine N-demethylation (11% in the particular example illustrated). Large increases in N-demethylation which have been reported by Kamataki and Kitagawa (1973) in adult rats, were not observed in the present experiments in microsomes prepared from 25-day old rats. When adult rat microsomes were used, 25 μM EDTA had no effect on aminopyrine N-demethylation in any preparation used in the

FIGURE 12. The effect of 25 μM EDTA on N-demethylation of aminopyrine in two preparations of hepatic microsomes from 25 day old rats and a preparation from adult rats.

N-demethylation of 1 mM aminopyrine was determined in incubation mixture containing 25 μM EDTA. Each bar represents the mean ± S.E. of 5 individual rats. Approximately 50% of preparations yielded results similar to preparation 1 and 50% yielded results similar to preparation 2.


n Moles HCHO/mg Prot./Minute
4.8
4.0
3.2
2.4
1.6
0.8
Control
EDTA
Control
EDTA
Control
EDTA
Adult
25 Day
Prep. 1
25 Day
Prep. 2

present studies. In the example illustrated in figure 12, incubation carried out in the presence of 25 μM EDTA resulted in an N-demethylation activity of 4.866 ± 0.171 nmoles HCHO/mg protein/minute, which was not significantly different from the activity of 4.724 ± 0.203 nmoles HCHO/mg protein/minute found in control incubations.

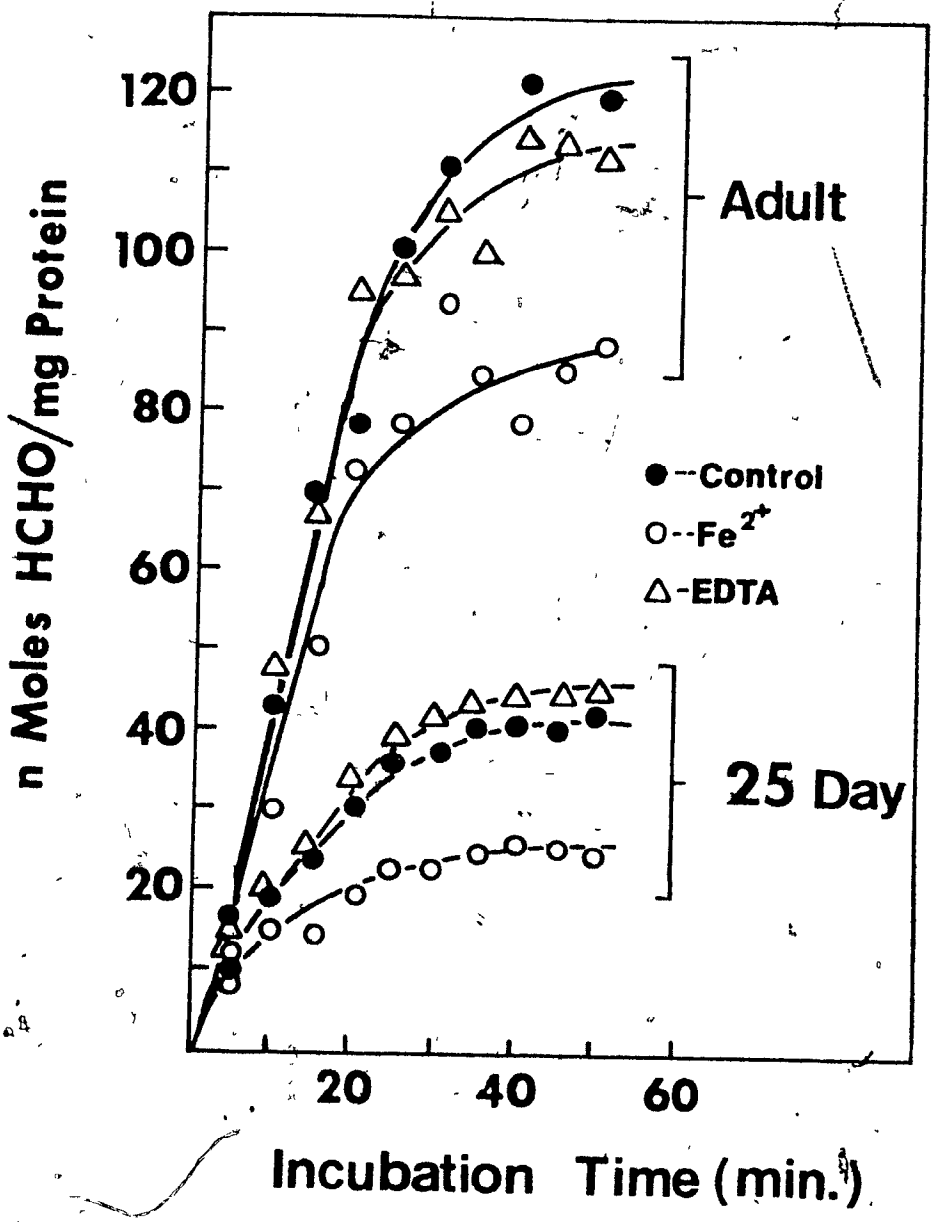
The linearity of 1mM aminopyrine N-demethylation reactions with respect to time is shown in figure 13 in 25 day old and adult rat microsomes incubated in the presence of EDTA and ferrous iron. In control incubation mixtures containing the standard aminopyrine N-demethylation reaction mixture, the reaction was linear for 20-25 minutes in both 25 day and adult aged rats. When EDTA was added to the incubation mixtures, linearity was similar to untreated microsomes. When 25 μM ferrous iron was added to incubation mixtures, the linearity of the N-demethylation reaction was shortened to less than 10 minutes in 25 day old rats and to 15-20 minutes in adult microsomes. With respect to all the conditions tested, a plateau of total formaldehyde formation was reached in less than 50 minutes reaction time. The amount of N-demethylation occurring within 50 minutes in iron treated microsomes was 50% of the value observed in both control and EDTA treated microsomes for 25 day old rats. In adult rats the amount of N-demethylation which occurred in 50 minutes in iron treated microsomes was about 70% of that found in both control and EDTA treated microsomal preparations.

C. THE EFFECT OF NADPH DEPENDENT LIPID PEROXIDATION ON TYPE I AND TYPE II SUBSTRATE BINDING SPECTRA AND ON SPECTRAL DISSOCIATION CONSTANTS IN MICROSOMES FROM ADULT RATS

Substrate induced binding spectra for aniline (type II substrate)

FIGURE 13. The linearity of aminopyrine N-demethylation reactions in control incubation mixtures and in the presence of 25 μM iron or 25 μM EDTA in hepatic microsomes prepared from 25 day old and adult rats.

Each point represents an individual incubation mixture containing microsomes obtained from a pool of 6 rats. Reactions were terminated at 5 minute intervals up to a total of 60 minutes. ●———● control incubation mixtures, o———o 25 μM Fe^{2+}, Δ———Δ 25 μM EDTA


120
100
80
60
40
20
n Moles HCHO/mg Protein
Adult
25 Day
●--Control
○--Fe2+
△-EDTA
20
40
60
Incubation Time (min.)

and cytochrome P-450 of microsomes incubated with an NADPH generating system to induce NADPH dependent lipid peroxidation, are illustrated in figure 14 (A and B). Without preincubation, 3 mM aniline demonstrated a typical type II difference spectrum when added to a microsomal suspension containing 2 mg protein/ml (figure 14A). λmax was at 430 nm and λmin was 405 nm. Preincubation for 10, 20 and 30 minutes in the presence of an NADPH generating system had no effect on either the qualitative or quantitative aspects of the difference spectrum. λmax and λmin remained about 430 nm and 405 nm, respectively, during the incubation. Twenty-five μM ferrous iron added to the incubation mixture had no effect on the difference spectrum of aniline when added immediately prior to the determination of the spectrum (figure 14B). λmax remained 430 nm and λmin remained 405 nm. Aniline difference spectra, in microsomes preincubated with an NADPH generating system, and 25 μmoles ferrous iron, are shown in figure 14B. Preincubation for 10, 20 and 30 minutes had no effect on the qualitative aspects of the difference spectra with λmax remaining about 430-435 nm and λmin remaining about 400-410 nm throughout the incubation period. In contrast, the magnitude of the binding spectra at 430 nm and 400 nm declined during the 30 minute incubation period. These quantitative aspects of the difference spectra are illustrated in figure 15, where the difference in extinction between λmax at 430 and λmin at 400 nm ($\Delta OD_{430-400}$) is shown after various periods of preincubation. In control microsomes, preincubation in the presence of an NADPH generating system, had no effect on $\Delta OD_{430-400}$ for at least 30 minutes. In contrast, there was a gradual decline of $\Delta OD_{430-400}$ during the 30 minute preincubation period in

FIGURE 14. The effect of incubation of microsomes with an NADPH generating system on aniline induced spectral changes of adult rat liver microsomes.

The difference spectrum of 3 mM aniline in microsomes was determined after incubation with NADPH generating system for various times: ——— 0 minutes, ------ 10 minutes, ... 20 minutes, -.-.-.- 30 minutes. In A, incubation was carried out in control incubation mixtures and, in B, incubation was carried out in the presence of 25 μM iron.

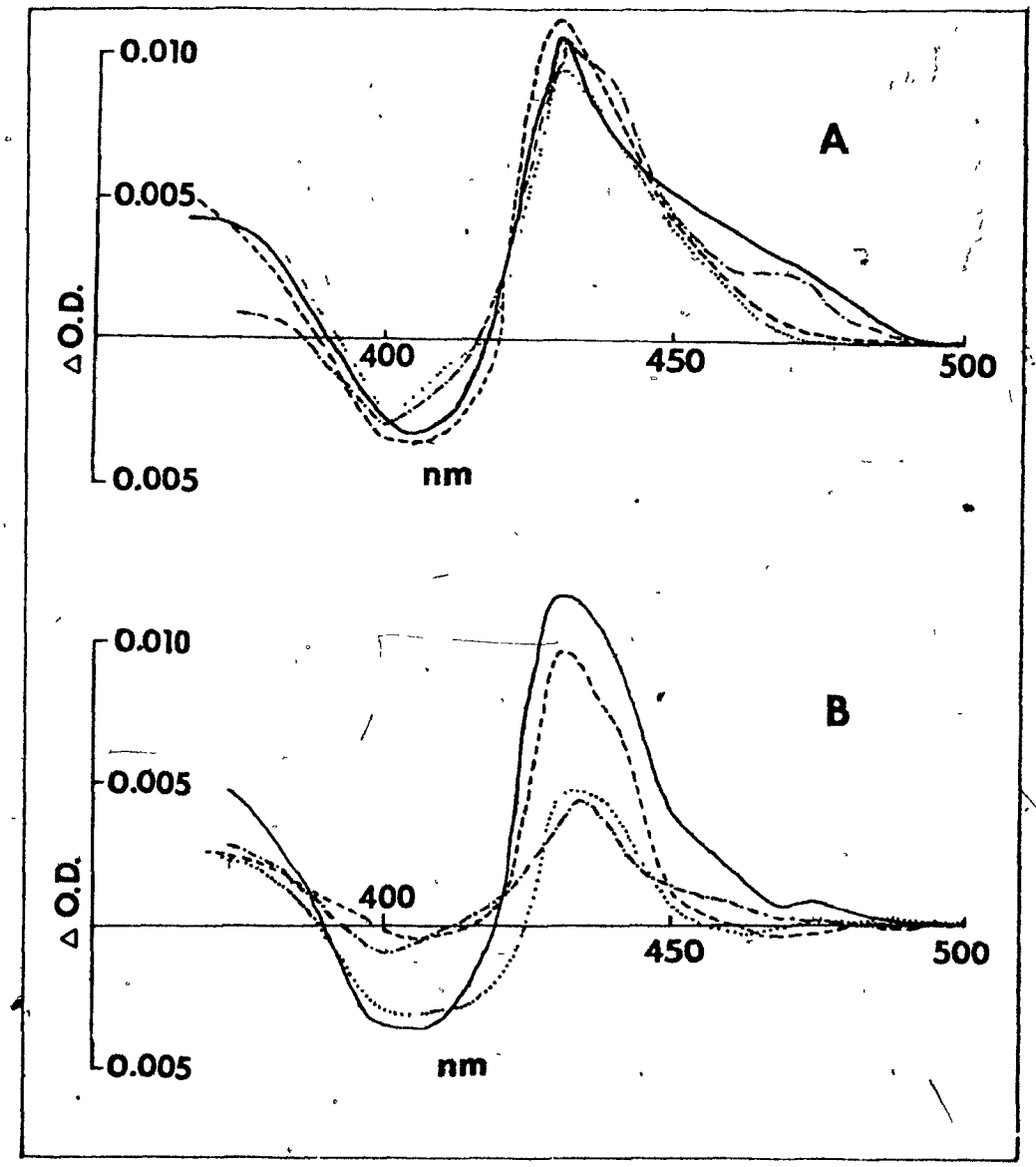
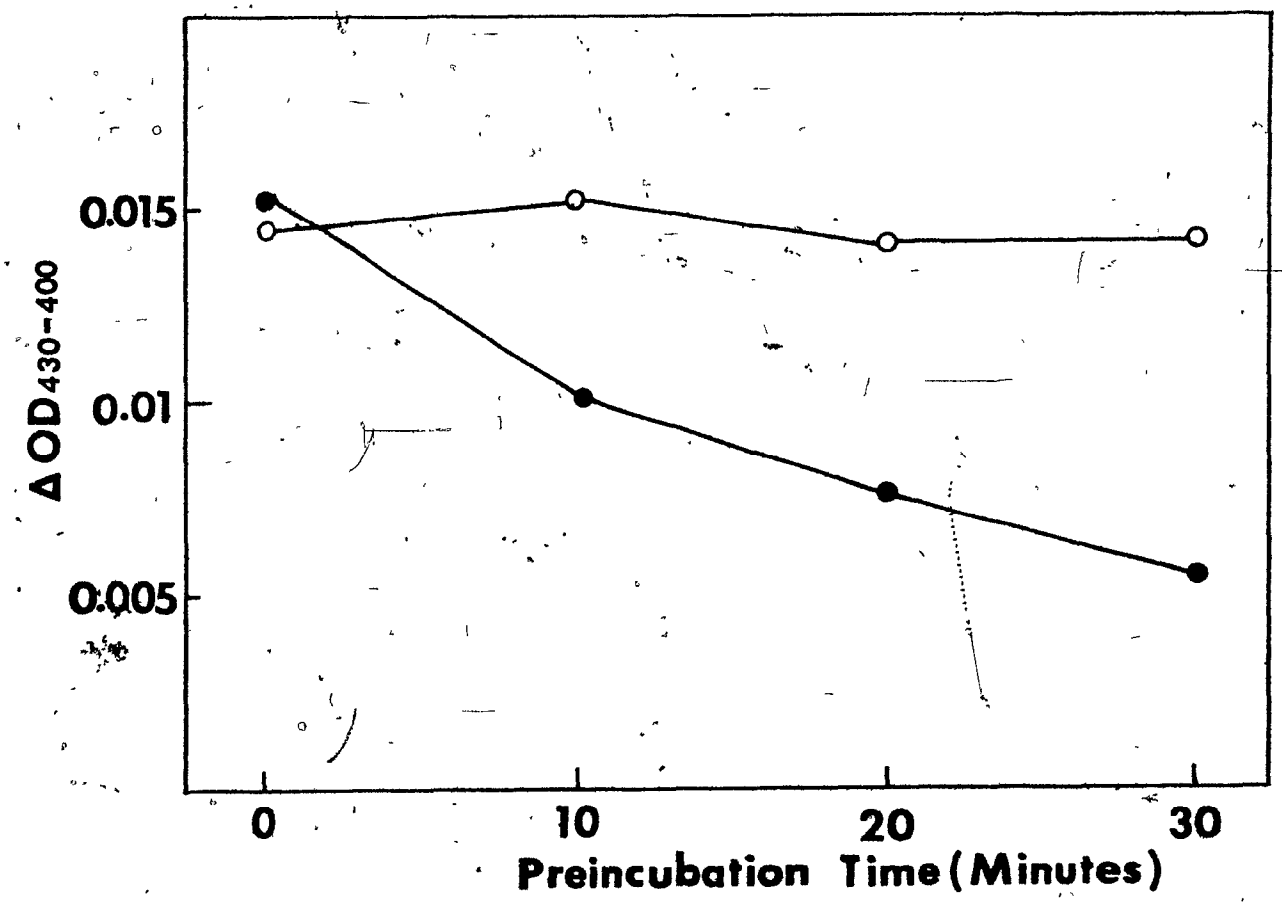

0.010
0.005
ΔO.D.
0.005
400
450
500
nm
A
0.010
0.005
ΔO.D.
400
450
500
0.005
nm
B

FIGURE 15. The effect of incubation of microsomes with an NADPH generating system on the $\Delta OD_{430-400}$ induced by aniline in adult rat liver microsomes.

Incubations were carried out in control reaction mixtures o———o, and in reaction mixtures containing 25 μM iron •———•. Each point represents the mean of two determinations of $\Delta OD_{430-400}$ from separate incubation mixtures containing microsomes pooled from 3 rats.


0.015
0.01
0.005
ΔOD430-400
0
10
20
30
Preincubation Time (Minutes)

microsomes preincubated in the presence of an NADPH generating system and 25 μM ferrous iron. After 30 minutes preincubation $\Delta OD_{430-400}$ had declined to 36% of its original value when compared to the control incubation mixture which was not preincubated.

The effect of preincubation of microsomes for 20 minutes with an NADPH generating system on the magnitude of spectral binding of varying concentrations of aniline and the spectral dissociation constant (Ks) for aniline are shown in tables 2 and 3. The magnitude of the binding spectra increases with increased substrate concentration. Using a double reciprocal plot ($1/\Delta OD_{430-400}$ *vs* 1/mM aniline) and varying the concentration of aniline from 0.1 to 1.0 mM, a linear double reciprocal plot was obtained (figure 16). Using a computer program to fit the data to the equation for a rectangular hyperbola, a spectral dissociation constant of $5.12 \times 10^{-4} \pm 0.57 \times 10^{-4}$ M was obtained for control microsomes. In microsomes preincubated in the presence of an NADPH generating system and 25 μM iron for 20 minutes, a linear double reciprocal plot was obtained using aniline concentrations of 0.1 to 1.0 mM (figure 16). The binding constant was $4.37 \times 10^{-4} \pm 1.97 \times 10^{-4}$ M. The spectral binding constant for aniline after 20 minutes preincubation was not significantly different from the control spectral binding constant. The goodness of fit of the data to the rectangular hyperbola equation, decreased after preincubation as reflected by an increase in the standard errors of Ks compared to control as shown in table 3.

Substrate induced difference spectra for hexobarbital (type I substrate) in microsomes incubated with an NADPH generating system to promote

Table 2

Change in Absorbance in Difference Spectra of Microsomes After The Addition of Various Concentrations of Aniline and Hexobarbital and Preincubation with an NADPH Generating System and 25 μM Iron.

Preincubation Time (minutes)	Substrate	Concentration mM	ΔAbsorbance
0	Aniline	0.10	0.004
		0.20	0.008
		0.30	0.010
		0.50	0.013
		0.70	0.015
		1.00	0.018
20	Aniline	0.10	0.001
		0.20	0.005
		0.30	0.007
		0.50	0.008
		0.70	0.009
		1.00	0.010
0	Hexobarbital	0.05	0.006
		0.10	0.010
		0.20	0.014
		0.30	0.018
20	Hexobarbital	0.10	0.003
		0.20	0.005
		0.30	0.007
		0.50	0.008

ΔAbsorbance: Aniline = λ430-400 nm
Hexobarbital = λ500-425 nm

Table 3

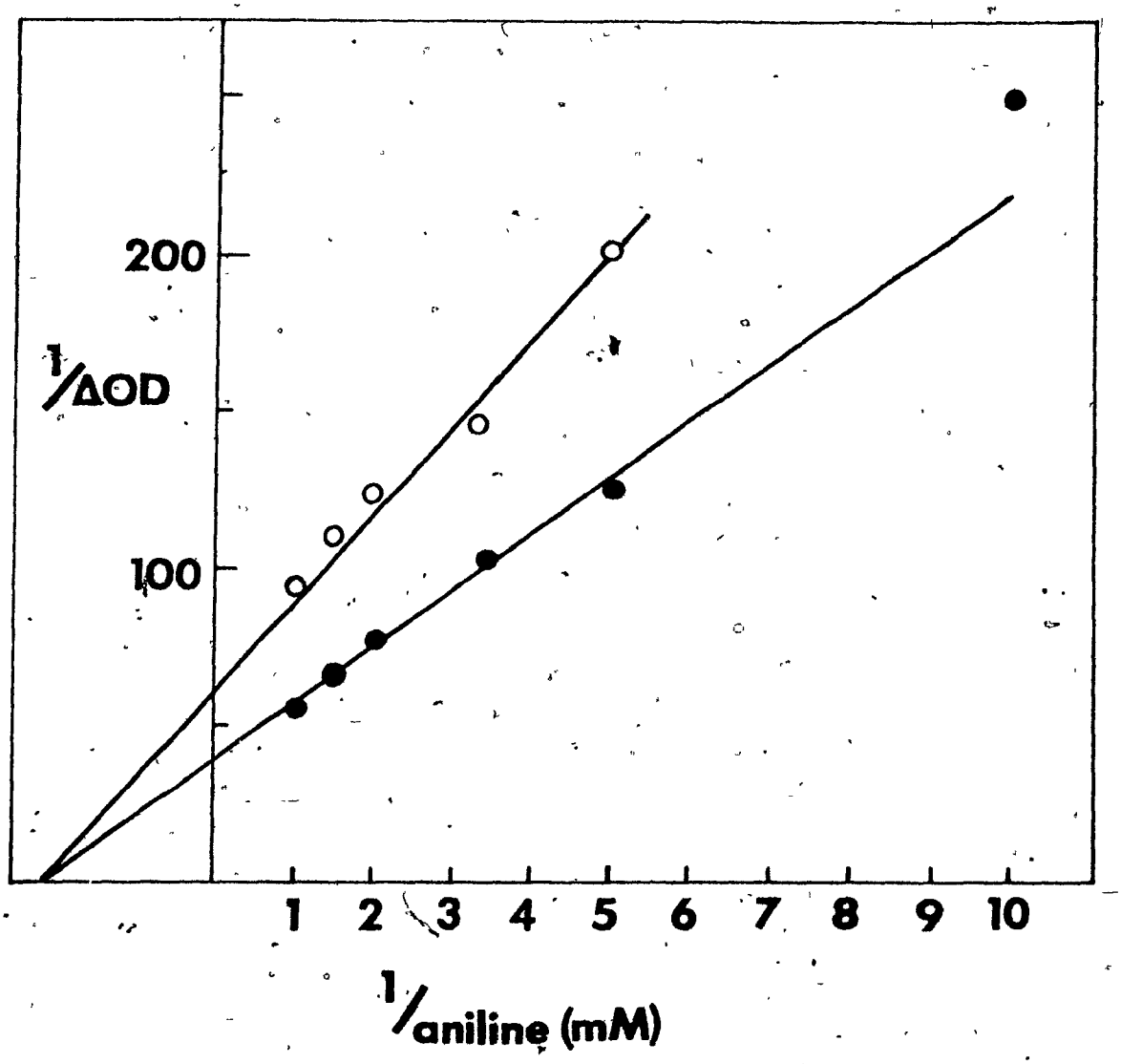
Spectral Dissociation Constants for Aniline and Hexobarbital in Microsomes Preincubated with an NADPH Generating System and 25 μM Iron

Substrate	Preincubation Time (minutes)	Spectral Dissociation Constant (Ks) (mM)
Aniline	0	0.512 ± 0.057
Aniline	20	0.437 ± 0.197
Hexobarbital	0	0.203 ± 0.040
Hexobarbital	20	0.326 ± 0.100

Values represent Ks calculated using a computer program to fit the data to a rectangular hyperbola equation.

FIGURE 16. Double reciprocal plot of changes in absorbance ($\Delta OD_{430-400}$) resulting from the addition of aniline to microsomes containing an NADPH generating system and 25 μM iron and incubated for 0 and 20 minutes.

Each point represents a single determination of $1/OD_{430-400}$ for concentrations of aniline from 0.1 to 1.0 mM. The lines of best fit are constructed using the constants generated by the computer program which fits the data to a rectangular hyperbola equation. ●——● no incubation, o——o 20 minute incubation.


200
100
1/ΔOD
1 2 3 4 5 6 7 8 9 10
1/aniline (mM)

NADPH dependent lipid peroxidation is shown in figure 17 (A and B). Without preincubation (figure 17A), 1 mM hexobarbital demonstrated a typical type I spectrum when added to a microsomal suspension containing 2 mg protein/ml. λmin was 425 nm and λmax was approximately 390 nm. Preincubation for 10, 20 and 30 minutes with a NADPH generating system had no effect on either the qualitative or quantitative aspects of the difference spectrum (figure 17A). λmin and λmax remained at about 425 nm and 390 nm respectively. Twenty-five μM ferrous iron added to the reaction mixture immediately prior to the determination of the difference spectra, had no effect on the hexobarbital difference spectra, with λmin remaining at 425 nm and λmax remaining at 390 nm. Preincubation with ferrous iron and an NADPH generating system for 10, 20 and 30 minutes, had no effect on the qualitative aspects of the difference spectrum with λmin remaining at 425 nm and λmax remaining at 390-395 nm (figure 17B). In contrast, the magnitude of the binding spectra declined during preincubation period, with the extinction λmin at 425 nm gradually declining over the 30 minute incubation time (figure 17B). The reduction in magnitude of the spectrum at λmin 425 nm, is illustrated in figure 18, where the difference in absorbance between 500 nm and 425 nm ($\Delta OD_{500-425}$) is shown after increasing preincubation times. In control microsomes, preincubation with an NADPH generating system has no effect on $\Delta OD_{500-425}$ for at least 30 minutes. In contrast, in microsomes preincubated with an NADPH generating system and 25 μM ferrous iron, $\Delta OD_{500-425}$ had declined to 47% of its original value prior to incubation.

The effect of preincubation of microsomes for 20 minutes with an NADPH generating system, on the spectral binding of varying concentrations

FIGURE 17. The effect of incubation of microsomes with an NADPH generating system on hexobarbital induced spectral changes of adult rat liver microsomes.

The difference spectra of 1 mM hexobarbital in microsomes was determined after preincubation with NADPH generating system for varying times. ——— 0 minute, ----- 10 minutes, ... 20 minutes, -.-.-.-.- 30 minutes. In A, preincubation was carried out in control incubation mixtures and, in B, incubation was carried out in the presence of 25 μM iron.

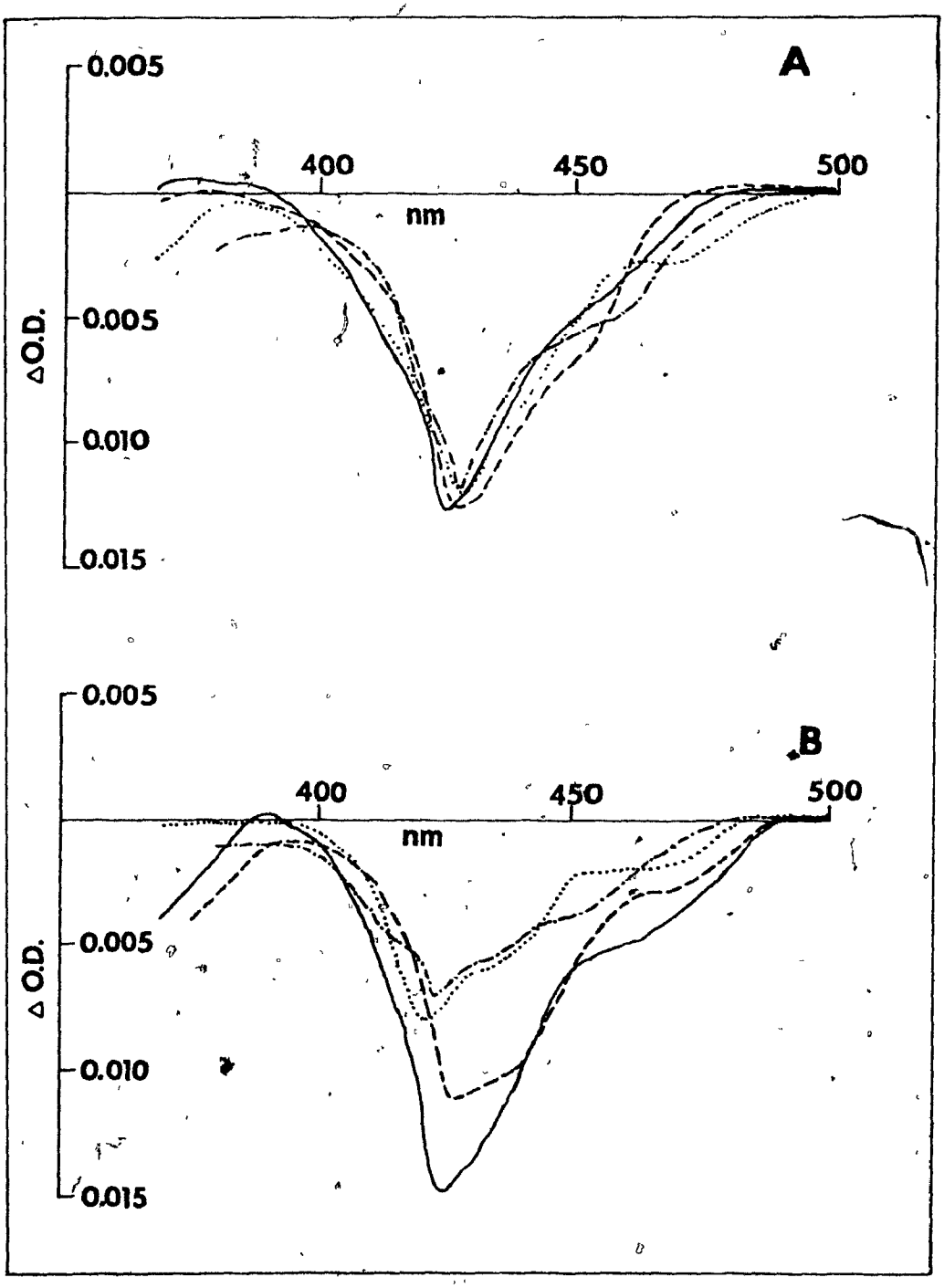
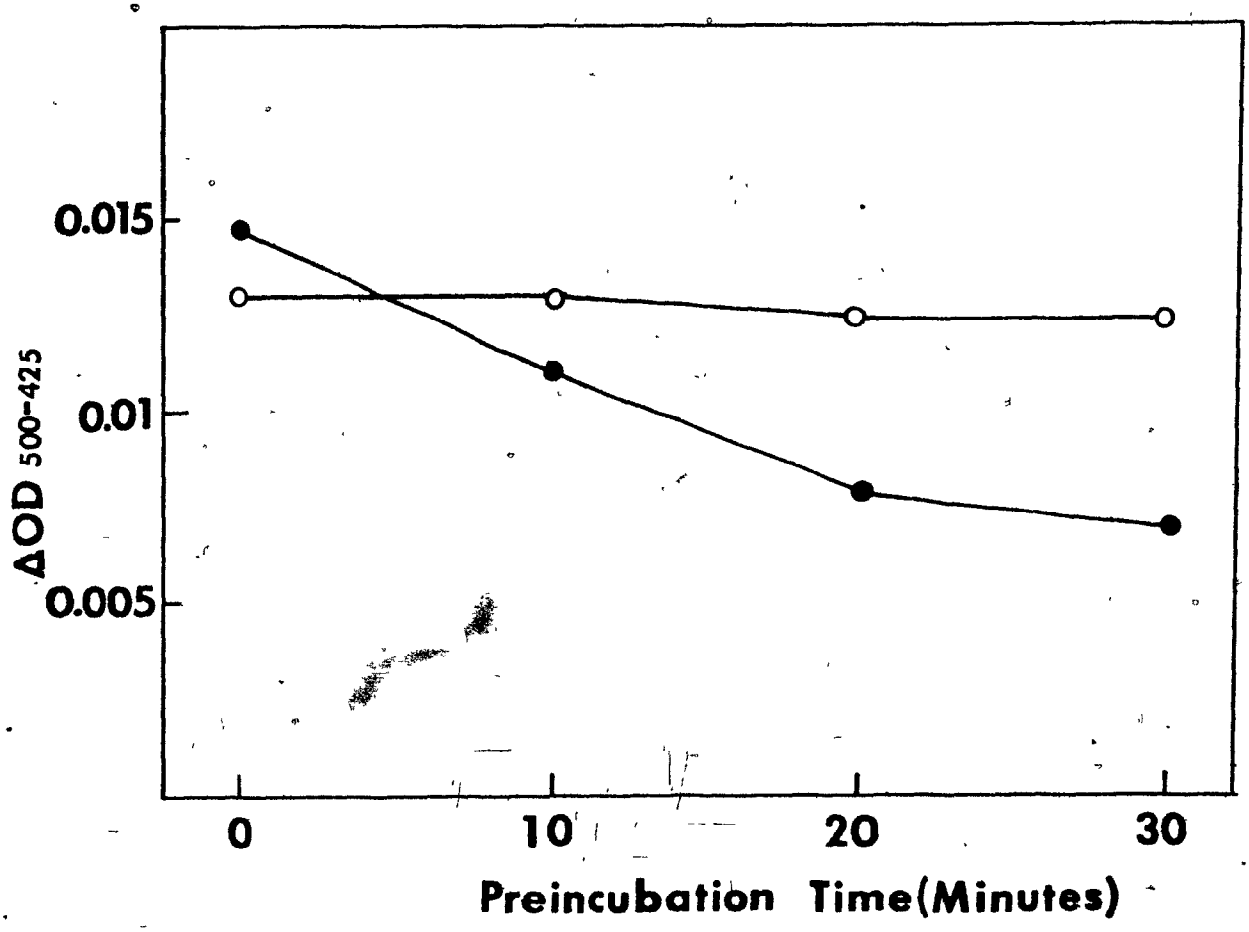

A
0.005
400
450
500
nm
ΔO.D.
0.005
0.010
0.015
B
0.005
400
450
500
nm
ΔO.D.
0.005
0.010
0.015

FIGURE 18. The effect of incubating of microsomes with an NADPH generating system on $\Delta OD_{500-425}$ induced by hexobarbital in adult rat liver microsomes.

Incubations were carried out in control reaction mixtures o——o, and in reaction mixtures containing 25 μM iron ●——●. Each point represents the mean of two determinations of $\Delta OD_{500-425}$ from separate incubation mixtures containing microsomes from a pool of 3 rats.


0.015
0.01
0.005
ΔOD 500-425
0
10
20
30
Preincubation Time(Minutes)

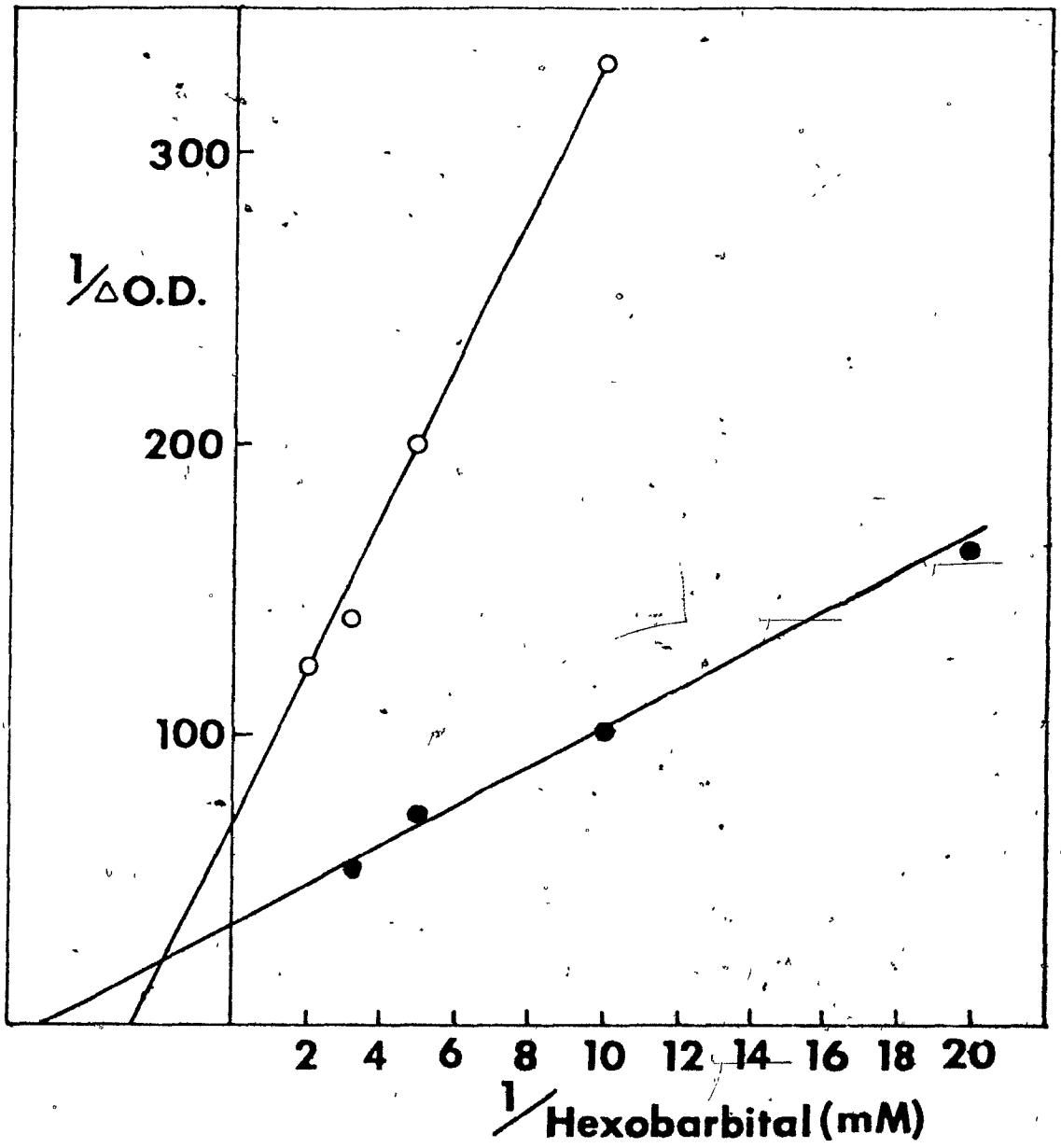
of hexobarbital and the spectral binding constant (Ks) for hexobarbital, are shown in tables 2 and 3. The magnitude of the difference spectrum increased with increased substrate concentration. Using a double reciprocal plot $1/\Delta OD_{500-425}$ *vs* 1/hexobarbital concentration, and concentrations of hexobarbital varying from 0.05 mM to 0.3 mM, a linear double reciprocal plot was obtained, as shown in figure 19. Using a computer program to fit the data to the equation for a rectangular hyperbola, a spectral dissociation constant of $2.03 \times 10^{-4} \pm 0.40 \times 10^{-4}$ M was obtained for hexobarbital in control microsomes. In microsomes preincubation in the presence of an NADPH generating system and ferrous iron for 20 minutes, a linear double reciprocal plot was obtained using hexobarbital concentrations of 0.1 to 0.5 mM. The binding spectral dissociation constant was $3.26 \times 10^{-4} \pm 1.00 \times 10^{-4}$ M for hexobarbital in preincubated microsomes. The spectral binding constant for hexobarbital after 20 minutes incubation was not significantly different from the spectral binding constant determined in control microsomes. As was the case with aniline binding spectra, the goodness of fit of the experimental data to a rectangular hyperbola, was decreased after incubation for 20 minutes as reflected by an increase in the standard errors of Ks compared to the control standard errors of Ks (table 3).

D. THE EFFECT OF NADPH DEPENDENT LIPID PEROXIDATION ON CYTOCHROME P-450 LEVELS IN MICROSOMES PREPARED FROM 25 DAY AND ADULT RATS

Microsomal content of cytochrome P-450 was determined after microsomes were incubated with an NADPH generating system to promote lipid

FIGURE 19. Double reciprocal plot of changes in absorbance ($\Delta OD_{500-425}$) resulting from the addition of hexobarbital to microsomes containing an NADPH generating system and 25 μM iron, and incubated for 0 and 20 minutes.

Each point represents a single determination of $1/\Delta OD_{500-425}$ for concentrations of hexobarbital from 0.05 - 0.50 mM. The lines of best fit are constructed using the constants generated by the computer program which fits the data to a rectangular hyperbola equation. ●——● no incubation, o——o 20 minutes incubation.


300
200
100
1/△O.D.
2
4
6
8
10
12
14
16
18
20
1/Hexobarbital (mM)

peroxidation. Incubations were carried out in reaction media with and without the addition of 25 μM ferrous iron. The method used for the determination of cytochrome P-450 measured the reduced cytochrome P-450-CO complex without interference from endogenously formed carbon monoxide.

Incubation of 25 day old microsomes with an NADPH generating system, had no effect on the cytochrome P-450 level for at least 30 minutes, with the value remaining between 0.39 and 0.41 nmoles cytochrome P-450/mg microsomal protein throughout (figure 20). Addition of 25 μM iron to the incubation reaction mixture, had no effect on the cytochrome P-450 content of microsomes during 30 minutes incubation. As shown in figure 20, the range of values at the various incubation times are identical to that found in the control incubation reaction

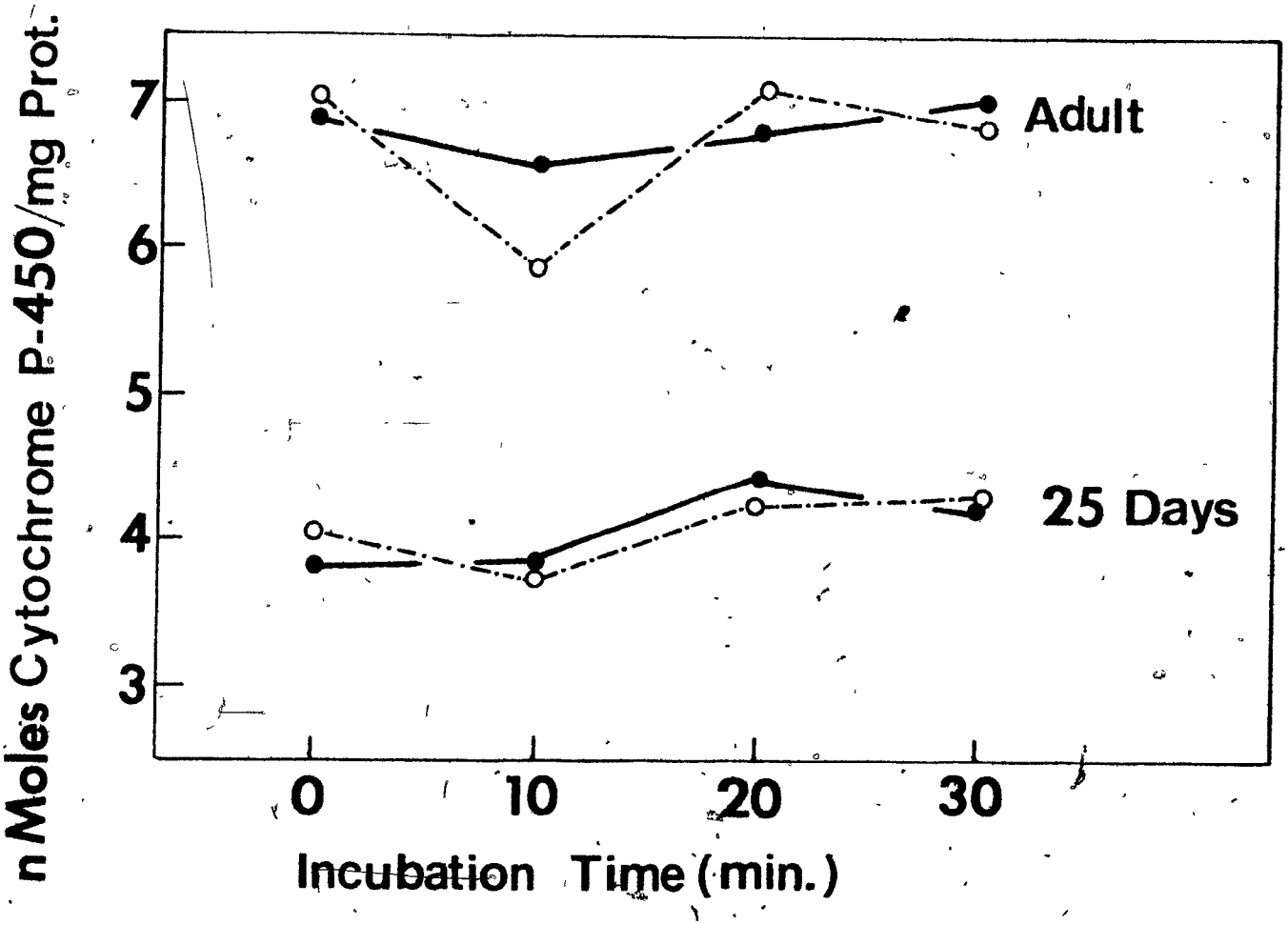
Incubation of adult microsomes for periods up to 30 minutes also had no effect on the cytochrome P-450 content of microsomes, with the values remaining between 0.65 and 0.70 nmoles cytochrome P-450/mg microsomal protein (figure 20). The increased cytochrome P-450 levels of adult microsomes compared to 25 day old microsomes, is due to the normal difference found in the developing rat. Addition of 25 μM iron to the incubation media had no effect on the cytochrome P-450 content of microsomes. The results illustrated in figure 20 are those obtained in a typical experiment. In three other similar experiments, incubation of microsomes with an NADPH generating system and iron, had no effect on cytochrome P-450 content.

E. MALE-FEMALE DIFFERENCE IN NADPH DEPENDENT LIPID PEROXIDATION, AMINOPYRINE N-DEMTHYLATION AND ELECTRON TRANSPORT COMPONENTS IN ADULT RATS

Microsomal suspensions prepared from adult male and female rats

FIGURE 20. The effect of incubation of microsomes with an NADPH generating system and 25 μM iron on hepatic microsomal cytochrome P-450 concentration in 25 day old and adult rats.

Incubations were carried out for the times indicated in an incubation mixture containing an NADPH generating system: ●——● control incubation, o-.-.-o incubation containing 25 μM iron. Each point represents the mean of two separate incubation mixtures containing microsomes obtained from a pool of 3 rats.


n Moles Cytochrome P-450/mg Prot.
7
6
5
4
3
Adult
25 Days
0
10
20
30
Incubation Time (min.)

(250-300 gm) contained similar amounts of protein. Female rats contained 27.64 ± 1.69 mg microsomal protein/gm wet liver and 18.43 ± 1.13 mg protein/ml microsomal suspensions compared to male rats which contained 28.08 ± 2.31 mg microsomal protein/gm wet liver and 18.72 ± 1.54 mg protein/ml microsomal suspension as shown in table 4. The differences in NADPH dependent lipid peroxidation in microsomes from female and male rats are shown in figure 21. NADPH dependent lipid peroxidation in female rats was 0.933 ± 0.052 nmoles malonaldehyde/mg protein/15 minutes, which was significantly higher than the male activity of 0.394 ± 0.029 nmoles malonaldehyde/mg protein/15 minutes. The addition of 25 μM iron to the reaction mixtures stimulated NADPH dependent lipid peroxidation activity to 17.003 ± 2.107 nmoles malonaldehyde/mg protein/15 minutes in female rats and to 10.568 ± 1.170 nmoles malonaldehyde/mg protein/15 minutes in male rats.

Non-heme iron levels were significantly higher in the female compared to male rats. A concentration of 7.092 ± 0.142 nmoles iron/mg protein, was determined in the female compared to 3.862 ± 0.221 nmoles iron/mg protein in the male, as shown in table 4.

Aminopyrine N-demethylation in microsomes was significantly lower in female rats when compared to male rats, as shown in table 4. Female rats had an N-demethylation activity of 3.476 ± 0.235 nmoles HCHO/mg protein/minute, compared to an activity of 5.141 ± 0.290 nmoles HCHO/mg protein/minute, recorded in the male rat. When aminopyrine N-demethylation reactions were carried out in incubation mixtures containing 25 μM EDTA, the activities in both female and male rats remained unchanged and the differences between the two sexes was maintained.

Table 4

NADPH Dependent Lipid Peroxidation Activity, Drug Oxidation and Electron Transport Components in Microsomes Prepared From Adult Male and Female Rats

		Male	Female
Lipid Peroxidation (nmoles malonaldehyde/ mg protein/15 minutes)	Control	0.394±0.029	0.933±0.052*
	25 μM Fe^{2+}	10.568±1.170	17.003±2.107*
Aminopyrine N-Demethylation (nmoles HCHO/mg protein/ minute) [1mM aminopyrine]	Control	5.141±0.290	3.476±0.235*
	25 μM EDTA	5.089±0.191	3.547±0.280*
Cytochrome P-450 (nmoles/mg protein)	--	0.476±0.031	0.316±0.022*
Cytochrome c Reductase (nmoles cytochrome c reduced/mg protein/ minute)	--	90.64 ±7.16	72.35 ±4.62+
Non-Heme Iron (nmoles/mg protein)	--	3.862±0.221	7.092±0.142*
Microsomal Protein	mg/ml microsomes	18.72 ±1.54	18.43 ±1.13
	mg/gm wet liver	28.08 ±2.31	27.64 ±1.69

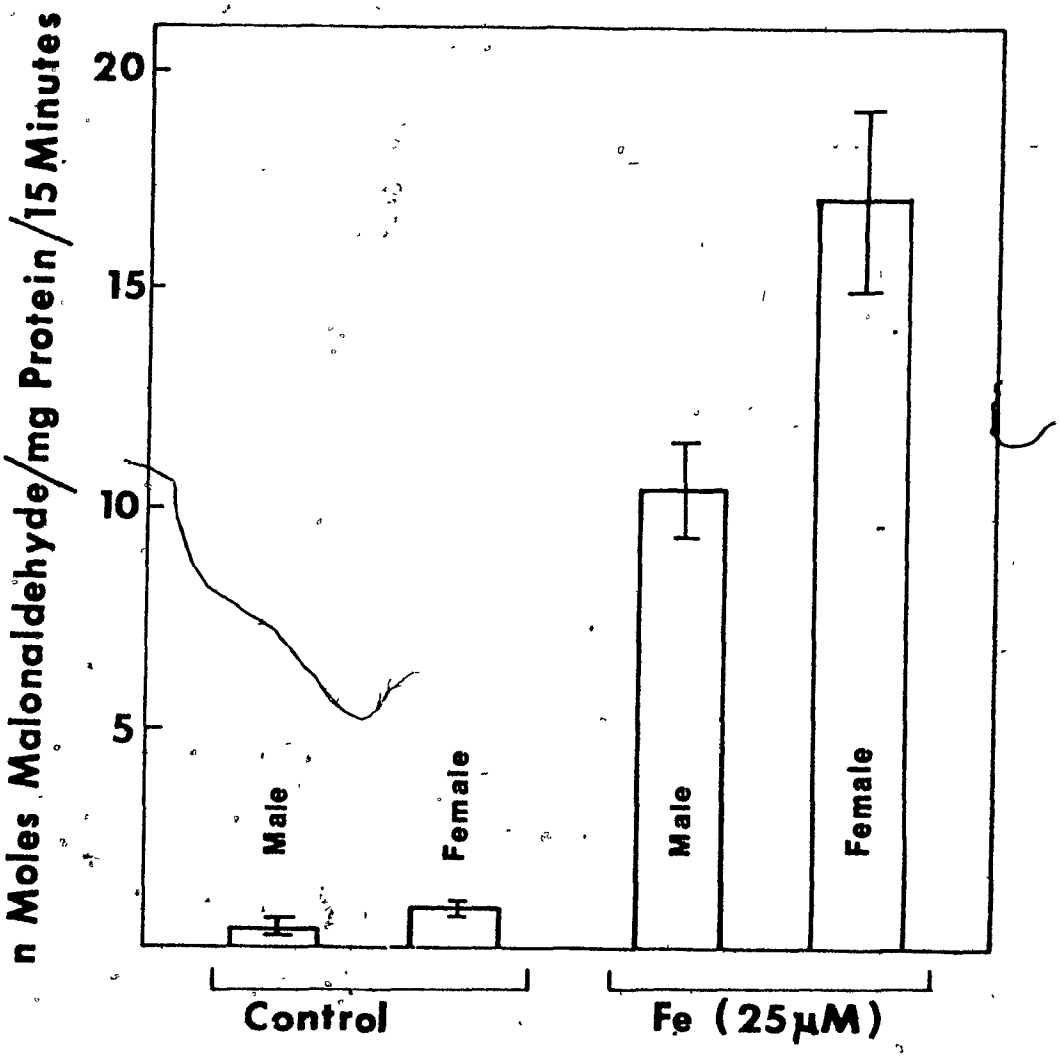
*Significantly different $p \leq 0.05$

+$p < 0.1$

Values represent the mean ± standard error of 6 individual rats

FIGURE 21. The difference in NADPH dependent lipid peroxidation in hepatic microsomes prepared from male and female rats.

Incubation mixtures contained 25 μM iron as indicated on the figure. Each bar represents mean ± S.E. of 6 individual rats.


n Moles Malonaldehyde/mg Protein/15 Minutes
20
15
10
5
Male
Female
Male
Female
Control
Fe (25μM)

Also, as detailed in table 4, microsomal cytochrome P-450 level was 34% lower in female rats compared to males, and microsomal cytochrome c reductase activity was 20% lower in female rats compared to males.

F. NADPH DEPENDENT LIPID PEROXIDATION AND ITS EFFECTS ON AMINOPYRINE N-DEMETHYLATION IN HEPATIC SUBCELLULAR FRACTIONS PREPARED FROM HUMAN LIVER OBTAINED AT AUTOPSY OF NEWBORN INFANTS

Microsomal NADPH dependent lipid peroxidation activity was determined in subcellular fractions prepared from livers of 3 human infants and one 19 week fetus. Table 5 records the relevant clinical data on the autopsy material studied. Samples 1-3 were obtained from infants of normal birth weight for their gestational age within 3 hours of death. Sample 4 was obtained from an aborted fetus, and though fetal body weight was not recorded, the crown-rump and crown-heel lengths were normal for a fetus of this gestational age. Table 6 summarizes the drugs received by each infant prior to death. Of particular note was the phenobarbital, used to control seizures, received by patient number 1 in 4 doses of 6 mg, each dose being given at 8 hour intervals.

NADPH dependent lipid peroxidation activities in the subcellular fractions of each sample are recorded in table 7. In sample 1, which is also illustrated in figure 22, highest activities were observed in the 100,000P fraction (microsomes). In this sample, lipid peroxidation activity was 0.686 nmoles malonaldehyde/mg protein/15 minutes. The addition of 25 μM iron to the reaction mixture, stimulated lipid peroxidation activity to 12.164 nmoles malonaldehyde/mg protein/15 minutes, while the addition of 25 μM EDTA reduced activity to 0.042 nmoles malonaldehyde/mg protein/15

Table 5

Clinical Data on the Human Infants Studied

Patient	Sex	Birth Weight gms	Gestational Age	Postnatal Age	Diagnosis
1	male	3026	40 wks	38 hrs	Asphyxia
2	male	3650	38 wks	36 hrs	Asphyxia
3	male	2215	31 wks	10 days	RDS, Sepsis
4	male	CR 17 cm CH 24 cm	19 wks	0	Abortion

CR = crown rump length

CH = crown heel length

RDS = respiratory distress syndrome

Table 6

Drugs Received by Human Infants Studies

Sample No.	Postnatal Age	Drugs Received
1	38 hours	Phenobarbital Dexamethazone Ampicillin Gentamycin Polybactracin
2	36 hours	Phenobarbital Diazepam
3	10 days	Vitamin K, Gentamycin Penicillin, Ca gluconate Kanamycin, Lasix Ampicillin
4	0 (fetus)	--

Table 7

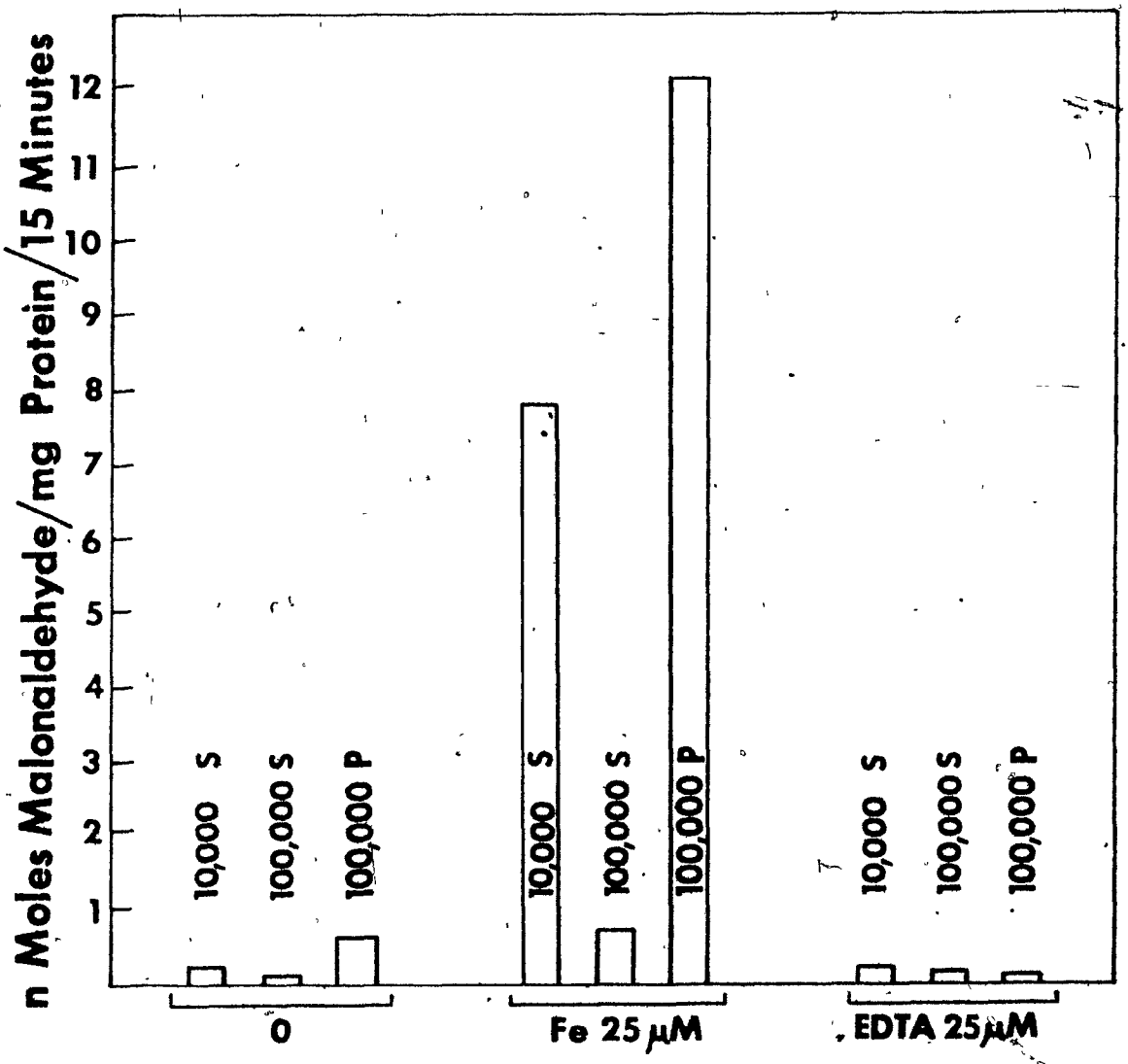
NADPH Dependent Lipid Peroxidation Activity in Subcellular Fractions Obtained from Human Liver Samples in Control Incubation Mixtures and Incubation Mixtures Containing 25 μM Iron or 25 μM EDTA

Sample	Fraction	Lipid Peroxidation (nmoles/mg protein/15 minutes) Control	25 μM Fe^{2+}	25 μM EDTA
1	10,000S	0.290 (0·290, 0·290)	7.851 (8·008, 7·694)	0.227 (0·235, 0·219)
1	100,000S	0.143 (0·078, 0·209)	0.765 (0·777, 0·759)	0.180 (0·157, 0·204)
1	100,000P	0.686 (0·619, 0·754)	12.164 (12·219, 12·109)	0.042 (0, 0·084)
2	10,000S	0.184 (0·192, 0·176)	1.128 (1·137, 1·118)	0.146 (0·175, 0·134)
2	100,000S	0.146 (0·154, 0·139)	1.185 (1·241, 1·129)	0.126 (0·139, 0·112)
2	100,000P	0.354 (0·400, 0·308)	1.639 (1·411, 1·867)	0.194 (0·197, 0·191)
3	100,000P	0.289 (0·339, 0·238)	0.353 (0·293, 0·412)	0.225 (0·174, 0·275)
4	10,000S	0.336 (0·225, 0·447)	1.347 (1·254, 1·439)	0.073 (0·077, 0·068)
4	100,000S	0.052 (0·039, 0·065)	1.628 (1·801, 1·456)	0.150 (0·000, 0·299)
4	100,000P	1.017 (0·827, 1·207)	2.946 (2·804, 3·089)	0.000 (0·000, 0·000)

Values represent mean of two determinations. Individual values are given in small type after each mean.

FIGURE 22. The effect of iron and EDTA on NADPH dependent lipid peroxidation in hepatic microsomes prepared from a human newborn infant.

Incubation mixtures contained 25 μM ferrous sulphate or 25 μM EDTA where indicated. Each bar represents the mean of the 2 determinations, which are given in table 7.


n Moles Malonaldehyde/mg Protein/15 Minutes
1
2
3
4
5
6
7
8
9
10
11
12
10,000 S
100,000 S
100,000 P
10,000 S
100,000 S
100,000 P
10,000 S
100,000 S
100,000 P
0
Fe 25 μM
EDTA 25μM

minutes. Lower activities in both control reaction mixtures and reaction mixtures containing 25 μM iron, were demonstrated in the 10,000S fraction. This fraction, however, was resistant to inhibition by 25 μM EDTA, with only a slight reduction in activity compared to the corresponding control reaction mixture. In the 100,000S soluble supernatant fractions, lipid peroxidation activities were much lower than in the other two fractions, and even in reaction mixtures containing 25 μM iron, only 0.765 nmoles malonaldehyde/mg protein/15 minutes was formed, a value which is only marginally higher than the control incubation mixture of the microsomal fraction.

In the subcellular fractions prepared from samples 2 and 4, the overall activities were much less than in sample 1. In both cases, highest activities were observed in the microsomal fraction for both control reaction mixtures and 25 μM iron reaction mixtures, and the lowest activities were recorded in the 100,000 S supernatant fraction. As noted in sample 1, EDTA was an effective inhibitor of the NADPH dependent lipid peroxidation in microsomes, but it was not a good inhibitor in the other two fractions. Only data for the 100,000 x g pellet fraction was determined for sample 3, and though control activity was similar to control activity in the other samples, stimulation by 25 μM iron was not as effective.

Protein content, non-heme iron content, and cytochrome c reductase activities for the subcellular fractions of samples 1, 2 and 4, are illustrated in table 8. Protein concentration in subcellular fractions was similar in the three samples, with protein concentrations in the microsomal fraction being comparable to the normal range of 20-35 mg/ml found

Table 8

Protein Yield, Non-Heme Iron Content and Cytochrome c Reductase Activity in Subcellular Fractions Obtained from Human Liver

	Subcellular Fraction	Sample 1	Sample 2	Sample 4
Protein (mg/ml)	10,000S	26.79	25.35	21.81
	100,000S	16.23	15.21	16.30
	100,000P	25.21	34.45	22.37
Non-heme iron (nmoles/mg protein)	10,000S	6.50	3.48	4.02
	100,000S	8.39	4.04	5.34
	100,000P	5.78	1.07	4.24
Cytochrome c reductase (nmoles cytochrome c reduced/mg protein/ minute)	10,000S	61.98	18.85	6.76
	100,000S	7.03	6.82	4.15
	100,000P	193.47	45.34	9.89

in preparations from adult rats. Non-heme iron content of the subcellular fractions from the three samples was similar, with the exception of the microsomal pellet from sample 2 which had a non-heme iron content of 25% of the other samples. Cytochrome c reductase activities were highest in the microsomal fraction and ranged from 9.895 nmoles cytochrome c reduced/mg protein/minute in sample 4 to 193.47 nmoles cytochrome c reduced/mg protein/minute in sample 1. Cytochrome c reductase activities in the 100,000 S supernatant fraction were almost negligible in all three samples.

Microsomal fractions from samples 1 and 2 were used to study the effects of NADPH dependent lipid peroxidation on aminopyrine N-demethylation in human infant liver. The effect of preincubating microsomes with an NADPH generating system to promote lipid peroxidation on the resulting ability of microsomes to subsequently N-demethylate aminopyrine is shown in figure 23 and 24 for sample 1 and 2 respectively. In microsomes obtained from sample 1, preincubation with an NADPH generating system for 30 minutes had no effect on aminopyrine N-demethylation in control reaction mixtures. When microsomes were preincubated with 25 μM iron and an NADPH generating system, aminopyrine N-demethylation was reduced from 2.205 ± 0.382 nmoles HCHO formed/mg protein/minute in the 0 time preincubation sample to 1.528 ± 0.210 nmoles HCHO formed/mg protein/minute after preincubation for 30 minutes. When microsomes were preincubated for 30 minutes with 25 μM EDTA, no effect on aminopyrine N-demethylation was observed. In a similar experiment in sample 2, preincubation of microsomes with an NADPH generating system for 30 minutes in the presence of 25 μM iron or 25 μM EDTA, had no effect on the ability of the microsomes to N-demethylate

FIGURE 23. The effect of preincubation of hepatic microsomes with an NADPH generating system and iron or EDTA on the subsequent N-demethylation of 5 mM aminopyrine in a human newborn infant (sample 1).

Preincubations were carried out for 0 and 30 minutes under the conditions detailed in Results before N-demethylation of aminopyrine was determined. Each bar represents the mean ± S.E. obtained from 5 individual incubation mixtures using microsomes prepared from sample 1.

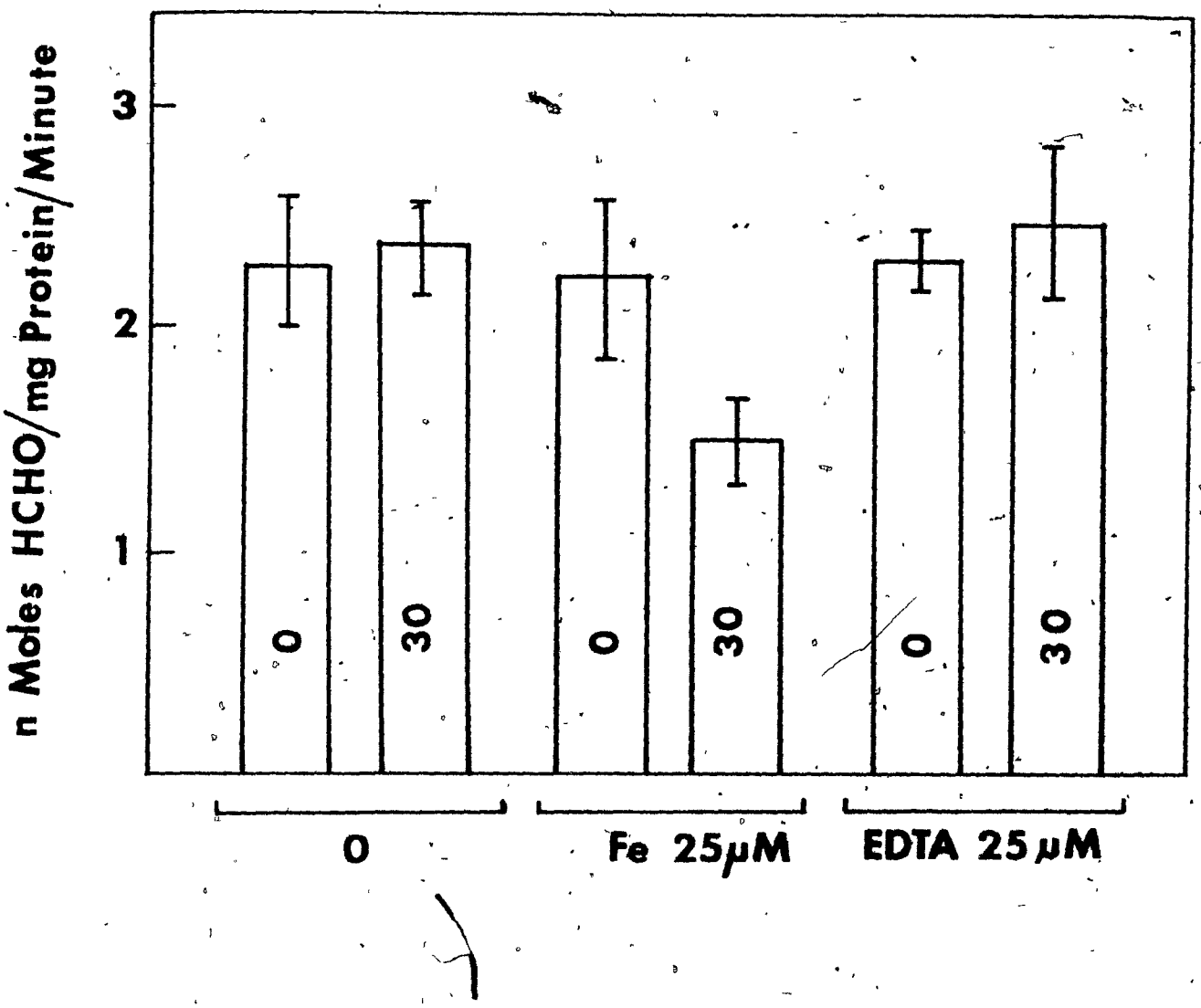
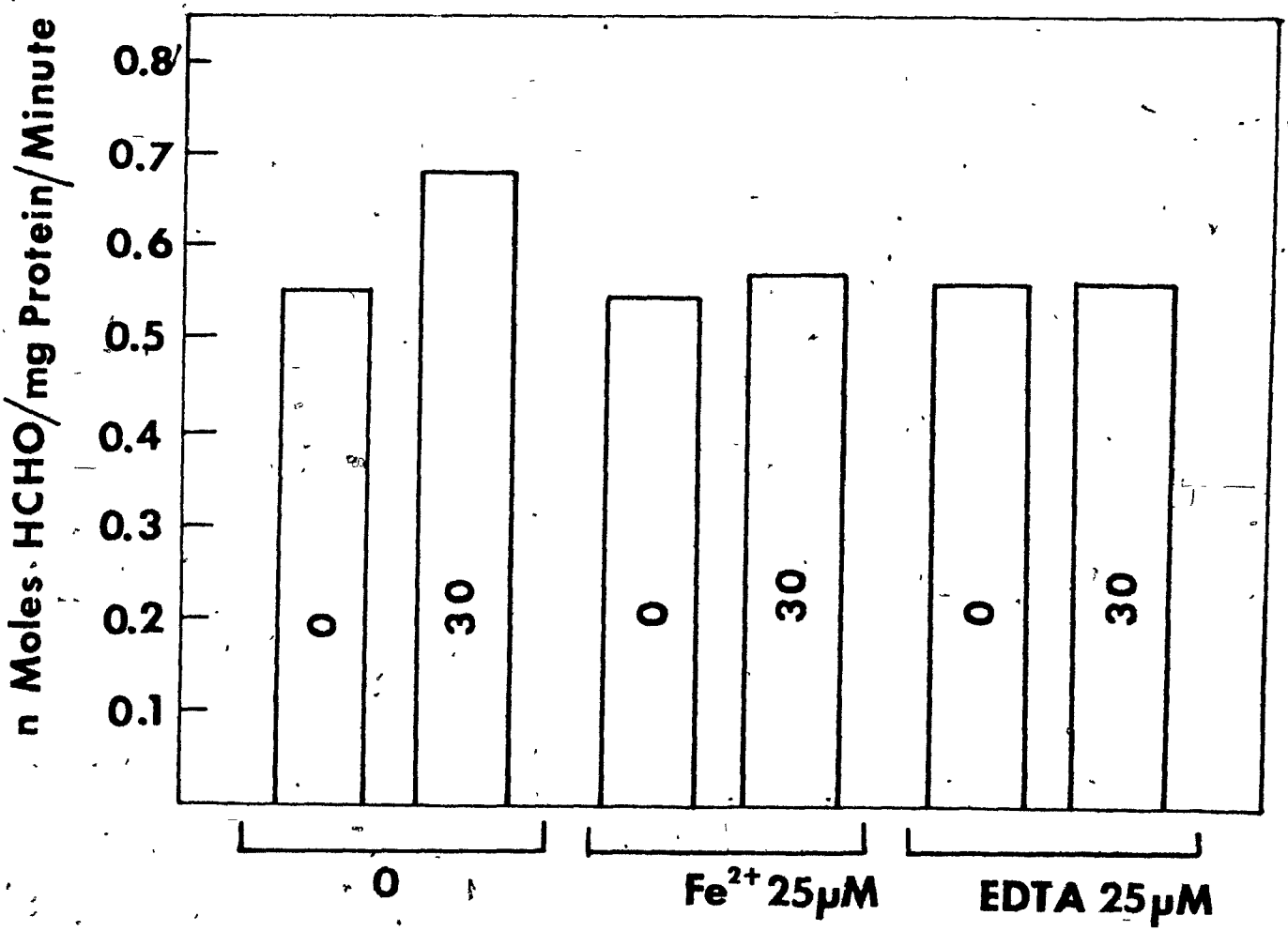

n Moles HCHO/mg Protein/Minute
3
2
1
0
30
0
30
0
30
0
Fe 25μM
EDTA 25μM

FIGURE 24. The effect of preincubation of hepatic microsomes with an NADPH generating system and iron or EDTA on the subsequent N-demethylation of 5 mM aminopyrine in a human newborn infant (sample 2).

Preincubations were carried out for 0 and 30 minutes under the conditions detailed in Results before the N-demethylation of aminopyrine was determined. Each bar represents a single incubation mixture using microsomes prepared from sample 2.

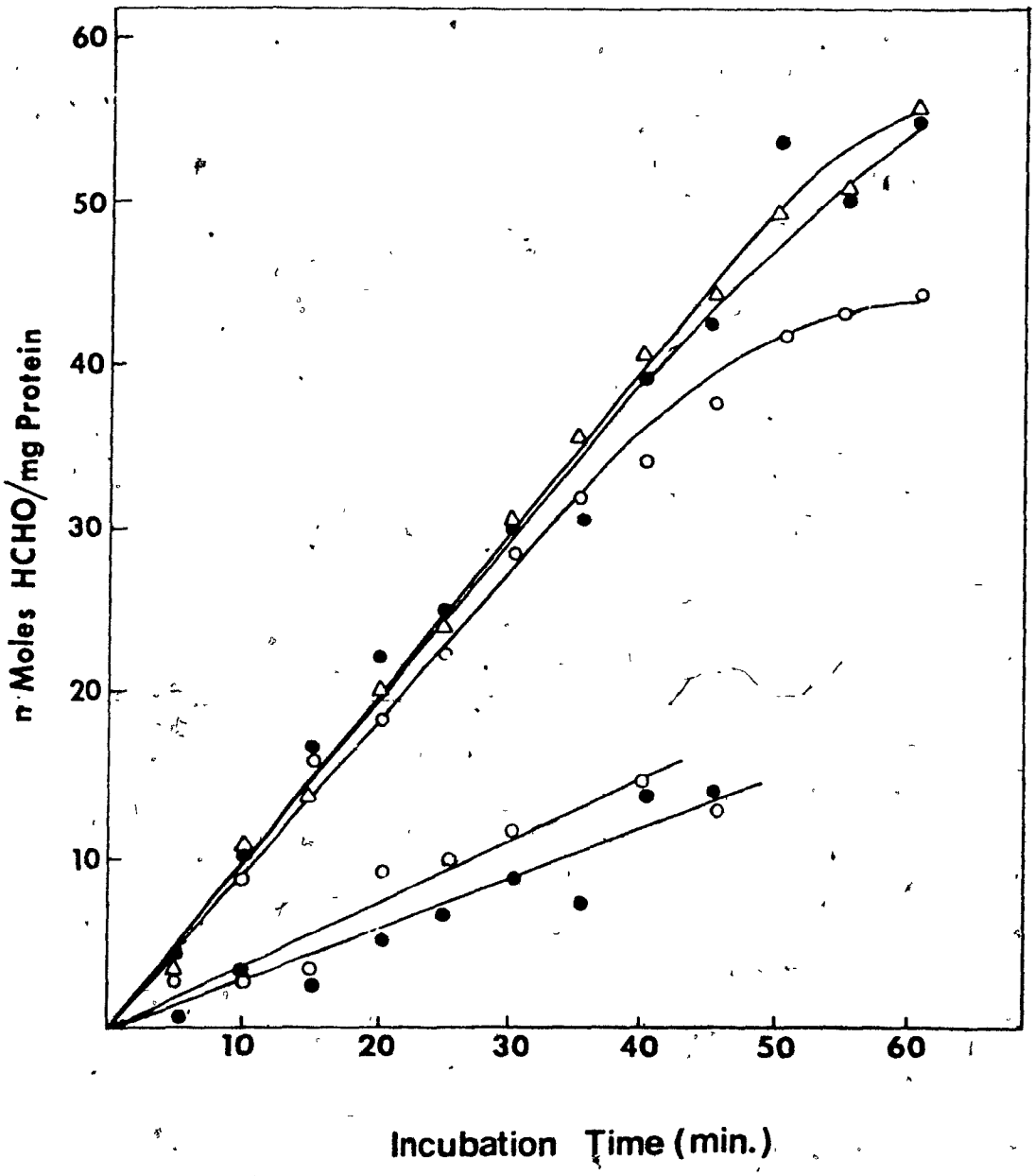

n Moles HCHO/mg Protein/Minute
0.8
0.7
0.6
0.5
0.4
0.3
0.2
0.1
0
30
0
30
0
30
0
Fe2+ 25μM
EDTA 25μM

aminopyrine (figure 24). This particular experiment was performed on single reaction mixtures and the activity throughout the experiment ranged from 0.55 - 0.69 nmoles HCHO/mg protein/minute. The data in figures 23 and 24 should be contrasted with the effect of preincubation in rat microsomes, which is shown in figures 8 and 9 for 25 day old and adult rats. In the rat, even the control preincubation reduced the ability of the microsomes to N-demethylate aminopyrine, which is in marked contrast with the resistance of human microsomes to such an effect, even in the presence of iron.

The linearity of the N-demethylation reaction in microsomes prepared from samples 1 and 2 is illustrated in figure 25. In sample 1, linearity of aminopyrine N-demethylation was maintained for at least 50 minutes in control reaction mixtures and in reaction mixtures containing 25 μM EDTA. When 25 μM iron was added to the reaction mixture, linearity was maintained for about 30 minutes and after 50 minutes the amount of formaldehyde formed was 83% of that in control reaction mixtures. In microsomes prepared from sample 2, overall N-demethylation activity was lower than in sample 1. In this preparation, reactions were linear for 45 minutes in control reaction mixtures and in reaction mixtures containing 25 μM iron. These experiments on the linearity of aminopyrine N-demethylation reactions in human microsomes, contrast with the results obtained in rats, in which linearity does not exceed 20 minutes and can be shortened considerably by the addition of 25 μM iron to the reaction medium, as illustrated in figure 13.

FIGURE 25. The linearity of 1mM aminopyrine N-demethylation reactions in control incubation mixtures and in the presence of 25 μM iron and 25 μM EDTA in hepatic microsomes prepared from human newborn infants.

Each point represents an individual incubation mixture containing microsomes obtained from sample 1 or sample 2. Reactions were terminated at 5 minute intervals up to 60 minutes for sample 1, and 45 minutes for sample 2. ●——● control incubation, o——o 25 μM Fe^{2+}, Δ——Δ 25 μM EDTA.


60
50
40
30
20
10
n Moles HCHO/mg Protein
10
20
30
40
50
60
Incubation Time (min.)

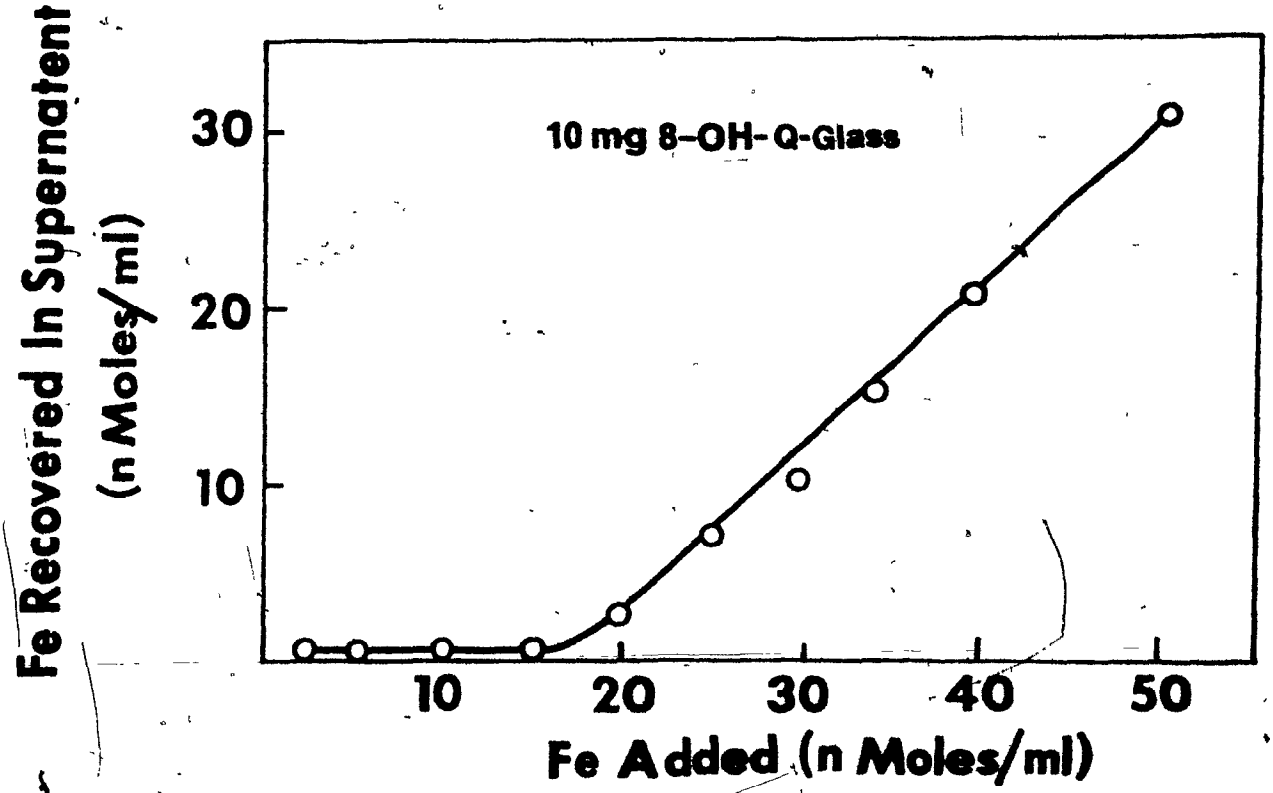
G. THE ABSORPTION OF NON-HEME IRON FROM MICROSOMES USING GLASS IMMOBILIZED 8-HYDROXYQUINOLINE AS CHELATING AGENT, AND THE RESULTANT EFFECT ON NADPH-DEPENDENT LIPID PEROXIDATION AND AMINOPYRINE N-DEMETHYLATION

The binding capacity of 8-hydroxyquinoline glass beads for ionic ferrous iron was determined by adding 8-hydroxyquinoline immobilized on glass beads to solutions containing known concentrations of iron and determining the iron concentration remaining in solution after an incubation period of 15 minutes at 37°C. 8-Hydroxyquinoline glass beads (10 mg wet weight) were added to 1 ml phosphate buffer (0.1 M, pH 7.4) containing iron in concentrations of 1 - 50 nmoles ferrous iron/ml. After 15 minutes incubation at 37°C, iron remaining in the aqueous medium was determined and plotted as a function of the iron concentration originally contained in the medium (figure 26). In solutions containing up to 15 nmoles iron/ml, 10 mg 8-hydroxyquinoline glass beads totally absorbed the iron from solution. When 8-hydroxyquinoline glass beads were added to solutions containing greater than 20 nmoles iron/ml, a constant quantity of 20 nmoles iron was absorbed. The capacity of the 8-hydroxyquinoline glass beads for iron was, therefore, 2 nmoles iron absorbed per mg 8-hydroxyquinoline glass beads.

Iron content, NADPH lipid peroxidation, and aminopyrine N-demethylation were determined in microsomal suspensions containing about 2 mg per ml microsomal protein, which were incubated for 15 minutes at 37° after the addition of 10 mg 8-hydroxyquinoline glass beads. In these experiments, 25 day old rats were used exclusively to provide microsomes which had a high lipid peroxidation activity. Incubations of microsomes with

FIGURE 26. The binding capacity of 8-hydroxyquinoline glass beads for ferrous iron.

Ten mg 8-hydroxyquinoline glass beads were incubated at 37^{0} with 1 ml phosphate buffer containing varying concentrations of iron. Each point represents the aqueous fraction iron content of a single incubation mixture after incubation.


10 mg 8-OH-Q-Glass
Fe Recovered In Supernatent (n Moles/ml)
30
20
10
10
20
30
40
50
Fe Added (n Moles/ml)

8-hydroxyquinoline immobilized on glass beads had no significant effect on the non-heme iron content of microsomes as shown in figure 27. Similarly, incubation of microsomes for 15 minutes with 8-hydroxyquinoline glass beads had no significant effect on NADPH dependent lipid peroxidation activity or aminopyrine N-demethylation activity (figures 28 and 29), when compared to similar activities in control microsomes incubated without 8-hydroxyquinoline glass beads. 8-Hydroxyquinoline glass beads were also added to iron stimulated NADPH dependent lipid peroxidation reactions in microsomes, as shown in figure 30 and table 9. NADPH dependent lipid peroxidation was 11.362 ± 0.537 in a reaction mixture in which microsomes and 10 μM iron were added together and an NADPH generating system was added 2 minutes later to initiate the reaction. This activity was defined as the control for this experiment. When iron was added to the reaction mixture 1 minute prior to the addition of microsomes, NADPH dependent lipid peroxidation was not significantly different from the control reaction mixture, in which iron and microsomes were added together to the mixture. Similarly, when 10 mg 8-hydroxyquinoline glass beads were added to the reaction mixture 1 minute after the addition of microsomes and iron, there was no significant difference compared to the control activity in the reaction mixture in which microsomes and iron were added simultaneously. When iron and 8-hydroxyquinoline glass beads were added together 1 minute prior to the addition of microsomes, NADPH dependent lipid peroxidation was significantly reduced to 19% of the control activity. This activity was only 1.6 times greater than NADPH dependent lipid peroxidation activity in microsomes incubated without the addition of iron.

FIGURE 27. Non-heme iron content of hepatic microsomes of 25 day old rats preincubated with 10 mg 8-hydroxyquinoline glass beads.

Each bar represents the mean ± S.E. for 5 incubation mixtures containing microsomes from a pool of 3 rats. Microsomes were preincubated for 15 minutes with 10 mg 8-hydroxyquinoline. Control incubation mixtures were preincubated without 8-hydroxyquinoline glass beads.

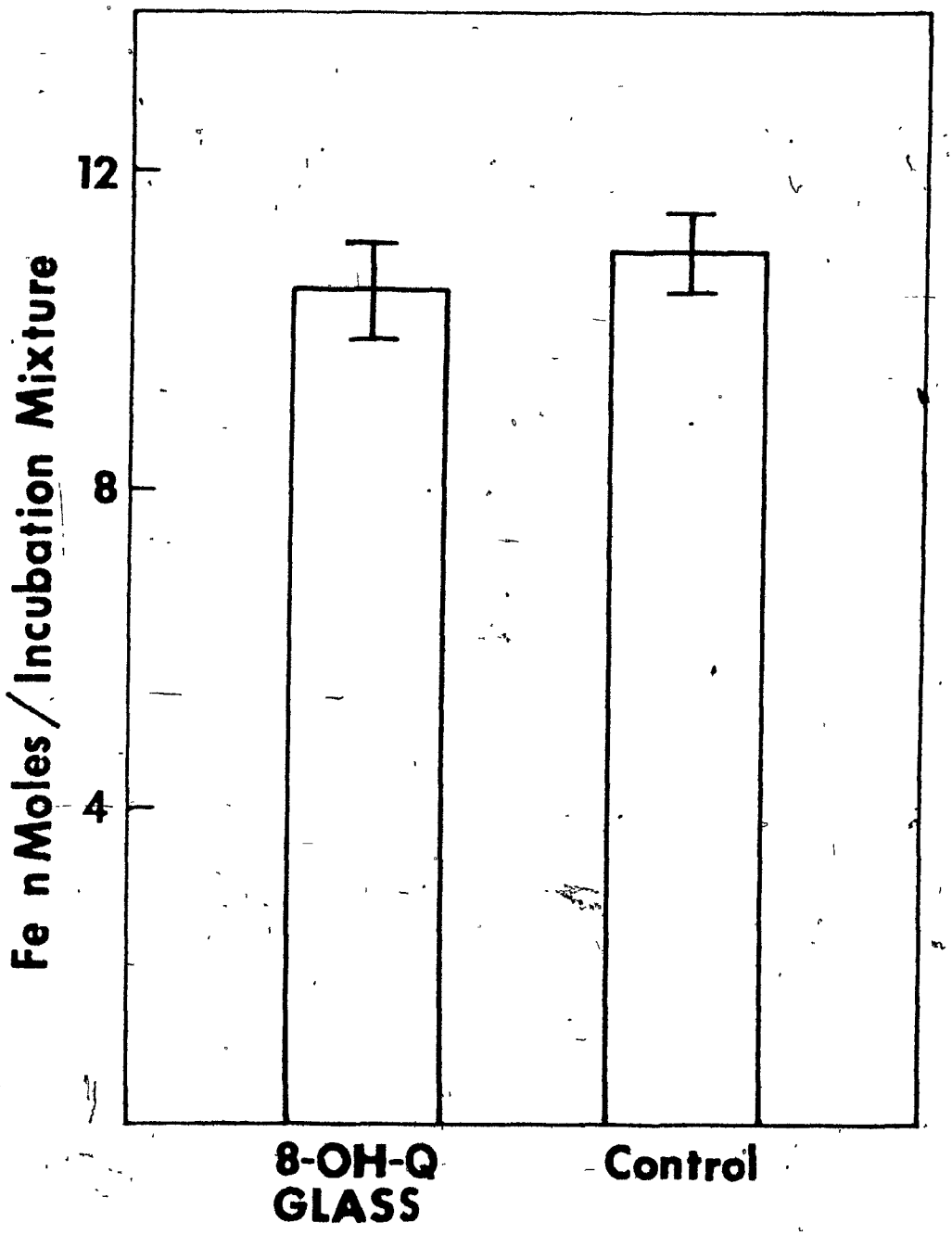

12
8
4
Fe n Moles / Incubation Mixture
8-OH-Q
GLASS
Control

FIGURE 28. NADPH dependent lipid peroxidation in hepatic microsomes of 25 day old rats preincubated with 10 mg 8-hydroxyquinoline glass beads.

Each bar represents mean ± S.E. for 5 individual incubation mixtures containing microsomes prepared from a pool of 3 rats. Microsomes were preincubated for 15 minutes with 10 mg 8-hydroxyquinoline. Control incubation mixtures were preincubated without 8-hydroxyquinoline glass beads.

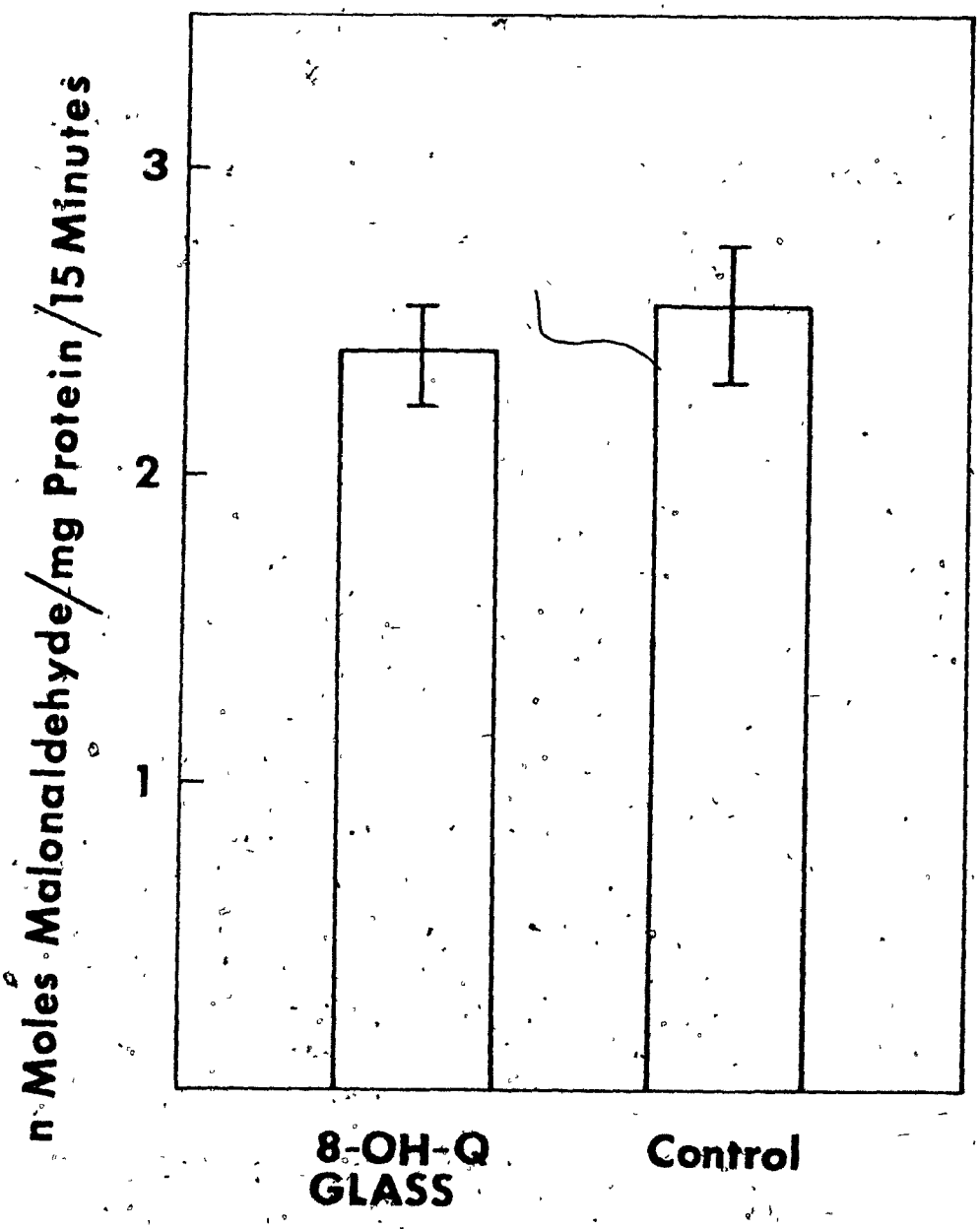

n Moles Malonaldehyde/mg Protein/15 Minutes
3
2
1
8-OH-Q
GLASS
Control

FIGURE 29. Aminopyrine N-demethylation activity in hepatic microsomes of 25 day old rats preincubated with 10 mg 8-hydroxyquinoline glass beads.

Each bar represents mean ± S.E. for 5 individual incubation mixtures containing microsomes prepared from a pool of 3 rats. Microsomes were preincubated for 15 minutes with 10 mg 8-hydroxyquinoline. Control incubation mixtures were preincubated without 8-hydroxyquinoline glass beads.

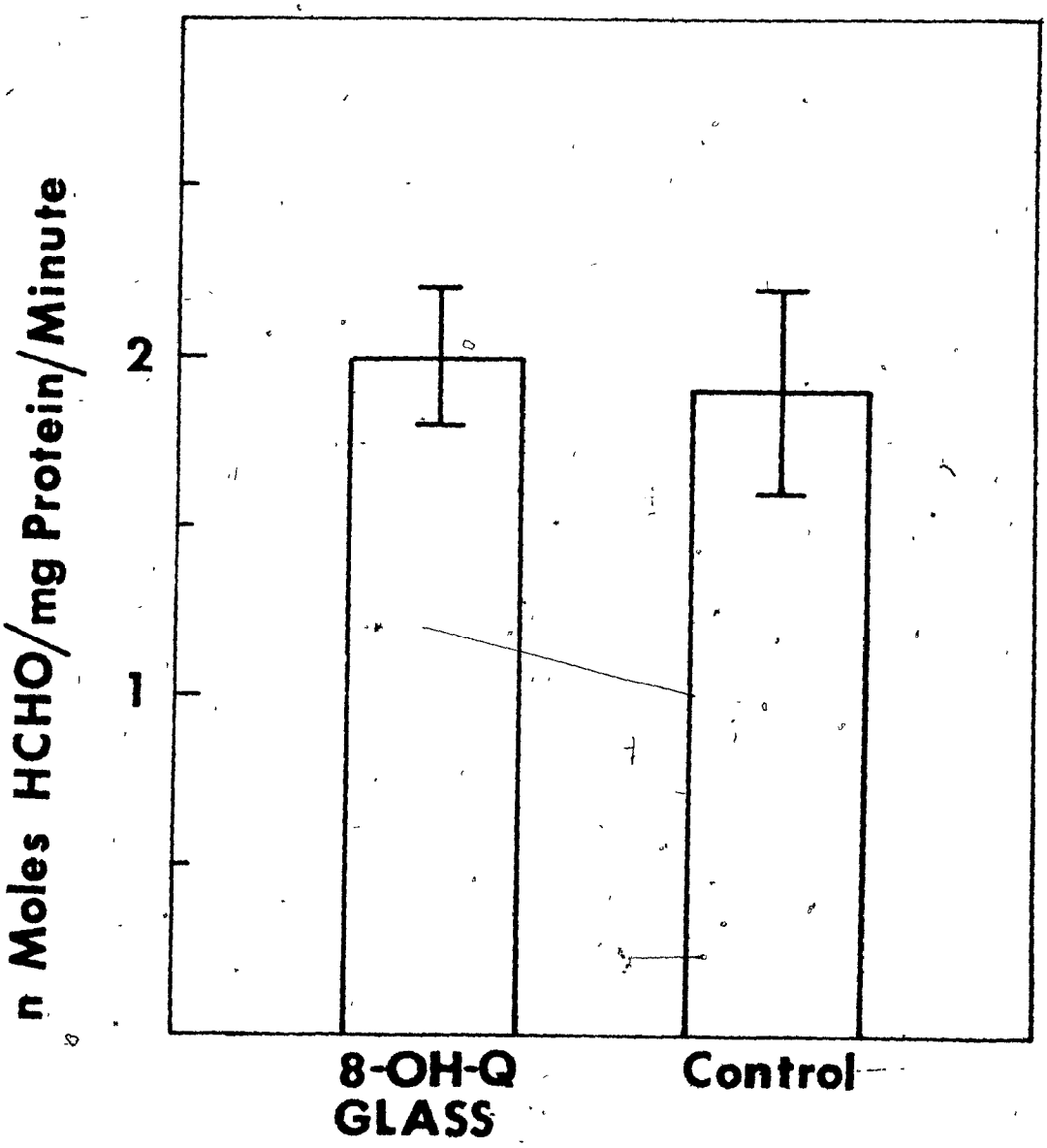

n Moles HCHO/mg Protein/Minute
2
1
8-OH-Q
GLASS
Control

FIGURE 30. The effect of 8-hydroxyquinoline glass beads on the stimulation of NADPH dependent lipid peroxidation by iron in microsomes of 25 day old rats.

Standard reaction mixtures for the determination of NADPH dependent lipid peroxidation, except for the addition of microsomes and NADPH generating system, were placed on an incubation bath at 37°. At 0 time or 1 minute, microsomes, 10 μM iron or 8-hydroxyquinoline glass beads, were added as indicated in the figure. At 2 minutes, NADPH generating system was added and NADPH dependent lipid peroxidation activity determined using an incubation time of 15 minutes. Each bar represents the mean of 5 individual incubation mixtures containing microsomes prepared from a pool of 3 rats.

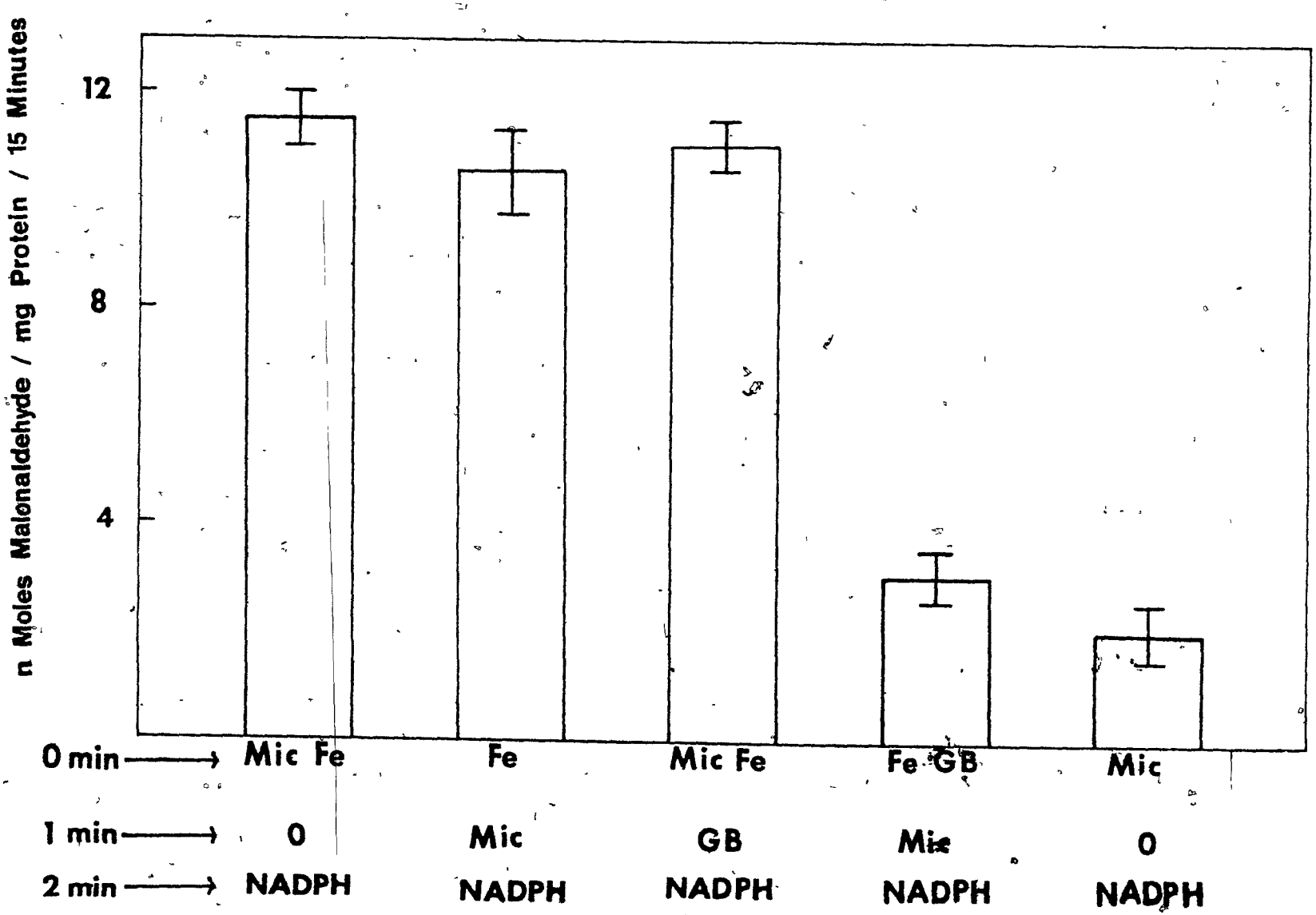

n Moles Malonaldehyde / mg Protein / 15 Minutes
12
8
4
0 min →
1 min →
2 min →
Mic Fe
0
NADPH
Fe
Mic
NADPH
Mic Fe
GB
NADPH
Fe GB
Mic
NADPH
Mic
0
NADPH

Table 9

The Effect of Ferrous Iron and 8-Hydroxyquinoline Glass Beads on the Activity of NADPH Dependent Lipid Peroxidation in Microsomes Prepared from 25 Day Old Rats

Time of Addition (minutes)			Lipid Peroxidation
0	1	2	(nmoles malonaldehyde/mg protein/15 minutes)
MIC + Fe	0	NADPH	11.362 ± 0.537
Fe	MIC	NADPH	10.093 ± 0.651
MIC + Fe	GB	NADPH	10.783 ± 0.436
Fe + GB	MIC	NADPH	2.153 ± 0.306*
MIC	0	NADPH	1.305 ± 0.147*

*Significantly different from incubation in which MIC + Fe were added at 0 time without GB. $p < 0.05$

MIC = Microsomes (0.2 ml)

Fe = Ferrous iron (final concentration 10 μM)

GB = 8-hydroxyquinoline glass beads (10 mg)

NADPH = NADPH generating system (0.1 ml)

Values represent the mean ± standard error of 5 incubation mixtures.

H. THE EFFECTS OF IRON OVERLOAD ON NADPH DEPENDENT LIPID PEROXIDATION AND DRUG OXIDATION IN MICROSOMES PREPARED FROM 25 DAY OLD RATS

An attempt to demonstrate *in vivo* lipid peroxidation and its resulting effects, was made by duplicating the conditions required for high *in vitro* lipid peroxidation in live rats. Twenty-five day old rats, which have the highest NADPH lipid peroxidation activity, were treated with 20 mg/kg, I.P. iron-dextran daily for 4 days. After 2 days, the skin of the rats became distinctly brownish in colour. This was particularly noticeable in the ears and skin surrounding the eye socket, which became bronze in colour. On dissection, the lining of the abdominal cavity was a deep brown-bronze colour. This is presumably similar to the bronzed or suntanned look which is used to describe patients who suffer from iron overload. Control rats which were treated with equivalent volumes of 0.9% saline, remained normal in colour. On the 5th day, 24 hours after the last iron-dextran injection, rats were killed and microsomes prepared in phosphate buffer containing 25 μM EDTA, in an attempt to inhibit lipid peroxidation activity during preparation of the microsomes. Microsomes prepared with EDTA are termed microsomes (EDTA).

In microsomes prepared without EDTA, NADPH dependent lipid peroxidation activity was 1.643 ± 0.203 nmoles malonaldehyde/mg protein/15 minutes in iron-dextran treated rats, which was significantly higher than the activity of 1.277 ± 0.087 nmoles malonaldehyde/mg protein/15 minutes, found in saline treated control rats, as shown in table 10. When 25 μM iron was added to the reaction mixtures, NADPH dependent lipid peroxidation was stimulated to 15.021 ± 0.811 nmoles malonaldehyde/mg protein/15 minutes

Table 10

Microsomal NADPH Dependent Lipid Peroxidation Activity and Content of Thiobarbituric Acid Reacting Material in Control and Iron Overloaded 25 Day Old Rats

	Control	Fe/Dextran
Lipid Peroxidation[+] (nmoles/malonaldehyde/mg prot/min)	1.277±0.087	1.643±0.203*
Lipid Peroxidation[+] (nmoles malonaldehyde/mg prot/min)	14.344±0.833	15.021±0.811
Thiobarbituric Reacting Material[X] (nmoles malonaldehyde/mg prot)	0.176±0.005	0.542±0.017*

*Significantly different from control $p < 0.05$

[+]Microsomes were prepared without EDTA in buffer

[X]Microsomes were prepared with EDTA in buffer

Values represent the mean ± standard error of 5 individual rats

in iron-dextran treated rats compared to a value of 14.344 ± 0.833 nmoles malonaldehyde/mg protein/15 minutes found in control rats (table 10).

In microsomes (EDTA), the content of material which reacts with thiobarbituric acid was equivalent to 0.542 ± 0.017 nmoles malonaldehyde/mg protein, which was significantly higher than the malonaldehyde equivalent of 0.176±0.005 nmoles malonaldehyde/mg protein, found in microsomes prepared from control rats, as illustrated in table 10.

As shown in table 11, N-demethylation of 1 mM and 5 mM aminopyrine in microsomes (EDTA) prepared from iron-dextran treated rats, was identical to the activity in microsomes prepared from control rats. Similarly, there was no difference in NADPH oxidase activity or in cytochrome P-450 levels in microsomes (EDTA) prepared from iron-dextran treated rats compared to control rats, as shown in table 11. Non-heme iron content of microsomes from iron treated rats was 12.738 nmoles/mg protein compared to 5.341 nmoles/mg protein in microsomes of control rats.

I. THE EFFECTS OF IRON OVERLOAD ON THE ELECTRON MICROSCOPY OF MICROSOMAL PELLETS PREPARED FROM 25 DAY OLD RATS

Microsomal pellets (EDTA) from iron treated and saline treated control rats, were examined by electron microscopy. In control preparations, electron micrographs showed vesicles which had membranes with a triaminar structure as illustrated in figure 31. Most vesicles were either round or slightly oval in shape. Adhering to many of these vesicular structures were large numbers of ribosomes as indicated by the arrow in figure 31. Several broken vesicles and other smaller membrane fragments could also be

Table 11

Aminopyrine N-Demethylation Activity, Electron Transport Components and Protein Yield in Microsomes Prepared from Control and Iron Overloaded 25 Day Old Rats

		Control	Fe/Dextran
Aminopyrine N-demethylation (nmoles HCHO/mg protein/minute)	5 mM	2.516±0.086	2.614±0.141
	1 mM	1.497±0.042	1.494±0.093
Cytochrome P-450/ (nmoles/mg protein)		0.215±0.007	0.195±0.008
NADPH oxidase (nmoles NADPH oxidized/mg protein/minute)		11.158±0.735	11.237±0.940
Non-heme iron (nmoles/mg protein)		5.341±0.289	12.738±0.670
Microsomal protein (mg/ml)		24.34 ±0.43	22.38 ±1.09

Values represent the mean ± standard errors of 5 individual rats.

FIGURE 31. Electron micrograph of microsomes prepared from a 25 day old saline treated control rat.

Magnification = 44,880

observed.

In microsomes (EDTA) prepared from iron-dextran treated rats, round or oval vesicles could be seen as shown in figure 32. A major difference, however, compared to the control microsomes, was that the vesicle membranes were almost completely devoid of ribosomes as indicated by the arrow in figure 32. Membranes which did carry ribosomes on their surface, had greatly reduced numbers. Also apparent in these microsomes were several membrane fragments and some broken vesicles, as seen in the control. Of particular note was the greatly increased numbers of flat or linear membrane fragments in rats treated with iron-dextran compared to the almost complete absence of membranes of this shape in the control preparations.

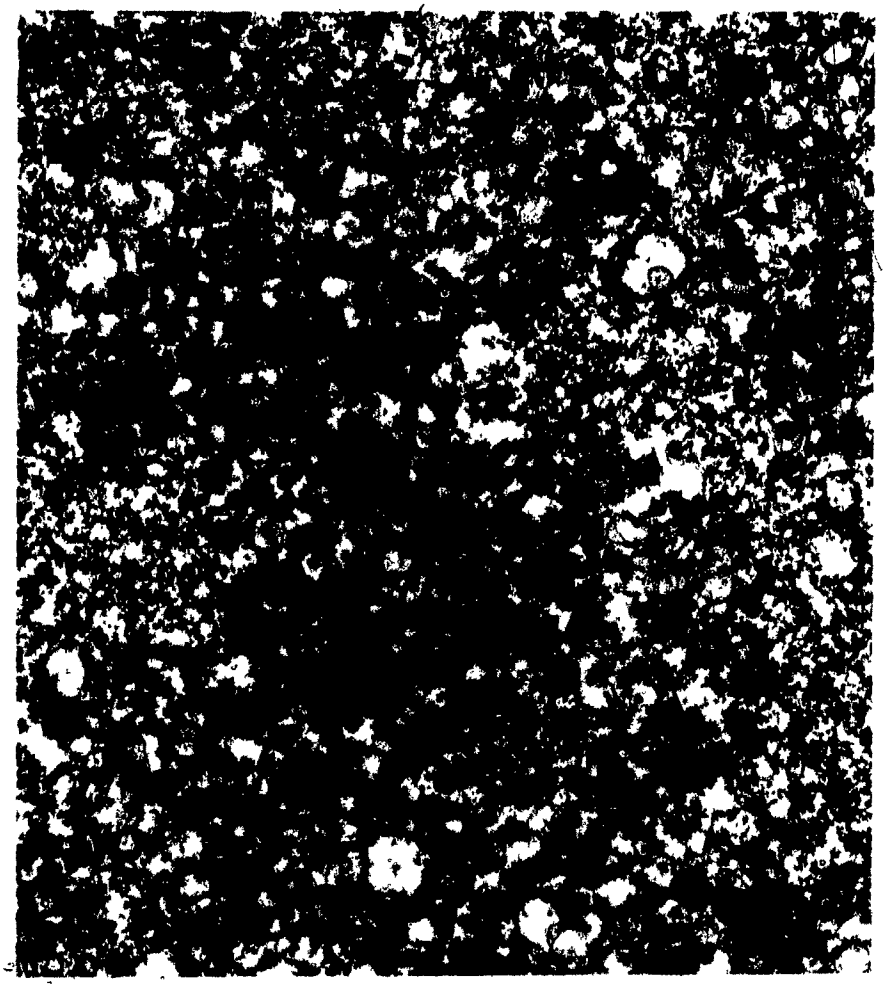
A normal microsomal preparation was incubated with 25 μM iron and an NADPH generating system, to promote lipid peroxidation, and recentrifuged at 100,000 x g to yield a second pellet. Figure 33 illustrates an electron micrograph of the second pellet. It is apparent that most of the vesicle structures have been destroyed and that most of those present do not demonstrate a trilaminar structure. These vesicles tended to clump together and become associated with a diffuse area of dense material. Such an area is seen slightly below the centre of the electron micrograph in figure 33.

FIGURE 32. Electron micrograph of microsomes prepared from a 25 day old rat treated with 20 mg/kg iron-dextran for 4 days.

Magnification = 44,880

FIGURE 33. Electron micrograph of microsomes subjected to *in vitro* lipid peroxidation.

Microsomes were incubated for 15 minutes with an NADPH generating system and 25 μM iron. This incubation mixture was recentrifuged at 100,000 x g to yield a second pellet which was examined in the electron microscope. Magnification = 31,930



DISCUSSION

The results described, show that hepatic microsomal NADPH dependent lipid peroxidation activity is present in rats aged from birth until adulthood. Activity increases from birth to a postnatal maximum at 25 days of age, and then declines to the normal adult range by 35 days of age. In a recent report by El Defrawy *et al* (1974), it was shown that between 21 and 56 days of age, NADPH dependent lipid peroxidation was constant when expressed as activity per g of liver but when activity was expressed as activity per mg microsomal protein, lipid peroxidation decreased with advancing age. The decrease was less in magnitude than that found between 25 days of age and adult in the present experiments which have been reported as activity per mg microsomal protein. These authors did not study rats younger than 21 days and the fact that they added iron and ADP to the reaction mixtures probably accounts for the differences in the two studies. All other studies of microsomal NADPH dependent lipid peroxidation have been carried out using rats of adult ages. The only other study of peroxidation in immature animals, showed that in hepatic mitochondria, spontaneous lipid peroxidation (presumably of a non-enzymatic nature) was increased in the rat fetus compared to that found in adult rats (Williams 1966). In a subsequent study, the same investigator demonstrated that newborn and adult rats had identical spontaneous lipid peroxidation activity in hepatic mitochondria (Williams 1973). Ages between newborn and adulthood, however, were not included in the study.

Several mechanisms can be postulated to explain the increased activity of NADPH dependent lipid peroxidation in developing rats. Lipid peroxidation requires cytochrome c reductase as an electron carrier (Wills

1969a, Pederson and Aust 1972, Pederson *et al* 1973), and several reports have demonstrated that the flavoprotein cytochrome c reductase is deficient at birth and increases to adult activity during the perinatal period in the rat (MacLeod *et al* 1972, El Defrawy *et al* 1974). Increasing lipid peroxidation activity may, therefore, be related to the increasing availability of cytochrome c reductase in developing rat microsomes. This postulate, however, is unlikely for two reasons. First, MacLeod *et al* (1972) demonstrated that cytochrome c reductase activities continued to increase for 14 days after the maximal lipid peroxidation activity was reached. When cytochrome c reductase activity was at its highest in the adult rat, lipid peroxidation was at its lowest activity. Second, the fact that in both 25 day old and adult rats, NADPH dependent lipid peroxidation was stimulated several fold by the addition of iron to the reaction mixtures, suggested that there is sufficient flavoprotein in reserve for normal function of lipid peroxidation at both the ages studied. It would be unlikely that cytochrome c reductase would be rate limiting under the low lipid peroxidative conditions of the experiments described. Also, though it does not apply directly to the experiments in immature rats, a poor correlation between lipid peroxidation activity and cytochrome c reductase activity was found in microsomes prepared from a variety of species which had different cytochrome c reductase activities (Kamataki and Kitagawa 1974). This also supports the contention that cytochrome c reductase is not the rate limiting factor in determining the activity of NADPH dependent lipid peroxidation. Further support for this argument was given in the results presented by Jacobson *et al* (1973) who reported that in the

rabbit, microsomal NADPH dependent lipid peroxidation was about 10% of that found in the rat but that cytochrome c reductase activities in the two species are identical (MacLeod 1972).

As iron has been shown to stimulate NADPH dependent lipid peroxidation (Hochstein and Ernster 1963, Wills 1969b, Kamataki and Kitagawa 1973), control of lipid peroxidation activity in rats of different ages could result from variations in the content or availability of non-heme iron in microsomes at different ages. Evidence from three separate experiments, provide arguments against such a hypothesis. First, total non-heme iron content of microsomes was identical in preparations from 25 day old and adult rats, although it is fully recognized that if the difference in lipid peroxidation at the two ages was due to differences in a small specific pool of iron, then this would not be apparent by measuring total non-heme iron. Second, when lipid peroxidation was maximally stimulated by the addition of iron to reaction mixtures, the maximally stimulated activity was still higher in 25 day old rats compared to adult rats. If the differences in lipid peroxidation activity were due to a simple deficiency of iron in the adult compared to 25 day old rats, then the addition of iron to the reaction mixtures would have equalized the maximally stimulated activities in the two ages. Third, it was demonstrated that the I_{50} for the inhibition of lipid peroxidation by EDTA was identical in 25 day old and adult rats. This suggests that equivalent amounts of iron involved in the lipid peroxidation reaction were present in the two ages, as different concentrations of iron would almost certainly have produced different I_{50}'s.

Several authors have suggested that the iron involved in NADPH dependent lipid peroxidation reactions in microsomes is complexed in some way and that the iron complex is involved in the transfer of electrons required by the reaction (Orrenius *et al* 1964, Wills 1969b, Bidlack and Tappel 1973). Experiments described here have shown that glass immobilized iron chelating agent was incapable of reducing microsomal iron content and has no effect on the stimulation of lipid peroxidation activity which results from the addition of iron to reaction mixtures. These experiments support the idea of an iron complex being involved in the lipid peroxidation reaction and it is possible that the increased lipid peroxidation activity of 25 day old rats was due to differences in this complex at this age period.

Another possible mechanism for increased lipid peroxidation in young rats could be the lack of the specific anti-oxidants proposed by Wills (1972b) which are responsible for protection of the endoplasmic reticulum membrane. Williams (1966) considered a deficiency of the anti-oxidant vitamin E being responsible for the increase of spontaneous lipid peroxidation in fetal mitochondria. Such an explanation for the present observations is only theoretical as the anti-oxidants of Wills have not been defined and, therefore, cannot be tested in this system.

Kamataki *et al* (1974) has reported that a factor present in the 100,000 x g soluble supernatant fraction could inhibit NADPH dependent lipid peroxidation. If microsomes are regarded as closed vesicles which are created when endoplasmic reticulum is pinched off, soluble supernatant would be trapped inside. If this soluble fraction contains an-

inhibitor of lipid peroxidation, then the relative amounts of this component will control lipid peroxidation activity in microsomal preparations. If this factor is reduced or absent in young rats, then this could explain increased lipid peroxidation at these ages. However, in experiments attempting to investigate this possibility, the addition of soluble fraction to microsomal lipid peroxidation mixtures, resulted in variable and inconsistent results and, therefore, these results are not reported in this thesis. In the original report of Kamataki *et al* (1974) great variability was also found in their adult rat experiments which they stated were due to unknown physiological factors or seasonal variations.

The present experiments have demonstrated an increase in NADPH dependent lipid peroxidation in microsomes at certain ages in the developing rat. The exact reason for the increased activities was not determined but cytochrome c reductase activity and non-heme iron levels were not directly responsible.

A close relationship between the activity of the NADPH dependent lipid peroxidation and drug oxidation in hepatic microsomes of the rat has been proposed by several investigators (Hochstein and Ernster 1963, Orrenius *et al* 1964, May and McCay 1968a, Wills 1969c). In general, it has been demonstrated that the rate of drug oxidation in isolated microsomes is inversely related to the activity of NADPH dependent lipid peroxidation, and factors which increase lipid peroxidation activity, reduce the capacity of microsomes to metabolize drugs (Orrenius *et al* 1964, Wills 1969c, Jacobson *et al* 1973, Kamataki and Kitagawa 1973), and inhibition of lipid peroxidation has been shown to increase the capacity of microsomes

to metabolize drugs (Lewis *et al* 1967, Kamataki and Kitagawa 1973). The close relationship between lipid peroxidation and drug oxidation might suggest that lipid peroxidation activity could play a role in the reduced levels of microsomal drug oxidation which are present in the young rat (Quinn *et al* 1958, Mac leod *et al* 1972). However, the present study would indicate that the influence of lipid peroxidation in determining the deficient drug oxidation system in the young rat is of minor importance. Drug oxidation in microsomes was almost absent at birth in the rat and increased to adult activity during the first six weeks of life (MacLeod *et al* 1972) whereas NADPH dependent lipid peroxidation had a low activity except between 2 and 6 weeks of age and the general pattern was not inversely related to the pattern of the development of drug oxidation. Lipid peroxidation, however, may have some effect on the developmental pattern of drug oxidation, as both MacLeod (1972) and Aranda (1975) demonstrated a plateau or slowing in the developmental pattern of aminopyrine N-demethylation and aniline hydroxylation at the ages where lipid peroxidation was maximal in the studies reported here. In rabbit microsomes which had a low NADPH dependent lipid peroxidation activity (Gram and Fouts 1966, Jacobson *et al* 1973), this plateau in the development of drug oxidation in microsomes appeared to be absent (Fouts and Devereux 1972, MacLeod 1972).

The decreased activity of drug oxidation in hepatic microsomes which results from lipid peroxidation activity in microsomes, has been attributed to competition for available electrons from the common electron pathway (Orrenius *et al* 1964, Wills 1969c), destruction of the membrane structure of the microsomes (Wills 1969c, Hogberg *et al* 1973, Arstila 1972), and/or

to a breakdown in the structure of the hemoprotein cytochrome P-450 or its heme group (Schacter *et al* 1972, 1973, Levin *et al* 1973).

The effects of different activities of lipid peroxidation on the ability of microsomes to N-demethylate aminopyrine was tested in 25 day old and adult rats. N-demethylation was determined by adding 5 mM aminopyrine to microsomes after they had been preincubated for varying times with an NADPH generating system to promote lipid peroxidation of the microsomes. As the concentration of aminopyrine utilized, inhibits NADPH dependent lipid peroxidation by at least 90% in both 25 day old and adult microsomes, N-demethylation activity was measured with a minimum interference from electron competition which would normally occur if lipid peroxidation was operating simultaneously. These experiments, therefore, effectively measured the effects of NADPH dependent lipid peroxidation which had taken place during the preincubation period, before the substrate for the drug oxidation enzyme system is added.

Preincubation of microsomes with an NADPH generating system markedly reduced the ability of the microsomes to N-demethylate aminopyrine in adult rats with the maximum effect occurring when lipid peroxidation activity was increased by adding 25 μM iron to the reaction mixtures. Similar findings were reported by Kamataki and Kitagawa (1973) who described a reduction in codeine N-demethylation in microsomes which had been incubated with an NADPH generating system. As predicted in 25 day old rats which have a higher lipid peroxidation activity, the effect of preincubation of microsomes with an NADPH generating system was greater than that found in adult rats in both control incubation mixtures and incubation mixtures

containing iron. As competition for available electrons was not a factor in these experiments, the reduction in the ability of microsomes to N-demethylate aminopyrine resulted from another effect. Destruction of cytochrome P-450 or destruction of the microsomal membrane would be the most likely explanation. Strong evidence has been presented to relate decreases in drug oxidation to cytochrome P-450 breakdown occurring as a result of lipid peroxidation (Levin *et al* 1973, Schacter *et al* 1972, 1973). These investigators showed that during lipid peroxidation in microsomes, cytochrome P-450 content decreased and was accompanied by the evolution of carbon monoxide, heme breakdown products and a reduction in drug oxidation activity. All of these effects could be prevented by adding EDTA to the reaction mixture. General membrane destruction (Wills 1969c, Hogberg *et al* 1973, Arstila 1972) is likely to be part of the same phenomenon or results from more severe lipid peroxidation conditions. Other agents such as detergents or phospholipases which destroy membranes, lead to cytochrome P-450 destruction and reduced drug oxidation (Wills 1971, Omura and Sato 1964a). Recently, however, in a discussion following a report at the Second International Symposium on Microsomes and Drug Oxidation, Mannering (1973) reported that in microsomes which were incubated with NADPH, he recorded a decrease in drug oxidation and the evolution of carbon monoxide but no reduction in cytochrome P-450 and that measurements of protoheme indicated that heme remained intact. Also, Carpenter and Howard (1974) showed that preincubation of microsomes with NADPH resulted in only a slight loss of cytochrome P-450 (14%). Most of this loss occurred in the first 5 minutes of preincubation. In a similar experiment, Mannering (1971)

reported loss of cytochrome P-450 but the method used for the determination of the hemoprotein did not correct for endogenously formed carbon monoxide. The loss of cytochrome P-450 was only an apparent loss and was in fact really due to the appearance of carbon monoxide interfering with the assay method. Although this explanation can be considered to explain the loss of cytochrome P-450 observed in the experiments of Schacter *et al* (1972, 1973) and the slight decrease in the experiments of Carpenter and Howard (1974) who used the method of Omura and Sato (1964a), this did not apply to the experiments of Levin *et al* (1973) who recognized the problem and showed that the method of Raj and Estabrook (1970) could be applied to determine cytochrome P-450 levels in the presence of endogenously formed carbon monoxide. As in the experiments of Mannering (1973) and Carpenter and Howard (1974), in the present experiments repeated attempts to reduce cytochrome P-450 levels in microsomes by inducing high lipid peroxidation activity in reactions containing NADPH generating system and iron were unsuccessful when cytochrome P-450 was measured by a method which is valid in the presence of endogenously formed carbon monoxide in microsomes (Raj and Estabrook 1970). We have concluded that the destruction of cytochrome P-450 and/or its heme group is not a necessary requirement for the decrease in drug oxidation activity in microsomes which are subjected to conditions promoting lipid peroxidation activity. The destruction of cytochrome P-450 and membrane reported by some authors, may be due to further stages of the same reaction process or due to some completely different unrelated effect, but they are not an absolute requirement for the decrease in drug oxidation which results from the process of lipid

peroxidation.

In relative terms, the use of the reduced cytochrome P-450-carbon monoxide complex and its measurement by spectral means is a rather gross method to assess the function of cytochrome P-450 and its role in the endoplasmic reticulum as the central component in the hepatic mixed function oxidase system for the metabolism of drugs. The ability of the hemoprotein to form a complex with carbon monoxide, may exist even after a disturbance of cytochrome P-450 structure, its relationship with its electron transport system or its relationship to the endoplasmic reticulum membrane has rendered the system incapable of metabolizing drug substrates. In isolated fractions of microsomal enzymes, Lu *et al* (1969a, 1969b) isolated a fraction containing cytochrome P-450 which demonstrated a reduced cytochrome P-450-carbon monoxide spectra but which was incapable of oxidizing drugs by itself.

When drug substrates for the oxidizing enzymes are added to microsomes, they display characteristic difference spectra (Remmer *et al* 1966, Imai and Sato 1966), which can be divided into two main groups (type I and type II spectra). Although the exact nature of the binding is not absolutely clear, there is a school of thought that interprets these spectra as representing the binding of drug substrates at or near the enzymic site for the oxidation of the substrates (Schenkman *et al* 1967a). Whether this relationship between spectral binding and the enzymatic site proves to be correct or not, these spectral binding spectra do represent a specific reaction between drugs and cytochrome P-450 which may in fact be a much more sensitive measurement in determining the ability of cytochrome P-450

to participate in drug oxidation reactions than the measurement of cytochrome P-450-carbon monoxide complex spectra.

In the experiments described here, NADPH dependent lipid peroxidation had no effect on the qualitative aspects of aniline and hexobarbital difference spectra with the λmin and λmax remaining at the same wavelengths even after 30 minutes incubation. Low activities of lipid peroxidation had no effect on the magnitude of the spectra but when high activities of lipid peroxidation were induced in microsomes by adding iron, the magnitude of the absorbance at the λmax and λmin for both aniline and hexobarbital was markedly reduced. Also, it was shown that after a period of peroxidation in microsomes, the kinetics of the binding of the substrates as measured by the difference spectra were much more variable but it did appear that the spectra dissociation constants (ks) remained unchanged and that the magnitude of the maximum absorbance was reduced. In terms of classical Michaelis-Menten enzyme kinetics, this is equivalent to the Km remaining unchanged and the Vmax being reduced. The unchanged Ks for spectral binding and the fact that the spectra were qualitatively unchanged, suggests that after NADPH dependent lipid peroxidation the number of binding sites was decreased but those which remain bind drug with an unchanged affinity. The ability of NADPH to reduce the magnitude of substrate binding spectra had been observed previously by Schenkman *et al* (1967a) but at that time this was thought to be a direct chemical reduction of the cytochrome P-450 complex. Such an explanation cannot operate in the system reported here, as in reaction mixtures which had low activity of lipid peroxidation no effects were observed on spectral binding even

after incubation with an NADPH generating system for 30 minutes. If the reduction in magnitude of the binding spectra was due to chemical reduction by NADPH, then it would occur in both low and high peroxidation reaction mixtures.

We interpret the data to indicate that NADPH dependent lipid peroxidation reduces drug oxidation by affecting the ability of the hemoprotein cytochrome P-450 to bind substrates which are oxidized by the hepatic mixed function oxidase system. Total destruction of cytochrome P-450 or its heme moiety, or total destruction of the membrane are not a requirement for a decrease in drug oxidation activity, and these effects probably occur as a later stage in the action of lipid peroxidation in microsomal membranes. It is also possible that in the cases where destruction of cytochrome P-450 and its heme have been reported, that it occurs by a mechanism other than lipid peroxidation.

Increases in microsomal lipid peroxidation activity in this study and others have shown that drug oxidation in microsomes is decreased but the opposite effect of inhibition of lipid peroxidation activity resulting in an increase in microsomal drug oxidation has not been consistently shown. Several investigators have found that inhibition of lipid peroxidation did not result in increases in drug oxidation in microsomes (Gram and Fouts 1966, Anders 1969, Wills 1969c, Lewis *et al* 1967, Peters and Fouts 1970). Stimulation of epoxidation in microsomes was observed by Lewis *et al* (1967) after inhibition of lipid peroxidation. Recently, Kamataki and Kitagawa (1973) not only observed a reduction of N-demethylation after lipid peroxidation was increased by adding iron to reaction mixtures but they

demonstrated an increase in N-demethylation when lipid peroxidation was inhibited with EDTA. In the present experiments, drug oxidation was not stimulated by the addition of EDTA to adult rat microsomes but in 25 day old rat microsomes the addition of EDTA to incubation mixtures slightly increased N-demethylation of aminopyrine in about half of the preparations tested. The magnitude of the increase in N-demethylation in these preparations was small in comparison to the stimulation reported by Kamataki and Kitagawa (1973) in adult preparations.

Drug oxidation reactions have a well defined incubation time for the maintenance of a linear reaction and the explanation for variable results for the stimulation of drug oxidation after inhibition of lipid peroxidation in different laboratories and in the two age groups of the present study may involve the incubation time and reaction conditions used by different laboratories. Kamataki and Kitagawa (1973) used a reaction time of 30 minutes for N-demethylation which is much longer than that normally considered the limit for a linear reaction. In figure 13, linearity of reaction never exceeded 20 minutes in 25 day old and adult rats for 1 mM aminopyrine. When lipid peroxidation was actively promoted by the addition of iron to the reaction mixture, linearity, particularly in the case of young rats, was greatly reduced. In the experiments of Kamataki and Kitagawa (1973) basal activity of lipid peroxidation was only 50% of the maximal activity achieved by the addition of iron which compared to a basal activity in the present experiments of about 5% of the maximal activity produced by the addition of iron. It is therefore likely that the effect of lipid peroxidation on N-demethylation reactions in the experiments

of Kamataki and Kitagawa are similar to the effect observed in our incubation mixtures containing iron. The effect of EDTA on lipid peroxidation in their experiments is comparable to the present control and EDTA incubation mixtures. In that case, by using incubation times of 30 minutes, differences in N-demethylation activity became apparent between control and iron added incubations, but when an incubation of 15 minutes or less was used then no difference in N-demethylation activity is observed. The observation of increases in drug oxidation resulting from inhibition of lipid peroxidation, appear to be a function of the linearity of the reaction as lipid peroxidation activity effects the linearity of reaction time. A similar conclusion can be drawn from the data of Jacobson *et al* (1973) who showed that lipid peroxidation activity has a marked effect on the linearity of pentobarbital oxidation and acetanilide hydroxylation in microsomes. EDTA was shown to reverse these effects. Jacobson *et al* (1973) used a phosphate buffer containing an iron contaminant to produce a high activity of lipid peroxidation. In their experiments, incubation periods greater than 10 minutes would yield an apparent stimulation of drug oxidation on the addition of EDTA. It is concluded that inhibition of lipid peroxidation in microsomes does not directly result in an increase in drug metabolism if the reaction times are within the period of linear reaction. Apparent increases in drug oxidation can be observed if the linearity of reaction time is exceeded for the reaction being examined, and lipid peroxidation activity is sufficiently high to account for the deviation in linearity. Reaction linearity times vary from laboratory to laboratory and as will be discussed later, microsomal

reaction mixtures contain varying amounts of iron which also appears to vary between laboratories. This would account for the variable ability of different investigators to demonstrate stimulation of drug oxidation reactions after inhibition of lipid peroxidation.

Increases in NADPH dependent lipid peroxidation in microsomes prepared from 25 day old rats compared to adults should also be reflected in differences in NADPH oxidation in microsomes at the two ages. However, in the present experiments, NADPH oxidase was identical in 25 day old and adult rats, which is in agreement with several reports that NADPH oxidase activity was relatively constant during the development of the rat (Dallner *et al* 1965, El Defrawy 1974, Aranda 1975). This is perhaps not surprising, as NADPH oxidation measured by the decrease in absorbance at 340 nm is an estimation of the total NADPH oxidized by microsomes, and NADPH utilized by enzyme systems such as drug oxidation or lipid peroxidation are likely to be only a fraction of the total. Assessment of NADPH oxidation specific for lipid peroxidation was estimated in the present investigation by determining NADPH oxidation which is sensitive to inhibition by EDTA. As 25 μM EDTA inhibits lipid peroxidation, it was reasoned that the portion of NADPH oxidized during the process of lipid peroxidation was that sensitive to EDTA inhibition. NADPH oxidase specific for lipid peroxidation was highest in 25 day old rats compared to adult rats in both control and iron stimulated incubation mixtures. The overall pattern obtained for NADPH oxidation used by lipid peroxidation was very similar to the pattern obtained when lipid peroxidation was measured using the thiobarbituric acid reaction.

Recently, Beuning and Franklin (1974) and Jeffery and Mannering (1974) have shown that a reduced nicotinamide mononucleotide is formed from NADPH by a pyrophosphatase in microsomes. This mononucleotide has an identical spectra to NADPH and therefore, leads to an underestimate in the amount of NADPH utilized by microsomes when the spectrophotometric method is used for its estimation. This is most likely to be responsible for the discrepancies and difficulties many investigators have encountered in attempting to show a stoichiometric relationship between drug oxidation and NADPH oxidized. NADPH can be correctly assessed by inhibition the pyrophosphatase with EDTA (Jeffery and Mannering 1974) or with 5'AMP (Beuning and Franklin 1974) with a resulting closer approach to stoichiometry. In the present experiments, the concentration of EDTA (25 μM) had no effect on pyrophosphatase (Jeffery and Mannering 1974) and therefore, did not have to be accounted for in our calculations. When higher concentrations of EDTA were utilized as pyrophosphatase inhibitors in stoichiometry experiments, NADPH oxidation for lipid peroxidation was also inhibited and therefore, effectively removed as a source of interference in these estimations.

Other than age, the most widely studied physiological difference in drug oxidation is the male-female differences in the rat. Since the original observation of Quinn *et al* (1958) several investigators have reported sex related differences in drug oxidation and the related electron transport system (Conney 1967, Gillette 1963, Gillette *et al* 1972). A very simplistic explanation for the decreased drug oxidation activity in female rats, which has not been previously considered, is that an increase

in NADPH dependent lipid peroxidation activity in microsomes of female rats could lead to an apparent decrease in drug oxidation. This could result from simple competition for electrons in the common electron transport system or from a destruction of the drug oxidation enzyme system. In the present experiments, identical incubation mixtures were used for both the determination of drug oxidation and lipid peroxidation in microsomes from male and female rats. NADPH dependent lipid peroxidation was highest in female rats in both control and iron stimulated microsomes, which was in keeping with the proposal that low drug oxidation in female rats was due to increased lipid peroxidation. However, when aminopyrine N-demethylation was determined in the presence of 25 μM EDTA to inhibit lipid peroxidation, the male-female difference in drug oxidation was maintained and the activities were identical to the corresponding activities determined without EDTA in the reaction mixtures. Because the difference in drug oxidation was maintained even in the absence of lipid peroxidation, it must be concluded that the contribution of NADPH dependent lipid peroxidation to the male-female differences in drug oxidation is negligible. A similar conclusion was reached by El Defrawy *et al* (1974) who showed that when lipid peroxidation was estimated in an incubation mixture which differed from that used for drug oxidation, lipid peroxidation was highest in the male rat. These investigators reasoned that increased lipid peroxidation did not exist in female rats compared to male rats and therefore, could not account for the decreased drug oxidation activity. This conclusion is also suggested by the fact that low lipid peroxidations (control reaction mixtures) in adult rat microsomes had only

a small effect on aminopyrine N-demethylation even after microsomes had been exposed to lipid peroxidation for 30 minutes. The relatively small difference in lipid peroxidation in male and female rats would therefore, be unlikely to result in a measurable difference in drug oxidation.

Microsomal drug oxidation is of major importance in the overall elimination of drugs and factors which increase or decrease the rate of oxidation become important in any considerations involving drug elimination. In both the present experiments and those of previous investigators, lipid peroxidation activity has been shown to reduce the ability of microsomes to oxidize drugs. An important question which is then raised is that if lipid peroxidation has such pronounced effects on drug oxidation in rat microsomes *in vitro*, does lipid peroxidation have the same effects *in vivo* in the live rat, and also, in man, is lipid peroxidation a factor to be considered as part of the overall drug elimination system? Before evaluating the possible relevance of lipid peroxidation in the practical use of drugs, two questions have to be answered. First, does lipid peroxidation exist in human microsomes and, second, does the lipid peroxidation reaction occur *in vivo* in the endoplasmic reticulum membrane of the liver or is it a reaction confined to isolated preparations of microsomes?

The results obtained from microsomes prepared from liver samples obtained from human newborn infants and fetus, indicate that NADPH dependent lipid peroxidation does in fact occur in human hepatic microsomes. Activities in the human microsomes were similar to that found in the rat with the activity being confined mainly to the microsomal

(100,000P) subcellular fraction with lowest activities occurring in the soluble supernatant subcellular fraction (100,000S). Except in the case of sample 1, iron did not increase lipid peroxidation to an activity comparable to the rat. Sample 1 was stimulated by iron to activities equivalent to that found in adult male rats after iron stimulation. This sample, however, was most likely induced by phenobarbital as the infant had received phenobarbital over a period of 36 hours, and cytochrome c reductase activities in this sample were much higher than in several newborn infants studied by Aranda *et al* (1974) working in the same laboratory. Lipid peroxidation can also be induced by phenobarbital treatment of rats (Nilsson *et al* 1964) so it is most likely that the values recorded in this particular sample represent those resulting from phenobarbital inducation. Overall, the activities of lipid peroxidation in human microsomes were similar to those in the rat, guinea pig, and mouse, and are higher than those in the rabbit (Jacobson *et al* 1973, Levin *et al* 1973, Kamataki and Kitagawa 1974). Unfortunately, in the present study difficulties were encountered in obtaining adult tissue samples from humans and, therefore, the present data only consists of immature human tissue.

The effect of NADPH dependent lipid peroxidation on N-demethylation of aminopyrine was also studied in human microsomal preparations. In sample 1 which had lipid peroxidation activity close to that found in the adult male rat, preincubation of microsomes with an NADPH generating system had no effect on drug oxidation. The addition of iron to the preincubation medium for 30 minutes, reduced drug oxidation by 25%. This compares dramatically to the effects of preincubating male rat microsomes with an

NADPH generating system which reduced N-demethylation of aminopyrine by 25% in control incubations and by 80% when iron was added to the incubation medium. In sample 2 which had a lower lipid peroxidation activity, preincubation with an NADPH generating system had no effect on the ability of microsomes to oxidize substrate even in reaction mixtures containing iron. It is concluded that the drug oxidizing system in human microsomes is resistant to the effects of lipid peroxidation when compared to the effects of equivalent lipid peroxidation activity in rat microsomes.

A similar conclusion can be reached by comparing the linearity of of N-demethylation reactions in human and rat microsomes. Linearity of reaction was maintained for much longer periods in human microsomes compared to the rat. When lipid peroxidation was increased to its maximum activity by the addition of iron to the incubation medium, only a slight deviation from linearity was observed in human microsomes. It is clear that to achieve equivalent decreases in drug oxidation in human and rat microsomes, human microsomes require a higher lipid peroxidation activity for a longer period of time.

Alteration of drug oxidation has also been shown resistant to conditions promoting lipid peroxidation in two other species. In the rabbit, reaction linearity is maintained in the presence of an NADPH generating system (Gram and Fouts 1966, Jacobson *et al* 1973) but these effects are due to low activity of lipid peroxidation in this species. On the other hand, drug oxidation in the guinea pig, which has a lipid peroxidation activity similar to the rat or mouse, is resistant to the effects of lipid peroxidation (Kamataki and Kitagawa 1974). It would appear

that effects in the guinea pig and those in the human are similar though the source of this apparent resistance to the effects of lipid peroxidation is yet undetermined.

As age related differences in lipid peroxidation activities have been shown in the rat and it is well established that age related differences in drug oxidation occur in both animals and humans (MacLeod *et al* 1972, Aranda *et al* 1974), the data recorded here for man must be regarded as preliminary and applied strictly to the age group studied.

The other major question which concerns the existence of *in vivo* NADPH dependent lipid peroxidation is much harder to answer. In general, it is always easier to demonstrate that an entity exists than to demonstrate its absence. Several laboratories have taken the former approach and attempted to demonstrate the existence of *in vivo* lipid peroxidation (Wills 1972a, Fujita 1973, 1974), by treating rats with iron to increase lipid peroxidation to a level which can be more easily detected. One of the major difficulties is that malonaldehyde and other breakdown products of lipid peroxidation are metabolized rapidly in the whole animal and are therefore, difficult to detect by existing methods, in tissues (Recknagel 1967). The other major difficulty with the experiments is the fact that the thiobarbituric acid reacting material and the decreases in drug metabolism in microsomes from iron treated rats, could well occur as a result of lipid peroxidation occurring during the preparation of the microsomes and not reflect what took place before the death of the animal. As iron treated animals have a higher tissue iron level and lipid peroxidation is very sensitive to iron concentration, the chances of lipid

peroxidation occurring during the preparation of microsomes is greatly increased over normal preparations which act as controls in the experiments of these investigators.

In the present experiments, *in vivo* lipid peroxidation was assessed by treating 25 day old rats with iron-dextran (Imferon^{R}) in an attempt to create the most favorable conditions for the promotion of *in vivo* lipid peroxidation. Microsomes from these rats were prepared in a buffer containing 25 μM EDTA to eliminate lipid peroxidation during the preparation of microsomes. In contrast to the experiments of Wills (1972a), who did not use EDTA during microsomal preparation, no decrease in drug oxidation was recorded in iron treated rats as reflected by aminopyrine N-demethylation, cytochrome P-450 levels and NADPH oxidase in microsomes in iron treated and saline treated control rats. However, the microsomal content of thiobarbituric acid reacting material was about three fold higher in iron treated rats compared to control rats. Provided this could not be accounted for during preparation of microsomes, this could result from *in vivo* lipid peroxidation reaction products. When microsomes from iron treated rats were prepared without the use of EDTA in the buffer, an increase in lipid peroxidation activity was found *in vitro* compared to control microsomes, which is similar to the results presented by Wills (1972a) in a similar experiment. These effects are most likely due to increased iron content of microsomes from iron treated rats and occur *in vitro* and are not due to effects taking place in the live animal. In the present study, electron micrographs of microsomal pellets from iron treated rats showed that ribosomes appeared to be absent from the surface of the

microsomal membrane and there was evidence of increased membrane fragments compared to control rats treated with saline. These changes which occurred in microsomal preparations derived from iron treated immature rats, which had favorable conditions for *in vivo* lipid peroxidation, were very similar to the changes observed by Arstila *et al* (1972) in microsomes exposed to low activities of *in vitro* lipid peroxidation. High activities of *in vitro* lipid peroxidation resulted in complete destruction of microsomal membrane structure in the present experiments and those reported by Arstila *et al* (1972) and Hogberg *et al* (1973).

The evidence is suggestive that lipid peroxidation took place in the living rat provided that one accepts that the use of EDTA eliminated sources of thiobarbituric acid reacting material during the preparation of the microsomes and that the data does not represent a preparation artifact which was not recognized. Recently, Hogberg *et al* (1974) have demonstrated the presence of NADPH cytochrome c reductase in 100,000S fraction of hepatocytes which were incubated as isolated cells with iron complexes. This enzyme can be released from isolated microsomes during the process of *in vitro* lipid peroxidation (Bidlack and Tappel 1973), suggesting that in the experiments in isolated cells, lipid peroxidation of the endoplasmic reticulum can occur. The occurrence of lipid peroxidation in the intact isolated cell is a step closer to *in vivo* conditions compared to subcellular fractions and tends to support evidence which suggests the occurrence of *in vivo* lipid peroxidation. In iron deficient rats, which reduces the possibility of *in vivo* lipid peroxidation, drug oxidation is increased (Catz *et al* 1970) and in vitamin E deficient rats, which increases lipid

peroxidation, drug oxidation is decreased (Carpenter and Howard 1974, Diplock 1974). These observations also agree with the predicted response if lipid peroxidation occurs *in vivo*. Strong evidence already exists to demonstrate *in vivo* lipid peroxidation in hepatic microsomes following carbon tetrachloride intoxication (Recknagel 1967, Reily and Cohen 1974). However, peroxidation of this type results from the action of free radicals generated during the metabolism of carbon tetrachloride.

Even though the present experiments tend to support the idea that NADPH dependent lipid peroxidation can take place, *in vivo* drug oxidation activity in animals treated with iron remained similar to the control activity, suggesting that even if it did occur, lipid peroxidation had no effect on the ability of the hepatocyte to metabolize drugs. This suggestion, however, does not entirely eliminate the hypothesis of Wills (1972b) that *in vivo* lipid peroxidation plays a role in the overall turnover of endoplasmic reticulum membrane. Such a role would not become apparent by measuring a function of the membrane such as N-demethylation as long as the rate of synthesis of the membrane kept pace with its breakdown rate.

The importance of iron in NADPH dependent lipid peroxidation in microsomes has been demonstrated by many independent investigators. The initial paper describing NADPH dependent lipid peroxidation in microsomes (Hochstein and Ernster 1963) reported an absolute requirement for ADP. This was later shown to be a dependence on iron contaminating the ADP rather than the ADP itself (Hochstein *et al* 1964). The required iron, which can be ferrous or ferric was non-heme in nature and could not be

replaced by other metal ions (Wills 1969b). As now suggested by several investigators, the apparent requirement for ADP or an ADP-iron complex for lipid peroxidation, is most likely due to the fact that ADP keeps ferric iron from precipitating from reaction mixtures kept at neutral pH (Poyer and McCay 1971, Pederson *et al.* 1973, Noguchi and Nakano 1974). Apart from establishing that the iron involved is non-heme, little is known about the iron which appears essential for lipid peroxidation.

Wills (1969b) suggested that iron is bound to the microsomes and does not act as a free iron in solution during lipid peroxidation. However, Pederson and Aust (1972), Pederson *et al* (1973) and Noguchi and Nakano (1974) have demonstrated lipid peroxidation in an isolated preparation of cytochrome c reductase which still required iron despite the absence of other microsomal components. Noguchi and Nakano (1974) concluded that the iron involved in the peroxidation of endogenous lipids of microsomes was free in solution. Also, EDTA was shown to be an essential component in lipid peroxidation using isolated cytochrome c reductase. As Wills (1969b) used EDTA and other chelating agents in the experiments designed to determine if iron was bound or free, the restoration of lipid peroxidation activity after washing EDTA treated microsomes may have been due to artifact introduced by the EDTA dependency described by Pederson and Aust (1978).

In the present experiments, in an attempt to establish if iron was bound or free, we utilized an iron chelating agent, 8-hydroxyquinoline, which was covalently bound to glass beads. Using these glass beads, microsomes could be incubated and after gentle centrifugation, all free iron,

iron chelate and free chelate, could be removed. Incubation of microsomes with 8-hydroxyquinoline glass beads had no effect on non-heme iron content, lipid peroxidation or aminopyrine N-demethylation. This suggests that the iron present in microsomes which contributes to the basal lipid peroxidation activity was bound to the microsomes and did not exist free in solution. This bound iron may be the non-heme iron which could not be removed from microsomes in the form of ferritin (Montgomery *et al* 1974). When iron was added to the microsomes to produce maximal activities of lipid peroxidation, glass beads were ineffective in reducing the activity if the beads were added after microsomes. When glass beads were added prior to microsomes, lipid peroxidation activity was reduced to a level close to the basal activity. This experiment demonstrated that even when iron is added exogenously, it is rapidly bound by the microsomes and does not remain free in solution. This evidence in conjunction with the similar conclusion reached by Wills (1969b) using a different technique, clearly establishes that the iron component involved in lipid peroxidation in intact microsomes is bound and does not exist free in solution even when added from an exogenous source. Chelating agents inhibit lipid peroxidation in microsomes by acting on iron at the bound source and do not remove iron from the microsomes. In isolated systems of lipid peroxidation using cytochrome c reductase as an electron carrier, this bound iron component is unnecessary and as suggested by Pederson and Aust (1974) it can be replaced or by-passed, by an EDTA-Fe complex.

As lipid peroxidation is extremely sensitive to iron concentration in microsomes, this has led to some confusion in the literature as even

slight contamination by iron in a reaction mixture can result in a marked stimulation of lipid peroxidation. Unfortunately, phosphates which are used widely in buffers, contain iron as a trace contaminant and depending on the extent of this contamination, basal lipid peroxidation activities will vary. As a result, some investigators (Levin *et al* 1973) could not demonstrate a stimulation of lipid peroxidation by iron, but their basal activity was high in comparison to the activities found in the present study. On the other hand, Wills (1969b) and Kamataki and Kitagawa (1973) reported that the maximal effect of iron was double that of the basal activity. In the present experiments, lipid peroxidation was stimulated to a maximal activity 20 times that of the basal activity after the addition of iron to reaction mixtures. As the maximal response to iron in all of these studies is similar, we conclude that the differences in basal activity in the different studies is due to different levels of iron contained in the microsomes of the different preparations. In our experiments, particular care was taken to minimize iron contamination of lipid peroxidation reaction mixtures. Such a conclusion is supported by the observations of Jacobson *et al* (1973) who showed that if phosphate buffer was passed through an ion exchange column to remove iron before using the buffer for lipid peroxidation reactions, their basal activity (which was similar to our maximum stimulated activity) was reduced by about 15 fold. Also, Vatsis *et al* (1974) found that lipid peroxidation activity was less in tris buffer compared to phosphate buffer.

In the present experiments, some day-to-day variation in lipid peroxidation was apparent which was most likely due to variations in

total iron content of microsomes. The problem of iron contamination and the fact that basal lipid peroxidation activities are a function of iron contamination in microsomes has not been widely recognized. Most of the inter-laboratory differences reported in the literature can probably be accounted for by iron contamination and makes comparison of results between different investigators difficult. The main source of this contamination is phosphate buffers used in reaction mixtures and from glassware which has been contaminated by trace amounts of iron in standard cleaning procedures.

CONCLUSIONS AND CONTRIBUTIONS TO ORIGINAL KNOWLEDGE

The results summarized below represent the original descriptions and contributions to new knowledge which are described in this thesis.

1. In immature rats, the pattern of activity of NADPH dependent lipid peroxidation in hepatic microsomes is described. Activities reached a maximum level 25 days after birth and then declined to adult levels by six weeks of age. The reason for this peak of activity during the development of the rat, was not determined but cytochrome c reductase activities and non-heme iron levels did not play a prominent role.

2. NADPH dependent lipid peroxidation plays only a very minor role in the diminished ability of immature rat microsomes to metabolize drugs. It appears that the only effect of lipid peroxidation on the general developmental pattern of drug oxidizing enzymes in microsomes, is a slight slowing in the rate of development at the time when NADPH dependent lipid peroxidation activity is highest.

3. In rat hepatic microsomes exposed to lipid peroxidation, drug oxidation activity was reduced by a much greater degree in 25 day old rats compared to adult rats. In both age groups, iron added to microsomes increased lipid peroxidation activity and resulted in a further decrease in the ability of microsomes to oxidize drugs.

4. Total NADPH oxidase activity was similar in 25 day old and adult rats. When NADPH oxidase activity which is specific for lipid peroxidation was calculated, the pattern of activities found in different age groups and under different conditions, was similar to the pattern obtained

for the activity of lipid peroxidation determined using the thiobarbituric acid reaction, *i.e.*, NADPH oxidase activity associated with lipid peroxidation was highest in immature rats compared to adult rats. Iron added to incubation mixtures, increased NADPH oxidation in both age groups.

5. Inhibition of NADPH dependent lipid peroxidation does not result in a direct increase in the activity of drug metabolizing enzymes in microsomes. The increases in drug oxidation reported by other investigators after lipid peroxidation activity was inhibited with EDTA, was due to incubation artifacts caused by utilizing reaction times which exceeded the time limit for a linear reaction. NADPH dependent lipid peroxidation activity had marked effects on reaction linearity times for drug oxidizing enzymes in both immature and adult rats.

6. Destruction of cytochrome P-450 or the microsomal membrane are not absolute requirements for the reduction of drug metabolizing enzyme activity following NADPH dependent lipid peroxidation in microsomes. In this study, type I and type II binding spectra were diminished following lipid peroxidation but cytochrome P-450 remained intact. It is proposed that drug oxidation is decreased following NADPH dependent lipid peroxidation activity in microsomes by an effect on the substrate binding sites. The reduction in substrate binding results in decreased drug oxidation.

7. Although increased NADPH dependent lipid peroxidation was found in female compared to male rats, this was not responsible for the lower activity of drug metabolism in females compared to males.

8. NADPH dependent lipid peroxidation has been demonstrated for the

first time in humans using subcellular fractions of liver homogenates obtained from newborn infants. As reported for other species, activity was concentrated in the microsomal fraction and is similar to that found in the rat. NADPH dependent lipid peroxidation activity in human microsomes reduced drug oxidation, but compared to the rat, human microsomes were much more resistant to this effect.

9. Evidence is presented which supports the idea that lipid peroxidation can occur *in vivo* in the rat.

10. The iron involved in NADPH dependent lipid peroxidation reactions was shown to be bound to the microsomes and does not act as a free ion. Also, exogenously added iron is rapidly taken up by microsomes and stimulates lipid peroxidation in a bound form rather than as a free ion.

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