ROLE OF LIPIDS IN THE CONTROL OF SEX DIFFERENCES IN THE PHASE I METABOLISM OF DRUGS

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SUMMARY.

Hepatic microsomal preparations from male and female rats were delipidated by column chromatography following cholate solubilisation. The enzyme activity was assayed using lignocaine as the substrate for the mixed function oxidase. The N-deethylation of lignocaine catalysed by delipidated microsomal proteins from male and female rat liver is greater when reconstituted in microsomal lipid than in dilauroylphosphatidylcholine (DLPC). The 3-hydroxylation of lignocaine is unaffected by this treatment. The above effect is mimicked by incorporation of dilauroylethanolamine (DLPE) into the DLPC vesicles with male- but not the female-derived enzymes. Microsomal lipids derived from the male were more effective than female-derived lipids in reconstituting enzyme activities with both male- and female-derived enzymes. There is, thus, a sex- and pathway dependent effect of the lipids: the male-specific N-deethylase pathway is more affected by lipid composition and then more so in the male-derived enzyme. It is possible, therefore, that some of the sex differences in drug metabolism may be related to changes in lipid composition. In addition, the sex-dependence of the metabolism of lignocaine was maintained in the reconstituted system, indicating that this is a suitable system for investigating the role of lipids in maintaining sex-specific drug metabolism. We have extended this work with the isolation of the isozyme of cytochrome P-450 responsible for the N-deethylation of lignocaine. The male-specific cytochrome P-450 isozyme, lignocaine N-deethylase, was purified to electrophoretic homogeneity from liver microsomes of untreated male rats with high retention of bioactivity. The purified N-deethylase was an efficient catalyst of the 16α–hydroxylation of androst-4 ene3,17-dione (specific activity of 10 nmole product/min/nmole cytochrome P-450). The isozyme, together with purified NADPH-cytochrome P-450 reductase, was reconstituted using known lipids and the drug-metabolising activity assayed using lignocaine as substrate. The results showed that the purified isozyme only N-deethylated lignocaine and led to the following conclusion, (I) Mixed

dilauroylphosphatidylcholine (DLPC)/dilauroylphosphatidylethanolamine (DLPE) vesicles gave a higher N-deethylase activity than DLPC vesicles. (II) The N-deethylation of lignocaine catalysed by a purified male-specific cytochrome P-450 from rat liver is greater when reconstituted in microsomal lipid than in DLPC. (III) Microsomal lipids derived from male were more effective than female-derived lipids in reconstituting enzyme activities. These data indicated that it is a direct interaction of the lipid with the enzyme (s) or an alteration of the protein-protein interactions caused by the lipid which leads to the change in enzyme activity and that it is the isozyme responsible for the N-deethylation that is particularly affected in this way. In order to assess which portion of the microsomal lipid causes these changes, the microsomal lipid has been fractionated into phospholipid and neutral lipid. Using delipidation and subsequent relipidation of microsomal lipid preparation, we have confirmed that the phospholipid is the most effective portion for the maintenance of the drug metabolism and the sex differences in drug metabolism. It is clear that the male-derived phospholipid was more efficient than the female-derived phospholipid when incorporated into both male- and female-derived delipidated microsomes. The mixture of phospholipid and neutral lipid fractions was very effective, indeed it restored the N-deethylase activity to the level of the microsomes. Experiments were carried out using the male-specific isozyme responsible for the N-deethylation of lignocaine and again it was shown that the phospholipid fraction was the most effective fraction when reconstituted with this isozyme, but it was less effective than the whole microsomal lipid. It is only when the combination of phospholipid and neutral lipid was used that the control activity was achieved. Again the male derived phospholipid was more effective than the female-derived phospholipid. We analysed the phospholipid composition and our analysis shows sex differences in microsomal phospholipid composition and in fatty acid acyl chain of the microsomal phospholipid. Also the analysis of the phospholipid from the diabetic animals showed that the streptozotocin treatment does alter the phospholipid composition and the ratio of

acyl chains found. All these changes might be associated with differences in enzyme activity. We have extended this work using delipidated solubilised microsomes, prepared from male Wistar rats, incorporated into vesicles prepared from various PCs (phosphatidylcholine) containing fatty acids of differing chain length and degree of unsaturation. All of the PCs were found to restore the N-deethylase activity of the cytochrome P-450 but to differing extents. Phosphatidylcholines having longer acyl chains were more efficient and the unsaturated PC (18:1) reconstituted the enzyme activity better than did the saturated PC of the same chain length (18:0). Diarachidoyl (20:4) PC gave the highest enzyme activity. It seems therefore that the longer the chain length and the more unsaturated the acyl side chain is, the better it is at reconstituting lignocaine N-deethylase activity. Lignocaine N-deethylase activity is, thus, activated preferentially by long chain, unsaturated fatty acyl side chains on the phospholipid. This would seem to correlate well with the higher content of linoleate (18:2) and arachidonate (20:4) and the lower content of stearate (18:0) in the male-derived lipid fraction, which also activates the N-deethylase activity. Our results indicated clearly that the arachidonic acid is the most likely candidate for further investigation to ascertain its role in maintaining the drug metabolising activity.

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Abbreviations:

AMP..... Adenosine-5'- monophosphate

CO..... Carbon monoxide

DAPC..... Diarachidoylphosphatidylcholine

DBPC..... Dibehenoylphosphatidylcholine

DCPC..... Dicaproylphosphatidylcholine

DEAE-cellulose..... Diethylaminoethyl-cellulose

DLPC..... Dilauroylphosphatidylcholine

DLPE..... Dilauroylphosphatidylethanolamine

DOPC..... Dioleoylphosphatidylcholine

DSPC ..... Distearoylphosphatidylcholine

DTT..... Dithiothreitol

FAD..... Flavin adenine dinucleotide

FMN..... Flavin mononucleotide

Hb..... Haemoglubin

HTP..... Hydroxylapatite

NADPH..... Nicotinamide adenine dinucleotide phosphate (reduced)

NL..... Neutral lipid

PC..... Phosphatidylcholine

PE..... Phosphatidylethanolamine

PEG..... Polyethyleneglycol

PI ..... Phophatidylinositol

PL..... Phospholipid

PMSF..... Phenylmethanesulfonyl fluoride

PS ..... Phosphatidylserine

TEMED..... N,N,N'N'-tetramethylenediamine

INTRODUCTION

1.1. HISTORICAL BACKGROUND.

The studies of Brodie et al. (1955) revealed the existence of an enzymatic system in the endoplasmic reticulum of the liver capable of converting drugs into more polar products by the use of the cofactor, NADPH, and it was established that the reaction can only proceed in the presence of molecular oxygen. Garfinkel (1958) and Klingenberg (1958) detected a CO-binding pigment in liver microsomes of pigs and rats respectively, which was shown to be reducible by either NADPH or dithionite. Omura and Sato (1964a,b) revealed the haemoprotein nature of the CO-binding pigment and its identification as a b-type cytochrome with a typical Soret band of the CO-complex at 450nm which made the authors designate it cytochrome P-450. The first step in the reaction cycle was determined as formation of an enzyme-substrate complex, the spectral characteristics of which depend on the substrate used (Narasimhulu et al., 1965; Remmer et al., 1966; Schenkman et al., 1967). Substrates, which are supposed to bind to a polar amino acid residue at the active site and are hydroxylated, induced a blue shift of the Soret band with absorption bands at 385-390 nm as maximum and 425-435 nm as minimum in the difference spectrum (type I). The other compounds which are assumed to be bound directly to the heme iron induce a red shift thus leading to an absorption maximum at 425-435 nm and a minimum at 390-410 in a difference spectrum which is denoted as a type II spectrum (Schenkman et al., 1967). Estabrook et al. (1963) demonstrated that cytochrome P-450 in the bovine adrenal mitochondrial system is indeed the terminal oxidase of the monooxygenase system. Somewhat later, in 1969, this result was confirmed for the enzyme system in the endoplasmic reticulum of mammalian liver by Diehl et al. (1969). It was simultaneously shown by Katagiri et al. (1968), Lu et al. (1969b) and Gunsalus and Sligar (1978) that the monooxygenase system is composed of a certain number of essential components. All of these systems work as electron transferring chains transporting the electrons from the cofactor, nicotinamide adenine dinucleotide phosphate

(NADPH), via an FAD/FMN-containing flavoprotein as electron transmitter to cytochrome P-450 as the terminal oxidase acting as electron acceptor.

1.2. THE STRUCTURE OF THE ENDOPLASMIC RETICULUM.

Most eukariotic cells are traversed by a membrane and tubular network which represent a basophilic component of the cytoplasm known as chromophilic substance or ergastoplasm. From its localisation and form it was called endoplasmic reticulum by Porter (1953). In 1954, Palade and Porter showed that it consisted of rough and smooth membranes. The particles attached to the rough membranes were named as ribosomes by Roberts (1958) (about 60% of the endoplasmic reticulum has been shown to be composed of ribonucleic acid) (Porter, 1953). That part of the endoplasmic reticulum which is involved in protein biosynthesis and occupied by ribosomes appears as rough membranes but in the peripheral part of the cell the ribosomes disappear and the endoplasmic reticulum morphologically appears as smooth membranes (Ruckpaul and Bernhardt, 1984). After homogenisation and differential centrifugation of cells a special fraction consisting of small particles, which were called microsomes because of their small size, was formed. Palade and Siekevitz (1956), by means of electron microscopy, showed that this microsomal fraction originates from endoplasmic reticulum. Because of the transversal rupture of the endoplasmic reticulum, microsomes have the same inside/outside orientation. Therefore they can be regarded as the biochemical equivalent of the endoplasmic reticulum and it seems reasonable to assume that the microsomes reflect functionally physiological conditions (DePierre and Dallner 1975; Dallner and Ernester 1968). The main constituents of the endoplasmic reticulum of hepatocytes - protein and lipids- are contained in 1g wet weight of liver in an amount of 40-50 mg protein and about 15mg phospholipid, meaning that the membrane of the endoplasmic reticulum consists of 60-70% protein and 30-40% phospholipid by weight. Based on a molecular weight of 800 for a typical phospholipid and 50,000 as the average molecular

weight of a microsomal protein, DePierre and Ernster (1977) roughly calculated that there are approximately 27-42 phospholipid molecules per protein molecule in the endoplasmic reticulum. Besides the monooxygenase system with cytochrome P-450 as a terminal oxidase the endoplasmic reticulum contains another electron transport system with cytochrome b_5 and an NADH-dependent reductase which apparently is involved in some hydroxylating reactions. Shimakata et al. (1971) have shown cytochrome b_5 to be the intermediate electron carrier in the microsomal NADH-dependent stearyl-Co-A desaturase system.

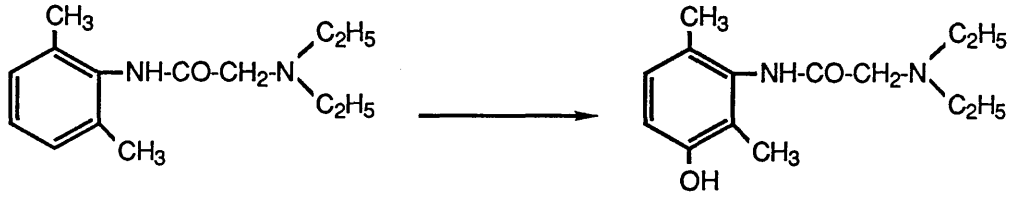
1.3. THE FUNCTION OF THE MIXED FUNCTION OXIDASE SYSTEM.

In 1968 Lu and Coon showed that the hepatic monooxygenase in the membrane of the endoplasmic reticulum is composed of three essential components:cytochrome P-450, NADPH-cytochrome P-450 reductase and a heat-stable lipid factor, later discovered to be phosphatidylcholine (Strobel et al., 1970), which can be reconstituted to an enzymatically active enzyme system. Due to the membrane-bound state and the presence of many other proteins in the endoplasmic reticulum, the purification of the monooxygenase system was rather difficult to achieve. The monooxygenase system can biotransform a variety of substrates, both endogenous and exogenous (Figure 1.1.). Due to the broad reaction specificity, many lipophilic drugs and xenobiotics are hydroxylated to more polar products making them more readily excreted than stored.

FIGURE 1.1.

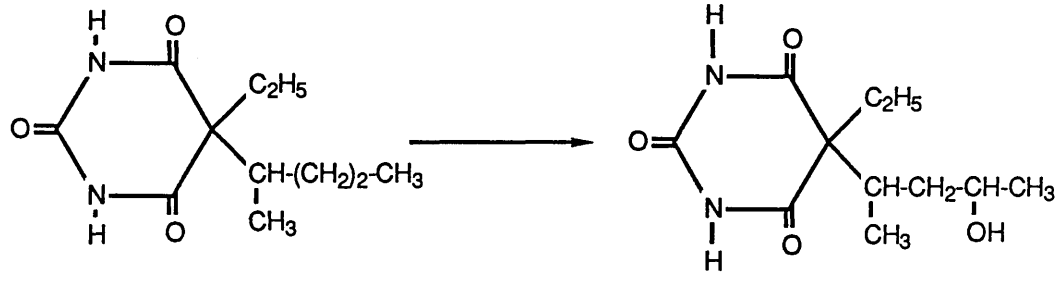
The mixed function oxidase system found in microsomes (endoplasmic reticulum) performs many different functionalisation reactions (summarized in figure 1.1.). An expamle of each reaction is given below.

1. AROMATIC HYROXYLATION



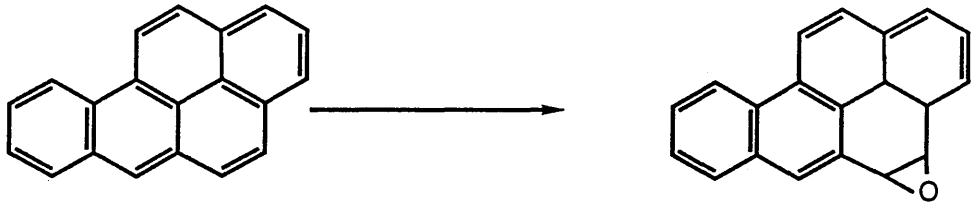
The 3-hydroxylation of lignocaine

2. ALIPHATIC HYDROXYLATION



The side-chain hydroxylation of pentobarbital.

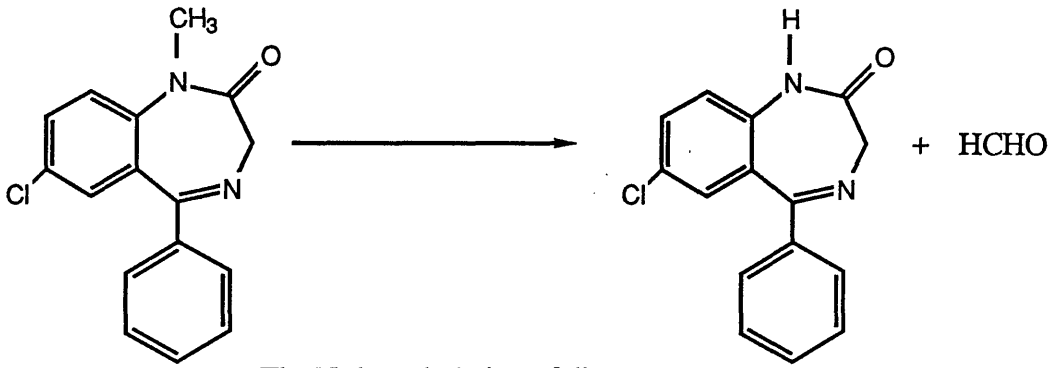
3. EPOXIDATION



The formation of benzo(a)pyrene-4,5-epoxide

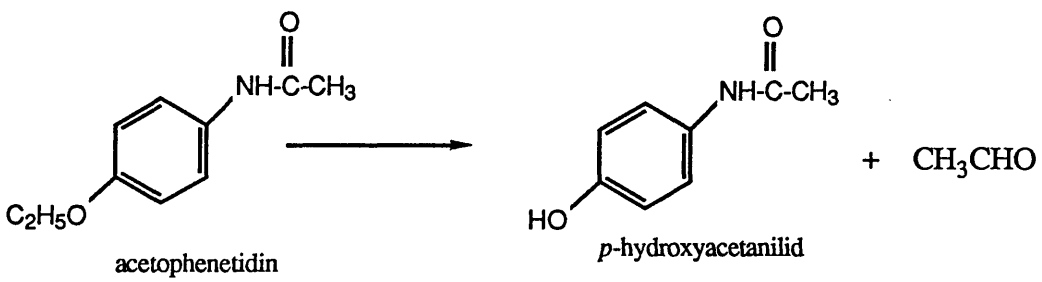
FIGURE 1.1. (continued).

4. N-DEALKYLATION



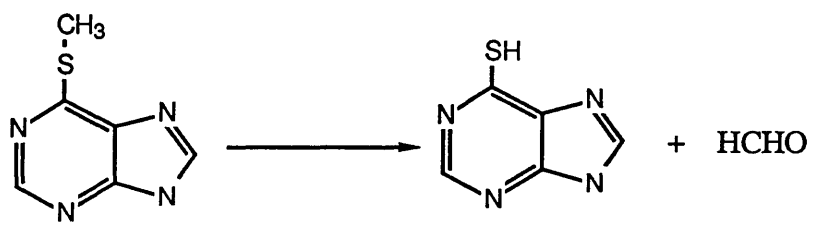
The N-demethylation of diazepam

5. O-DEALKYLATION



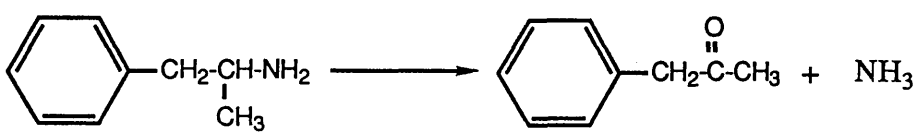
The O-de-ethylation of acetophenetidin

6. S-DEALKYLATION



The S-demethylation of S-methylthiopurine.

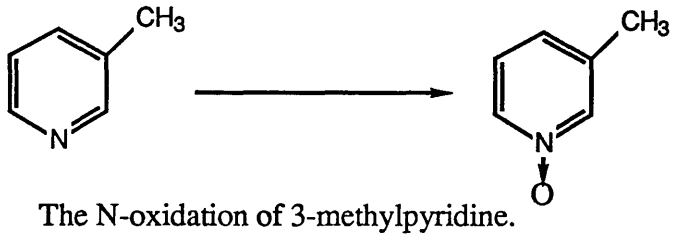
7. OXIDATIVE DEAMINATION



The oxidative deamination of amphetamine.

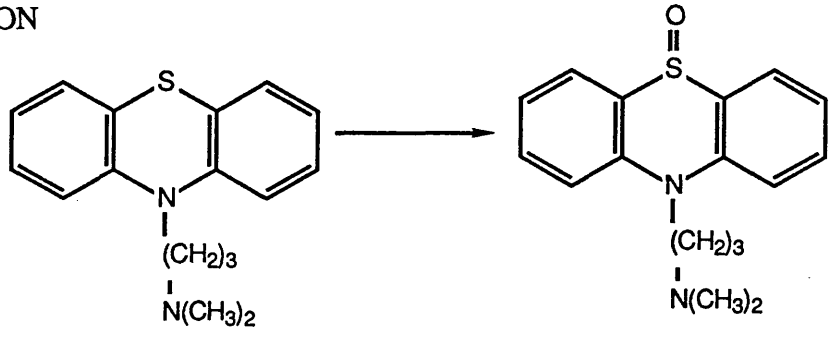
FIGURE 1.1. (continued).

8. N-OXIDATION



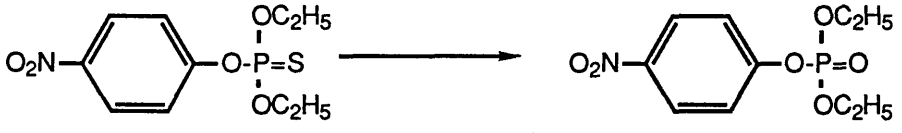
The N-oxidation of 3-methylpyridine.

9. S-OXIDATION



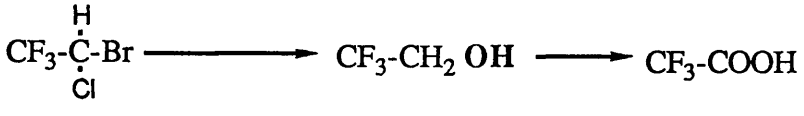
The S-oxidation of chlorpromazine.

10. PHOSPHOTHIONATE OXIDATION



The oxidation of parathion

11. DEHALOGENATION



The oxidative dehalogenation of halothane.

1.4. COMPONENTS OF THE MIXED FUNCTION OXIDASE SYSTEM.

1.4.1. Cytochrome P-450.

Cytochrome P-450 refers to a class of protoporphyrin IX-containing haemoproteins which show an unusual absorbing peak at 450nm when carbon monoxide is bound to the reduced form. Originally characterized by Omura and Sato (1964a,b), the enzyme was subsequently shown by Cooper et al. (1965) to participate in mixed function oxidation reactions. It does not appear to conform to the cytochrome b_5-type structural model. Rather, it is more intimately associated with the microsomal membrane, and no hydrophilic domain is released upon proteolysis. Proteolysis (as well as other harsh treatments i.e. a wide variety of detergents eg sodium deoxycholate and lysolecithin) can result in a conversion of cytochrome P-450 to a nonfunctional form termed cytochrome P-420. In a critical analysis of the manner in which these treatments convert cytochrome P-450 to cytochrome P-420, Imai and Sato (1967) concluded that the ultimate effect was to disrupt the association between haemoprotein and microsomal lipid. Using the combined approaches of proteolytic destruction of the native heme absorbance and derivitization with diazobenzene sulfonate, DePierre and Ernster (1980) have demonstrated that, like the other microsomal components, cytochrome P-450 is at least partially exposed to the cytoplasmic side of the membrane. At least twenty different forms of the cytochrome have been purified from detergent solubilization of microsomal membranes, and most, if not all, appear to be separate gene products (Waxman 1988). Apparent molecular weights (by SDS-PAGE) are in the 50,000-to 55,000-dalton range. The haemoprotein is present in very high concentrations in the microsomal membrane, and upon induction with phenobarbital, can comprise up to 15 to 20% of the microsomal protein. In its isolated form the protein self-associates to an aggregate of 500,000 daltons or more, and the size of the aggregate can be decreased by phospholipid (Guengerich and Holladay, 1979).

1.4.1.1. Multiplicity of Cytochrome P-450.

The existence of more than one liver microsomal drug-metabolising enzyme in different animal species was first postulated more than 28 years ago. An early study of Conney et al. (1959) suggested, not only that the drug metabolising enzymes of various species differ but that the liver of a single species contains several enzyme forms that catalyse the same reaction. Conney et al. (1959) found that the administration of benzo(a)pyrene to rats markedly increased the microsomal metabolism of substrates such as benzo(a)pyrene, acetanilide, zoxazolamine and 3-methyl-4-monomethylamino-benzene yet decreased the metabolism of meperidine and benadryl and had no effect on the metabolism of chlorpromazine. Since these initial observations, studies on the nature of the drug metabolising enzyme system have concentrated on the liver microsomal haemoprotein, cytochrome P-450. In 1965 Cooper et al., demonstrated that cytochrome P-450 is the terminal oxidase of the liver microsomal drug-metabolising enzyme system. Imai and Sato (1974) and Van der Hoeven et al. (1974) independently reported the successful purification and characterisation of hepatic microsomal P-450 from PB-treated rabbits to a gel-electrophoretically homogeneous state. Since then the data accumulated over the past years from studies with microsomes as well as purified cytochrome P-450 isozymes and the purification of cytochrome P-450's from a variety of induced and non-induced species supports the concept of multiple forms of cytochrome P-450. The use of different inducers to manipulate the biochemical and biophysical properties of the microsomal hydroxylation system has played a major role in establishing the existence of multiple forms of cytochrome P-450 i.e. liver microsomes prepared from phenobarbital (PB)-treated rats were shown to differ with respect to substrate specificity and the absorption maxima of their reduced difference spectra from liver microsomes prepared from 3-methylcholanthrene (3MC)-treated rats (Alvares et al., 1967;Sladek and Mannering, 1966; 1969a; Mannering, 1971; Thomas et al.,1976). Also immunological properties can be studied by the use of antibodies prepared against various purified cytochrome P-450

preparations which generally show high specificity and thus provide another means for distinguishing multiple forms of cytochrome P-450 i.e. antibodies produced against rat cytochrome P-450a, P-450b, P-450c (Haugen et al., 1977; Botelho et al., 1979) or B fractions of PB- and 3-MC-treated rats (Guengerich 1978) were highly specific.

The ultimate proof of different primary structures for different species of cytochrome P-450 will be a comparison of the total amino acid sequence of each isozyme. Partial sequences of rat P-450a, rat P-450b and rat P-450c forms are available (Haugen et al., 1977; Botelho et al., 1979) and the sequence of the first 19 amino acids on the N-terminal end of the three forms are quite different. All forms contain a high percentage of hydrophobic amino acids, the N- and C-terminal respectively are, methionine and methionine for P-450a, glutamic acid and serine for P-450b, and isoleucine and leucine for rat P-450c (Botelho et al., 1979; Haugen et al., 1977). Waxman and Walsh (1982), however, demonstrated that the P-450 PB-4 and P-450 PB-5 have identical primary structures for their first 31 and probably 34 amino acid residues. Most of these results, therefore provide further support for the multiplicity of cytochromes P-450 and suggest that most of the cytochromes P-450 are separate gene products rather than posttranslational modifications of a common precursor.

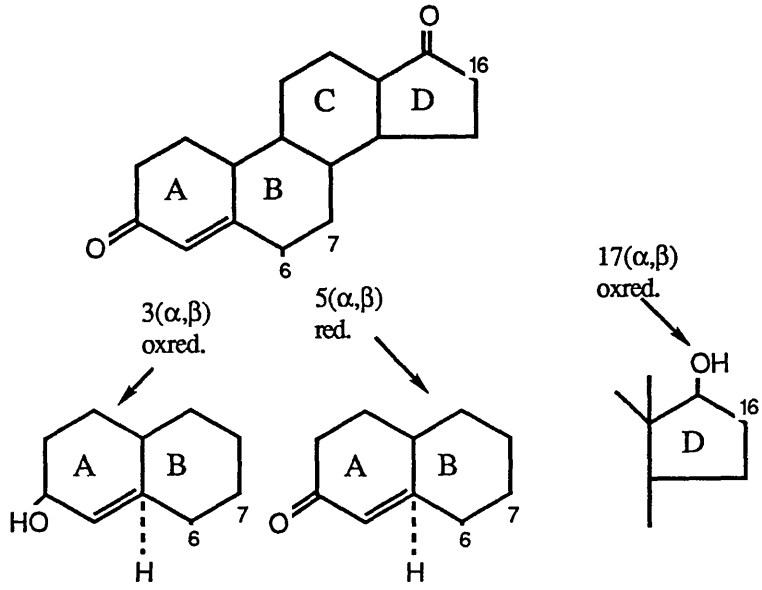
Over the last 2 decades workers in the field have used a variety of different nomenclatures for an increasing number of forms of P-450 isolated in their laboratories. This plethora of names has become more and more confusing. To rectify this problem a nomenclature for P-450 gene superfamily is proposed (Nebert et al., 1987) based on evolution. Recommendations include Roman numerals for distinct gene families, capital letters for subfamilies, and Arabic numerals for individual genes. An updating of this list, which presently includes 65 entries, will be required every 1-2 years. As segment of orthologous gene is presently uncertain in some cases-between widely diverged and especially in the P-450 II family due to large number of genes. For more details see Nebert et al., (1987).

1.4.1.2. Regioselectivity and Stereoselectivity of Steroid Hormone Hydroxylation.

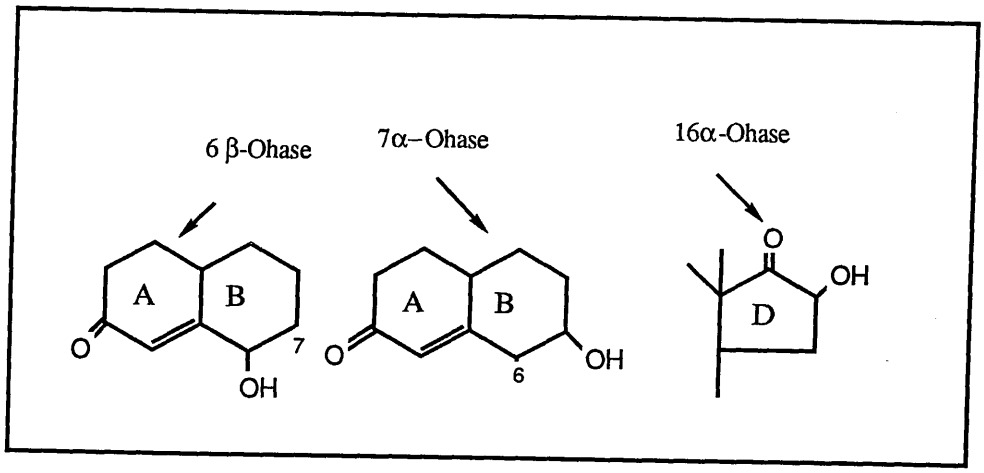
Many of the cytochromes P-450 metabolise the commonly studied compounds (i.e. 7-ethoxycoumarin, ethylmorphine, benzo(a)pyrene) with similar efficiencies and overlapping site specificities, making it difficult to uniquely characterise individual P-450 forms solely using these compounds as a substrate (Waxman 1988). In contrast, steroid hormones are frequently oxygenated by cytochrome P-450 enzymes with a high degree of specificity. Steroid hormones have proven most useful as substrates for distinguishing these multiple cytochrome P-450 forms (Sheets and Estabrook 1985) owing to the unique and characteristic metabolite profiles obtained (figure 1.2.) i.e. experiments carried out using electrophoretically homogeneous rat hepatic cytochrome P-450 form 2c (Waxman 1984) established that this enzyme can hydroxylate 4-androstene-3,17-dione from the alpha face on either the B-ring (yielding 7α–OH-androstenedione) or the D-ring (yielding 16αOH–androstenedione (for review see Waxman 1988)).

FIGURE 1.2.

Phase one Hepatic Microsomal Metabolism of 4-Androstene-3,17-Dione.



Isozyme	Product	Selectivity
6β–hydroxylase	6β–hydroxy (androstenedione)	female < male
7α–hydroxylase	7α–hydroxy (androstenedione)	female = male
16α–hydroxylase	16α–hydroxy (androstenedione)	female < male



1.4.2. NADPH-Cytochrome C (P-450) Reductase.

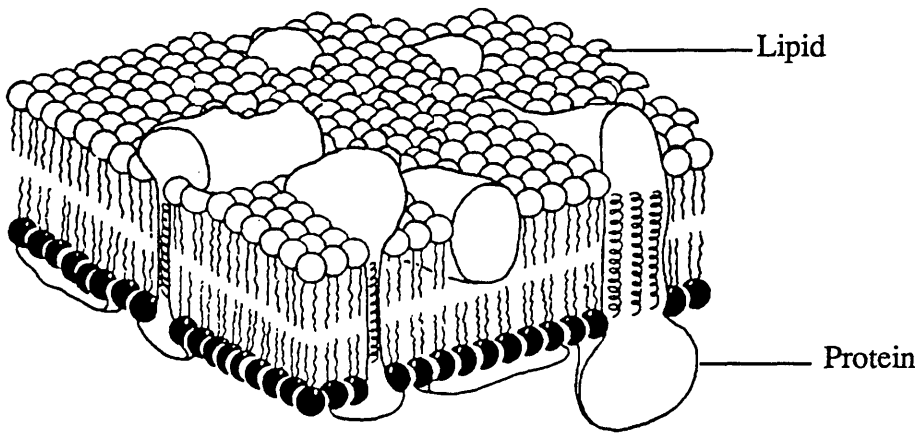
This flavoprotein was initially isolated and characterized by proteolytic treatment of microsomes. Williams and Kamin (1962) isolated and Kamin et al. (1966) characterized a 71000-dalton form which contained two flavins per molecule. The flavins were subsequently identified as flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN), (Iyanagi and Mason, 1973). In the native protein, the two flavins perform separate catalytic functions, (Vermillion et al., 1981); the flavin adenine dinucleotide (FAD) interacts with and accepts electrons from NADPH and donates them internally to the flavin mononucleotide (FMN). The latter then transfers electrons to the native electron acceptor, cytochrome P-450. The proteolytic form of the enzyme is monomeric and soluble in aqueous solutions, (Williams and Kamin, 1962; Kamin et al., 1966). This form is no longer capable of binding to microsomes, and does not function in the reduction of cytochrome P-450. The functional reductase was subsequently purified following detergent extraction of the microsomal membrane, (Dignam and Strobel, 1977; Vermillion and Coon, 1974). This form of the reductase had a molecular weight of 77, 000 with about 50 additional amino acids. With these additional residues, the isolated reductase aggregated to form a hexamer and could bind to both microsomes and artificial phospholipid membranes, (Gum and Strobel, 1979). The purified, functional form of the reductase required added phospholipid to support reduction of cytochrome P-450 and the associated oxidative reactions, (Miwa and Cho, 1976). Typsinization of the holoprotein allowed isolation of both the hydrophilic domain and a small hydrophobic peptide with a molecular weight of about 6000, (Black and Coon, 1982; Gum and Strobel, 1981). The sequence of the hydrophobic portion has been determined, (Black and Coon, 1982) and contains a long region of hydrophobic residues which promote interaction with the hydrophobic membrane. This peptide

sequence has also been proposed to function specifically in the interaction of the reductase with cytochrome P-450, (Black et al., 1979).

1.4.3. Microsomal Lipid.

Microsomal membranes are composed of a mixture of neutral lipid and phospholipid, with a large proportion of unsaturated fatty acyl residues, and cholesterol, (Dallner and Ernster, 1968). The observations by Kornberg and McConnell (1971) suggested that the membranes are fluid, allowing rapid lateral diffusion in the plane of the membrane, and that the membrane proteins are often inserted into and through the lipid bilayer, resulting in the Singer and Nicholson (1972) "fluid mosaic model" (figure 1.3.). Within this model, the functional role of lipids is related to their ability to self-assemble into bilayer structures on hydration, thus providing a permeability barrier as well as a matrix with which functional membrane proteins can be associated. Lipids in bilayers may undergo a temperature-dependent phase separation which produces patches of membrane with differing composition (Quinn, 1981).

FIGURE 1.3.
A refined mosaic model of a biological membrane.



Taken from Phospholipids and Cellular Regulation.
Volume I, ed. Kuo, J. F. (1985)

1.5. Examination of lipid in detail

1.5.1. Membrane Asymmetry.

Lipid phase transitions and lateral motions could occur in one or both layers of the bilayer. The composition of the two membrane bilayers appears to be very different but there is some movement of lipids between the outer and the inner layers of the membrane, usually referred to as "flip-flop". This transbilayer exchange has been reported to be as much as four times greater in biological membranes (Rothman and Kennedy, 1977) than in artificial bilayers (Roseman et al., 1975). Studies with phospholipase C have indicated that two-thirds of the phosphatidylethanolamine of rat liver endoplasmic reticulum is located in the inner leaflet of the membrane bilayer (Hutson and Higgins, 1982). They also suggest that phosphatidylethanolamine is synthesised at the cytoplasmic leaflet of the endoplasmic reticulum and subsequently transferred across the membrane to the cisternal leaflet of the bilayer. Valtersson et al. (1986) suggested that the phosphatidylethanolamine in microsomes is distributed in a non random fashion, and they demonstrated the existence of two pools of phosphatidylethanolamine. The two pools showed differences in fatty acid composition as well as in their sites of attachment. Phosphatidylcholine synthesised by the choline phosphotransferase pathway is preferentially located in the outer leaflet of the microsomal membrane (Higgins, 1979). In contrast, during synthesis of phosphatidylcholine by methylation of phosphatidylethanolamine, the first methylation step apparently occurs at the inner surface of the membrane and translocation of partly methylated intermediates across the membrane bilayer takes place so that phosphatidylcholine exists on both sides of the membrane (Higgins, 1981).

1.5.2. Membrane Fluidity.

Although several enzymes have specific phospholipid requirements for biological activity, it has been considered more likely that the role of membrane lipids is to provide an environment of proper viscosity and surface ionic milieu for optimal enzyme function (Thompson, 1973). The fluid mosaic model (figure 1.3.) of membrane structure (Singer and Nicholson, 1972) has provided a valuable model for protein-lipid interactions within the membrane. Studies of movement within membranes have suggested that phospholipids diffuse in the membrane at rates of approximately 10^{-8} to 10^{-7} cm^2/sec, (Stier and Sackman, 1973). It is well known that the artificial bilayers made of pure lipids of the same type found in membranes will undergo a transition from a relatively fluid state (generally termed the liquid crystalline phase) to a more rigid state (the gel phase) when the temperature is lowered to a characteristic point (Cullis et al., 1985). The membrane may also undergo transition from a bilayer form into a hexagonal phase (Thompson, 1973).

1.5.3. Model Membranes.

Due to the heterogeneous composition and the complexity of biomembranes for biochemical and biophysical studies, model membranes are often used which contain only one or a few essential components. The aim of such studies on model membranes is to find general principles according to which membranes are organized and thus to draw conclusions on biological membranes. The basis for such studies is the capability of phospholipids to arrange themselves as multiwalled rather than as single-walled vesicles. Many properties (e.g. permeability for ions and water, response to Ca^{2+}, incorporation of proteins) of bilayers resemble those of biomembranes. For biochemical and biophysical studies two types of bilayers can be used (I) multibilayer dispersions, which are formed spontaneously whenever phospholipids are dispersed in an aqueous medium, (II) homogeneous, small single-walled bilayer as vesicles which are readily obtained under appropriate conditions such as sonication. The formation of either multilayer or bilayer dispersions not only depends on the external physical conditions but also on the nature of the acyl residues.

Dispersion of lipids in water.

The simplest model system is obtained by depositing the lipid of interest as a film by evaporating from chloroform. The lipid is subsequently hydrated by mechanical agitation in the presence of aqueous buffer. Lipids that adopted the hexagonal H_{II} phase are usually much more difficult to disperse in water and form fine particulate suspensions. Water/Lipid dispersions are useful for studies on the structural preferences of lipids.

Small unilamellar vesicles:

These systems provide a more representative model system than the hexagonal H_{II} which do not exhibit a well-defined permeability barrier between external and internal environments. These vesicles can include reconstituted proteins if an understanding of lipid-protein interactions or the influence of a given lipid on protein function is required.These vesicles can be achieved by sonication (Huang, 1969).

Large unilamellar vesicles:

Large unilamellar vesicles can be achieved by detergent solubilisation followed by dialysis or gel filtration to remove the detergent, thus it produces vesicles which range in size from 50 to 200 nm diameter (Cullis et al., 1985; Schmitz, 1986).

1.5.4. Properties of individual lipid species.

Phosphatidylcholine:

At temperatures below the gel to liquid crystal transition temperature all species of PC adopt a lamellar gel phase where the acyl chains assume an all-trans configuration. Above the transition temperature the configurition is also lamellar but liquid crystalline where the acyl chains are disordered (Cullis et al., 1985).

Sphingomyelin:

Sphingomyelin adopts only the lamellar organisation over a wide range of temperatures and hydration (Cullis et al.,1985).

Phosphatidylethanolamine:

Phosphatidylethanolamine (PE) spontaneously adopts the hexagonal H_{II} phase structure in the presence of excess aqueous buffer at physiological temperatures. However unsaturated PE undergoes a transition between the lamellar and the hexagonal H_{II} phases with increasing temperature which, depending on the hydration, ionic strength and acyl chain unsaturation, can adopt both lamellar and hexagonal H_{II} configurations (Cullis et al., 1985).

Mixtures of phosphatidylcholine and phosphatidylethanolamine:

Phosphatidylcholine can stabilise the unsaturated PE into a lamellar configuration in a manner which is sensitive to the acyl chain unsaturation of the lipid, the temperature and the ratio of PC to PE (Cullis et al., 1985).

1.5.5. Influence of Cholesterol on Membrane Structure

Changes in the cholesterol content of the native membrane can cause alterations in its physico-chemical properties (e.g. fluidity, permeability, cell fusion properties) (Inoue, 1974; Block et al., 1977). The normal content of cholesterol in the liver and hepatic microsomes varies considerably (Bernhardt and Ruckpaul, 1981). Besides phospholipid-induced modification of the function of the monooxygenase system, there is an increasing amount of evidence that the incorporation of cholesterol also affects the drug metabolising enzyme activity (Bernhardt and Ruckpaul, 1981; Skett and Cuthill, 1986). It has been shown that cholesterol may induce incorporation of proteins into vesicles (Scotto and Zakim 1986, Scotto et al., 1987).The codeine N-demethylation is inhibited in guinea pigs fed for 72 day on cholesterol rich diet but when the rats are fed on the same diet for 137 day the codeine N-demethylation increases Tsia et al., (1977). On the other hand, the activity of NADPH cytochrome P-450 reductase in rats was not altered by the cholesterol rich diet (Lang et al., 1976) which indicates that either cytochrome P-450 or the interaction between the cytochrome P-450 and the reductase are influenced by the cholesterol.

1.6. LIPID INVOLVEMENT IN MICROSOMAL HYDROXYLATION.

In 1969 Lu and coworkers (Lu et al., 1969b) reported that one of the three components of their resolved and reconstituted fatty acid hydroxylation system was a heat stable factor. The same fraction was later found to also be required for the maximal hydroxylation of steroids, carcinogens and many drugs (Lu et al., 1969a; Lu et al., 1970; Lu et al., 1971; Lu et al., 1972). This factor was subsequently found to be phospholipid and phosphatidylcholine was found to be the most effective in reconstituting the enzyme system (Strobel et al., 1970). The result of phospholipase C treatment also indicated the essential role of the phospholipid in microsomal oxidation (Chaplin and Mannering, 1970; Eling and Di Augustine, 1971). This treatment removed approximately 70% of the total phospholipids, which led to a decrease in the metabolism of type I compounds and essentially eliminated the binding of type I compounds to cytochrome P-450. However the role of the phospholipids in drug hydroxylation was questioned by Cater et al. (1972) who found that the metabolism of type I compounds, inhibited by the deoxycholate which was still present in the reconstituted system of Lu and Coon (1968), could be overcome by the addition of phosphatidylcholine. Since the reconstituted system of Lu et al. (1969) still contained deoxycholate, it has been suggested that the role of lipid could be that of relieving the inhibition caused by the detergent. As for the phospholipase C treatment, Cater et al. (1972) found that the decrease in the aminopyrine demethylation after the phospholipase C treatment could not be restored completely by the addition of phosphatidylcholine. They suggested that fatty acids released by an endogenous acylhydrolase were responsible for the decrease in aniline hydroxylation, since fatty acids are also substrates for the cytochrome P-450 system, and because defatted serum albumin prevented this inhibition. Based on these studies they concluded that the liver microsomal cytochrome P-450-linked hydroxylation system is not phospholipid dependent. To resolve this controversy Vore et al. (1974) were able to remove lipids from microsomes without significantly affecting the other

components of the system. The enzymatic activity of the extracted microsomes, as measured by their ability to hydroxylate 3,4-benzpyrene, was 30-50% of the control activity. However, addition of a total lipid extract or synthetic phosphatidylcholine to the extracted microsomes fully restored the activity. These results are consistent with the idea that lipid is an essential part of the microsomal hydroxylation system and that its function is not related to the use of detergents.

1.6.1. Role of Phospholipid.

Several reports describing alterations of drug metabolism dependent on nutritional status, under physiological and pathological conditions which cause alterations in phospholipid composition, have been presented (Rumack et al., 1973; Paine and McLean, 1973; Laitinen et al., 1975; Hammer and Wills, 1978; Feuer et al., 1980). There are two different ways to modify the phospholipid structure (i) by changes in the fatty acid residues or (ii) by substituting the head group. In 1972, Norred and Wade demonstrated that a total fat deficiency in the diet caused a decrease in the activities of hydroxylation and glucuronidation of foreign compounds in microsomes. It was suggested that the fatty acid composition of a given phospholipid molecule exerts a signal function directing the lipid to its final intramembranous location (Valtersson et al., 1986). However Wade et al. (1978) showed that if the diet was supplemented with 3% corn oil, which increases the amount of linoleic (18:2) and arachidonic acid (20:4) in the phospholipid of hepatic microsomes, the ethylmorphine demethylase (Norred and Wade, 1972) increased. Fasting of rats leads to an overall increase of saturation of fatty acids in hepatic lipids which causes a decrease in the activity of 4-nitroanisole O-demethylation. Several authors (Ingelman-Sundberg and Glaumann, 1977; Ingelman-Sundberg et al., 1980) have demonstrated that the efficiency of phospholipids to enhance the monooxygenase activity increases with their acidity. The changes in the phospholipid concentration or composition could affect the functioning of the microsomal enzyme systems by a variety of mechanisms and thus affect the metabolism

of fatty acids, steroids, xenobiotics and carcinogens and such a change occurs during development. Feuer et al. (1980) demonstrated that at birth (in rats) there is a significant increase in the fluidity of the microsomal membrane, which correlates both with the membrane cholesterol content and unsaturation of the phospholipids. They also noted that the fatty acids become more unsaturated in the fetal to adult progression. In 1980, Dhami et al., noted that pregnancy causes a similar change in the lipid composition. Diet, as noted above, also causes changes in the phospholipid composition which in turn can affect the activity of the microsomal drug metabolising enzymes. Wills (1980) has demonstrated that the activity of benzo(a)pyrene metabolism was directly proportional to the fraction of unsaturated fatty acids in the membrane phospholipids. Microsomal phospholipids have been found to consist of about 55% phosphatidylcholine, 20-25% phosphatidylethanolamine, 5-10% phosphatidylserine, 5-10% phosphatidylinositol, and 4-7% sphingomyelin (DePierre and Dallner 1975). The fatty acid moieties of these phospholipids are mainly palmitic (16:0), palmitoleic (16:1), stearic (18:0), oleic (18:1), linoleic (18:2), arachidonic (20:4), and decosahexaenoic acid (22:6) species (Lee and Snyder 1973). In the endoplasmic reticulum the cytochrome P-450 and NADPH cytochrome P-450 reductase are embedded in a bilayer of membrane lipids with protein regions sticking out of the lipid (figure 1.3.). Delipidated proteins are incapable of performing hydroxylation reactions, but they can be reconstituted to an enzymatically active monooxygenase in the presence of phospholipid (Strobel et al., 1970; Van Der Hoeven et al., 1974).The early work on the mixed function oxidase system in the liver indicated that the lipid was the third essential component (Lu and Coon, 1968). Work with phospholipase C also indicated the essential role of phospholipids in microsomal oxidation (Chaplin and Mannering, 1970; Eling and Di Augstine, 1971). The requirement for lipid was further refined when dilauroylphosphatidylcholine (DLPC) was found to stimulate drug metabolism in a reconstituted system (Miwa and Lu, 1981). In contrast to earlier experiment with partially purified cytochrome P-450 (Lu et al.,

1970) it was proved that the binding of benzphetamine to cytochrome P-450LM2 increased about 5-fold in the presence of dilauroylphosphatidylcholine (Coon et al., 1976; Dawson et al., 1978; French et al., 1980). Although Parke (1981) suggested that this was an oversimplification of the endoplasmic reticulum and misrepresented the physiological situation as DLPC does not exist in vivo. Taniguchi et al. (1984) has suggested that protein insertion and interaction is affected by this artificial phospholipid as compared to the natural lipids. Indeed, Ingelman-Sundberg et al. (1980) have demonstrated that phosphatidylcholine (PC) can inhibit the activity of cytochrome P-450 LM2 and that this inhibition can be overcome by microsomal lipids. The idea is still prevalent, however, that the microsomal phospholipids are simply structural components to hold the enzymes in place.This view is challenged by findings of correlations between changes in lipid composition and drug metabolism. Decreased drug metabolism is seen in pregnancy in the rat (Neal and Parke, 1973) and this correlates with altered phospholipid content (Dhami et al., 1980). Similarly sex differences in drug metabolism have been shown to correlate to sex-dependent lipid differences (Belina et al., 1975). The binding of the substrate is paralleled by a shift to the high spin state, which can also be produced by phospholipid forming type I difference spectra (Tsong and Yang, 1978; Chiang and Coon, 1979; French et al., 1980). Whitmer et al. (1986) have suggested that the lipid composition and vesicle properties of the native membranes may modulate substrate disposition and biotransformation in hepatocytes, indeed they demonstrated that the lipid composition of membranes incorporating bilirubin appears to modulate the rate of glucuronidation and the relative rates of bilirubin mono- and diglucuronide formation. Spectrophotometric titrations and steady state kinetics have shown that the interaction between cytochrome P-450 reductase and cytochrome P-450 LM2 shifts the equilibrium of P-450 LM2 to the high spin state and that, in the presence of the phospholipid, the affinity of the cytochrome P-450 reductase for cytochrome P-450 is further increased (French et al., 1980). It has been shown that the reduction of

cytochrome P-450 by the cytochrome P-450 reductase as well as the relaxation rate of conformational changes of cytochrome P-450, are greatly enhanced by phosphatidylcholines with different fatty acid residues or a mixture of phospholipids (Taniguchi et al., 1979; Tsong and Yang, 1978; Yang and Tsong, 1980). Other reports (Haaparanta et al., 1980; Yang and Tsong, 1980) have demonstrated that the phospholipid-protein interaction is dependent on the fatty acid side chain of the phospholipid.

Phospholipids may function in at least 3 different ways:

1. Phospholipid mediated interactions between cytochrome P-450 and its corresponding reductase thus constituting a catalytically active arrangement.

2. Phospholipids modulate the electron transfer.

3. Phospholipids stabilise and/or induce a functionally active conformation of cytochrome P-450.

1.6.2. Role of the Fatty Acids.

The chain length and the degree of saturation of the fatty acid residues of the respective phospholipids determine the physical properties of the membrane, such as fluidity, and in this way modulate the activities of the membrane bound enzymes.The existence of two hydrocarbon chains per head group in the phospholipid helps the formation of bilayers whereas lipids which contain only one acyl chain, lysophospholipids, are unable to form bilayers and can even destroy bilayer structures (Cullis et al., 1985). The nature of the fatty acids can regulate oxidative drug metabolism in the liver endoplasmic reticulum (Rowe and Wills, 1976; Lambert and Wills, 1977a, b). In 1980, Wills demonstrated that the dietary lipid alters the rate of oxidative metabolism by modifications of the fatty acid composition of the endoplasmic reticulum, he suggested that the polyunsaturated fatty acids are important as a constituent of the membrane phospholipids, in holding the cytochrome P-450 in an active conformation in the membrane thus enhancing its efficiency. Maintaining rats on purified synthetic diets with decreased fat content markedly lowers cytochrome P-450 concentration and hydroxylating enzyme activity in liver microsomal fraction when compared to rats fed on stock pellets. When both groups are treated with phenobarbitone the difference is even greater, the purified fat free diets allowing only 50% of the cytochrome P-450 concentrations of the controls. However the addition of herring oil, containing linoleic acid to the diets allows induction of the cytochrome P-450 to take place (Mar shall and McLean, 1971).

1.7. TOTAL DEPENDENCE ON THE THREE ESSENTIAL COMPONENTS.

Since 1972 many studies have been carried out to isolate and investigate each component of the microsomal hydroxylation system individually.In the early 1970's Levin et al. (1974) and Coon et al. (1972) isolated cytochrome P-450 essentially free from reductase and phospholipids and reductase free from cytochrome P-450 and phospholipids. Levin et al. (1974) demonstrated a complete dependence on the three components (cytochrome P-450, NADPH-cytochrome P-450 reductase and phospholipid), for the hydroxylation of a variety of substrates. Early work in this laboratory has shown that all three components of the mixed function oxidase system are required for the full expression of sex dependent drug and steroid metabolism (Barr and Skett, 1984). They concluded that there is no one essential component of the microsomal drug hydroxylation system that can be ascribed with the responsibility of expressing sex differences in rat hepatic microsomal metabolism. Barr and Skett suggested that the choice of membrane in reconstitution studies of the system may be crucial to the quantitative and qualitative results obtained using partially pure and extensively purified cytochrome P-450 preparations. A role for the microsomal membrane lipids was postulated in the maintenance of sex differences in drug and steroid metabolism. This work showed a very complex interaction of proteins and lipids and proved difficult to interpret due to the three possible variables (Barr, 1985).

1.8. MECHANISM OF INTERACTION BETWEEN PHOSPHOLIPIDS AND THE PROTEIN COMPONENTS OF THE P-450 DEPENDENT MONOOXYGENASE.

Since Lu et al. (1969a) succeeded in the solubilization of the rabbit liver microsomal mixed-function oxidase it has been well established that lipids are essential for its hydroxylating activity. The reconstituted system, consisting of the terminal oxidase cytochrome P-450, NADPH-cytochrome C (P-450) reductase and a heat-stable, organic solvent extractable factor, which later could be identified as phospholipid (Strobel et al.,1970), was found to catalyze fatty acid ω-hydroxylation (Lu et al., 1969b) and also the hydroxylation of hydrocarbons and the demethylation of drugs (Lu et al., 1969a). Neutral fats proved ineffective for reconstituting an active system but synthetic phosphatidylcholine was found as active as the crude extract. Extending these results, Strobel et al. (1970) observed that the proportion of reduced cytochrome P-450 increased on addition of phospholipids to the assay mixture and that there existed a correlation between the extent of reduction and the lipid concentration. This relation has been quantitated with respect to hydroxylating activity by Ingelman-Sundberg and Glaumann (1977). All these data suggested that the most relevant function of phospholipids with respect to the substrate metabolism was in electron transfer processes. A lot of experimental data supported this working concept without solving the question of the mechanism of interaction. Ingelman-Sundberg (1977) demonstrated that phospholipids facilitate the NADPH-dependent, but also the sodium periodate supported, 6β-hydroxylation of androstenedione by a stabilization and/or induction of an active conformation of cytochrome P-450. He found that, in sodium periodate-supported 6β-hydroxylation of androstenedione, renaturable cytochrome P-450 was saturated with about 10 nmoles of phosphatidylcholine per nmole cytochrome P-450, whereas in NADPH-supported reactions saturation only occurs when a phosphatidylcholine/cytochrome P-450 ratio of 1000 was reached thus indicating

different requirements for phospholipids in the two types of reaction (peroxidatic or monooxygenatic).

A similar requirement for phospholipids was also found for other cytochrome P-450 dependent activities. Williamson and O'Donnell (1969) demonstrated that phospholipids can stabilize cytochrome P-450, thereby preventing decomposition to the inactive P-420 and enhancing the rate of deoxycorticosterone 11β-hydroxylation. Hydroxylation of testosterone in the 6β-, 7α- or 16 α-positions was also shown to depend highly on the presence of phospholipids in the assay system, as does the 3,4-benzpyrene hydroxylation (Lu and Levin, 1974). The relative unspecificity of the phospholipid/protein interaction is further supported by the well known fact that in reconstitution experiments cross substitutions, including phospholipids as well as reductase, are possible over a wide phylogenetic range (e.g. yeast-mammalian components) (Duppel et al., 1973; Duppell and Ullrich 1976). These results provide evidence for the functional importance of phospholipids. A role of the phospholipid component in substrate binding and control of substrate specificity was suggested by Lu et al. (1969a, b) who also reported on the influence of phospholipids on hydroxylation of benzphetamine and laurate (Lu et al., 1970) but could not detect any influence on the spectral binding constants of these two substrates. It was considered unlikely that the phospholipids are required for the binding of a substrate to cytochrome P-450 (Levin et al., 1974). Ristau et al. (1978) suggested that the phospholipids increase the affinity of cytochrome P-450 to bind substrates. DePierre and Ernster (1975) demonstrated that laurate and unsaturated fatty acids are able to compete with type I substrates for the binding sites thereby inhibiting the metabolism of type I compounds. No such effect was observed with aniline (a type II substrate). On the other hand, Haaparanta et al. (1980) demonstrated that the acidic phospholipids are more active than neutral ones in cytochrome P-450 LM2-catalysed O-demethylation of 7-ethoxycoumarin and hydroxylation of benzo(a)pyrene. Strobel et al. (1970) have reported that the lipid

component is essential for the electron transfer from NADPH to cytochrome P-450. One can conclude from these studies that the phospholipid, due to its highly ordered bilayer structure, not only mediates a functional linkage between proteins by its amphipathic environment but, rather more importantly, orientates the proteins in a specific manner within the bilayer. Therefore the phospholipid function in mediating the electron transfer is an indirect one and indicates protein-protein interactions involved therein. Negatively charged phospholipids may be responsible for recognition and regulation processed by charge compensation, which include the possibility to regulate enzymatic activity by metabolic processes (Ingelman-Sundberg et al., 1980).

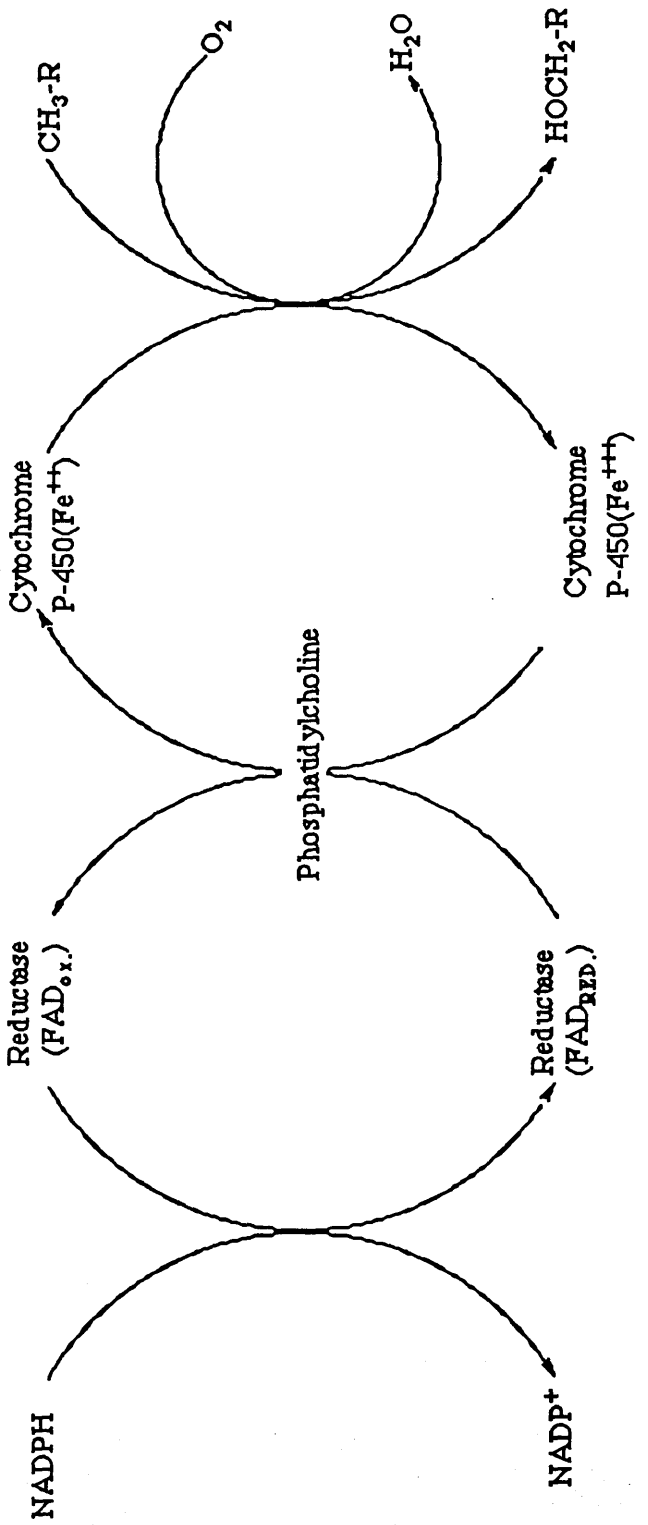
1.9. MECHANISM OF MIXED FUNCTION OXIDATION.

Phospholipid appears to be crucial to membrane activities requiring two or more protein components. For example, cytochrome c reduction can be reconstituted by recombining the two proteins (cytochrome P-450 and NADPH-cytochrome C (P-450) reductase) in the presence of phospholipid. Furthermore, acetone treatment of microsomes (which extracts phospholipids) was found to abolish the NADPH-cytochrome c reductase activity, but not the NADH-$K_3Fe(CN)_6$ reductase activity, indicating that phospholipid is required for the transfer of electrons from the flavoprotein to the cytochrome. This activity could be restored by the addition of a mixture of phosphatidylcholine and lysophosphatidylcholine to the delipidated preparation. These data thus indicated that the enzyme system described above requires the protein components plus microsomal lipid (Jones et al., 1969). Cytochrome P-450-dependent systems catalyze the oxidation of a variety of relatively hydrophobic compounds of both endogenous and exogenous origin. These include steroids, fatty acids, a large number of drugs and carcinogens. A typical hydroxylation reaction is shown below (Omura et al., 1967).

$$2H^+ + R\text{-}H + O_2 + 2e^- \rightarrow R\text{-}OH + H_2O$$

The aliphatic or aromatic substrate (R-H) is hydroxylated, requiring molecular oxygen plus two electrons. One atom of oxygen is inserted into the substrate (Coon et al., 1973a,b), while a second is incorporated into water. The system can also catalyze epoxidations, N-oxidations, sulfoxidations, peroxidations, and N-, S-, and O-dealkylations. There are a moderately large number of microsomal cytochromes P-450, each apparently with its own range of substrate specificities. In general, each of these microsomal cytochromes tends to be rather nonspecific, in contrast to some of the mitochondrial cytochromes P-450. The catalytic cycle for a generalised cytochrome P-450, a modification of that originally proposed by Estabrook et al. (1970), is shown in figure 1.4. (Lambeth 1985). Substrate binding to the oxidized (ferric heme) enzyme is followed by reduction to the ferrous heme enzyme. Oxygen becomes bound to the ferrous enzyme, and a second electron is donated to the system, resulting in the production of a powerful oxidising species at the active site which then hydroxylates the bound substrate. For more details about the proposals for the nature of the hydroxylating species see review by White and Coon, (1980). The electrons can be donated to the cytochrome by the flavoprotein NADPH-cytochrome P-450 reductase. In cytochrome P-450-containing reconstituted systems, this reductase, in the presence of phospholipid, can provide electrons for hydroxylating activity (Omura et al., 1966; Lu and Coon, 1968). Based upon a synergestic effect of NADPH and NADH in microsomes, Hildebrandt and Estabrook, (1971), proposed that the second electron in the catalytic cycle (figure 1.4.) can be provided by cytochrome b_5. In support of this proposal, Sugiyama et al. (1980) have purified a cytochrome P-450 from microsomes which shows an absolute activity requirement for both the NADPH-specific flavoprotein and the NADH-specific cytochrome b_5 system.

Figure 1.4.



Scheme of oxidative and reductive function of the microsomal drug metabolism system

1.9.1. Site of Lipid Function.

Lu et al. (1969b) have suggested that the phosphatidylcholine does not serve as a substrate when added to cytochrome P-450 and it does not cause any spectral changes. It has no effect on the dissociation constant of the cytochrome P-450-benzphetamine complex (Lu et al., 1970) or the cytochrome P-450-laurate complex (Lu et al., 1969b).It is unlikely that the phospholipid is required for the binding of substrate to cytochrome P-450 since the partially purified cytochrome P-450 has less than 1 nmole phospholipid per nmole P-450 (Levin et al., 1974). It still gives type I and II binding spectra with a variety of substrates (Strobel et al., 1970). Coon et al. (1973b) have reported that the lipid component is essential for electron transfer from NADPH to cytochrome P-450 (figure 1.4.). Since the phosphatidylcholine is obviously not an electron carrier, the mode of action is still unknown. Lu and Levin (1974) speculated that the phospholipid may function in some manner to facilitate the transfer of electrons from the reductase to cytochrome P-450.

1.10. SEX DIFFERENCES IN HEPATIC DRUG METABOLISM.

The action of a variety of drugs are more pronounced and persist longer in the female than the male rat (Kato,1974). The main mechanism of such sex differences is the differences in the activity of microsomal drug metabolising enzymes in rat liver (Conney et al., 1965; Kato and Gillette, 1965; Kato et al., 1962). Although the drug-metabolising enzymes from rat liver microsomes metabolise a variety of drugs, Kato and Gillette (1965) showed that the magnitude of sex differences is dependent on the substrates and metabolic pathways, i.e., the N-demethylation of aminopyrine and the aliphatic hydroxylation of hexobarbital are 3.5-fold higher in male rats than in females, whereas there is little or no sex differences in the hydroxylation of aniline. Björkhem and coworkers, (1974; 1976) and Kato and Takahishi (1968) showed that there is a slight sex differences in the NADPH

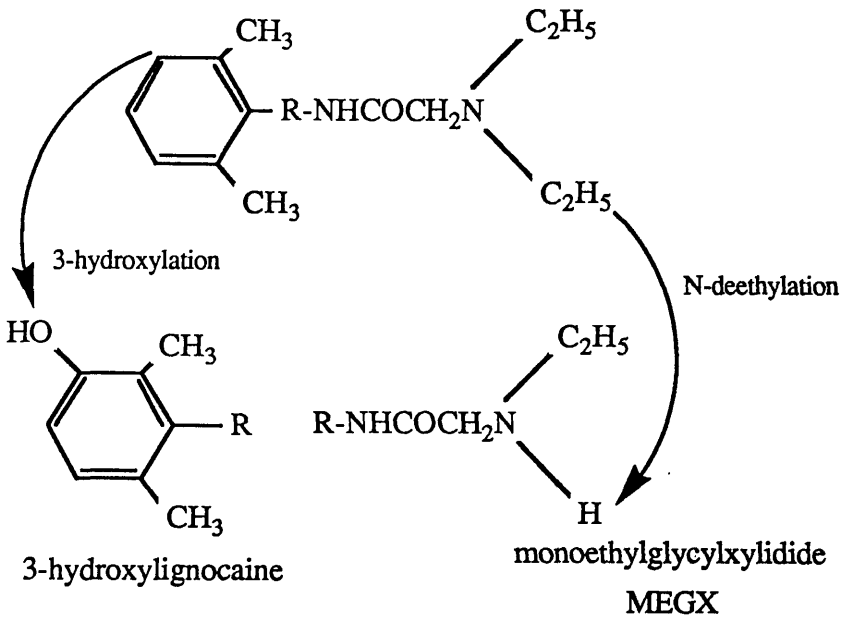
cytochrome C (P-450) reductase activity and cytochrome P-450 content of rat liver microsomes. It is generally thought that if differences in reductase activity do exist they are of secondary importance to the difference seen in cytochrome P-450 (Lu and West, 1980). Early work in this laboratory has shown that sex-specific NADPH cytochrome C (P-450) reductase is required for the full expression of sex-dependent drug and steroid metabolism (Barr and Skett, 1984). It has been suggested that androgen may induce some species of cytochrome P-450 capable of metabolising rapidly a variety of drugs, which show clear sex differences in their metabolism (Kato and Onoda 1970). Androgens were found to be involved in the regulation of sexually differentiated drug and steroid metabolism when Yates and associates (1958) found that castration of male rats reduced the activity of these enzymes towards the female level of metabolism. Androgenic control of metabolism has been reported using a number of substrates e.g. ethylmorphine N-demethylase (Castro & Gillette, 1967), hexobarbitone (Conney et al., 1965). Two sexually differentiated forms of cytochrome P-450 have been purified to homogeneity from untreated male and female rats respectively; 16α–hydroxylase (Morgan et al., 1985), and 15β–hydroxylase (MacGeoch et al., 1984). Of all the cytochrome P-450-dependent activities of rat liver microsomes that show sex differences, the steroid 16α–hydroxylase has been the most thoroughly characterised in terms of its hormonal regulation (Skett, 1977; Skett et al., 1979; Colby, 1980; Morgan et al., 1985) although the 15β-hydroxylase is also under hormonal regulation (MacGeoch et al., 1984). Almost all cytochrome P-450-catalysed reactions have been found to be more efficient in male than in female rat liver except the 15β-hydroxylase activity which was found to be present at high level in female microsomes and undetectable in the male and immature female (Gustafsson and Ingelman-Sundberg, 1974). Sex differences in the metabolism of steroids have also been well documented (Yates et al., 1958, Conney et al., 1965, Einarsson et al., 1973). Similarly sex differences in drug metabolism have been shown to correlate to sex-dependent lipid differences (Belina et al., 1975). Preliminary work has indicated that the lipid environment may affect the activity of these sex-dependent

enzymes and lead to a disappearance of the sex differences (Barr, 1985). It is well known that cytochrome P-450 requires phospholipids for activity (Lu et al., 1970; Strobel et al., 1970), and phospholipids affect the substrate binding of cytochrome P-450 (Chaplin and Mannering, 1970; Estabrook et al., 1971) and the interaction with NADPH-cytochrome P-450 reductase (Autor et al., 1973). Thus, the lipid component of liver microsomes can be regarded as a determinant of the activity of cytochrome P-450.

Generally, males exhibit higher oxidative metabolic activities than females. The hepatic metabolism of drugs and xenobiotics (e.g. ethylmorphine (Castro, and Gillette, 1967), aniline, (El Defrawy El Masry et al., 1974), 7-hydroxycoumarin, (Kamataki et al., 1980), lignocaine and imipramine (Skett et al., 1980)), as well as steroid hormones, (Gustafsson (1973); Gustafsson and Eriksson (1971)) in the rat, have been shown to be sex-differentiated (Gustafsson and Ingelman-Sundberg, 1975). Lignocaine is a substrate which has been shown to possess sex differences in metabolism; it exhibits a male-specific N-deethylation and a sexually independent 3-hydroxylation, (figure 1.5., von Bahr et al., 1977). It, thus, is ideal for the study of the maintenance of sex differences in drug metabolism as there is an inbuilt control activity for direct comparison. The use of lignocaine as a substrate in this project was suggested by the continuing research in this department into the hormonal control of drug metabolism, (Skett and Gustafsson, 1979; Gustafsson et al., 1983; Skett and Young, 1982; Skett and Weir, 1983; Skett et al., 1984; Skett and Joels, 1985; Skett and Barr, 1985) which shows that, the rate of N-deethylation was faster in the male microsomes than the female microsomes, whereas the rate of the 3-hydroxylation activity was similar in both sexes.

FIGURE 1.5.

The phase one hepatic microsomal metabolism of lignocaine.



1.11. EFFECT OF STREPTOZOTOCIN-INDUCED DIABETES MELLITUS ON HEPATIC DRUG METABOLISM IN RAT.

Insulin-dependent diabetes induced by either alloxan or streptozotocin alters drug metabolism in rat liver (Dixon et al., 1961; Dixon et al., 1963; Reinke et al., 1978; Past and Cook, 1982). Leaming et al. (1982) showed that conversion of dehydroepiandrosterone to androst-4-ene-3,17-dione and testosterone was enhanced in streptozotocin-treated animals, the same treatment can cause an increase in oestradiol metabolism. Cholesterol 7 α–hydroxylase activity is enhanced by STZ treatment (Subbiah and Yunker, 1984), on the other hand 5 α–reductase is unaffected by the same treatment (Warren et al., 1983). Steroid and drug metabolism by the rat liver is known to be sex-dependent (Kato, 1974; Gustafsson et al., 1980; Colby, 1980) and the effect of diabetes on hepatic metabolism of type I substrate such as aminopyrine and ethylmorphine (Schenkman et al., 1980) is also sex dependent (Kato and Gillette, 1965; Reinke et al., 1978; Faas and Carter, 1980). The effects of diabetes mellitus have been shown to be sex-dependent with regard to drug metabolism (Dixon et al., 1961; Kato and Gillette, 1965; Skett and Joels, 1984). In 1986, Skett demonstrated the effect of STZ-induced diabetes mellitus on 4-androstene-3,17-dione metabolism using male animals and showed that the 7α-hydroxylase activity was increased but the 6β- and 16α-hydroxylases were decreased and 5α-reductase was unaffected. Female animals were unaffected by the same treatment. Skett (1986) indicated that the effects seen in the male rats were always sex-dependent, i.e. only the male-specific activities were decreased and only in the male rat.

1.12. MICROSOMAL PHOSPHOLIPID COMPOSITION IN THE STREPTOZOTOCIN DIABETIC RAT.

Rat liver microsomal fatty acid composition and fatty acid desaturation were studied in streptozotocin diabetic rats (Faas and Carter, 1980;1983; Holman et al., 1983) and it was found that diabetes changes the fatty acid composition of rat liver microsomal phospholipid. It was demonstrated that fatty acids such as linoleic and docosahexaenoic acids were increased whereas palmitoleic, oleic and arachidonic acids decreased in the major microsomal phospholipids (Faas and Carter, 1980;1983). However, Faas and Carter in 1983 suggested that most of these changes in fatty acid composition in the diabetic rat are caused by the diminished fatty acid desaturase activities, since the fatty acid composition can be overcorrected by insulin therapy with the exception of the diminished arachidonic acid which was further decreased following insulin therapy. Several authors (Nelson, 1980; Mathur et al., 1983) have demonstrated that the phospholipid fatty acid composition plays an important role in the membrane fluidity and in the enzyme activities.

1.13. PURPOSE OF PROPOSED STUDY:

These may be summarised as follows:

1. To confirm the existence of sex differences in rat hepatic microsomal metabolism of lignocaine (Skett & Young, 1980).

2. To purify the essential components of the microsomal mixed function oxidase system from male and female rats and reconstitute a functionally active drug hydroxylation system.

3. To reconstitute the system in such a way as to investigate the role of cytochrome P-450 and microsomal lipid in the expression of sex differences.

4. To separate and analyse the microsomal lipids and use the separated fractions to reconstitute the enzyme activities to ascertain the role of sex dependent lipids in the modulation of microsomal enzyme activity.

5. To separate and analyse the microsomal lipids and use the separated fractions to reconstitute the enzyme activities to ascertain the exact nature of the protein-lipid interaction.

6. To separate and analyse the microsomal lipids from both male and female rats to establish whether the male microsomal lipid and the female microsomal lipid differ in their composition.

7. To investigate the effect of using lipids with different acyl chain (saturation and length) on the microsomal enzyme activity.

8. To study the effect of pathological conditions (eg diabetics) on the microsomal enzyme activity by modifying the microsomal lipid composition.

MATERIALS AND METHODS

2.1. GENERAL MATERIALS.

The materials used during the course of this study and their respective suppliers are described in the following section. All other chemicals were of analytical grade purity.

2.1.1. Autoradiography Materials.

X-ray Film X-Omat S (France), X-ray Developer D-19, X-ray Fixer FX-40 were supplied by Kodak (U.K.) Ltd., London.

2.1.2. Chromatography.

Bio-Gel P-30 and Hydroxylapatite (Bio-Gel HTP) were obtained from Bio-Rad Laboratories, California, USA.; Sephadex G-50 purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.; DEAE-Cellulose DE52 purchased from Whatman International Ltd, England and 2'5'-ADP Sepharose 4B obtained from Pharmacia Fine Chemicals, Sweden.

2.1.3. Electrophoretic Materials.

Acrylamide, 2-mercaptoethanol, Coomassie Brilliant Blue R-250, N, N'-methylene bis acrylamide, N, N, N'N'-tetramethylenediamine (TEMED) and ammonium persulphate were obtained from Sigma Chemical Co. Ltd., Poole, Dorset, U.K

2.1.4. Radioisotopes.

[Carbonyl-^{14}C]-lignocaine and 4-[4-^{14}C]-Androstene-3,17-dione purchased from Amersham International, Amersham, Bucks.

2.1.5. Fine Chemicals.

Dithiothreitol (DTT), Sucrose, Potassium chloride, di-Potassium hydrogen orthophosphate, Potassium di-hydrogen orthophosphate purchased from Koch Light Laboratory, Colnbrook. Ammonium Molybdate, Cholic Acid (Sodium Salt) and NADPH were obtained from Sigma, Chemical Co, . Ltd., U.K. EDTA was purchased from Hopkins and Williams and glycerol was from May and Baker. Emulgen 913 was the kind gift of Dr G. Gibson. Ecoscint was supplied by National Diagnostics, Manville, New Jersey. The kit for measurement of serum glucose was purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

2.1.6. Lipids and Fatty Acids.

L, α-dilauroylphosphatidylethanolamine, L, α-dilauroylphosphatidylcholine, L, α–dioleoylphosphatidylcholine, L, α–dicaproylphosphatidylcholine, L, α–dibehenoyl-phospha-tidylcholine, L, α–diarachidoylphosphatidylcholine, L, α–distearoylphospha-tidylcholine, arachidonic acid, palmitic acid, heneicosanoic acid and oleic acid, supplied by Sigma Chemical Co. Ltd., Poole, Dorset , U.K.

2.1.7. Proteins.

Cytochrome C, Bovine serum albumin (BSA), Albumin bovine 67000; Egg Albumin 45000; Pepsin 34700 and Trypsin 24000 were supplied by Sigma, Chemical Co, . Ltd., U.K.

2.1.8. Substrates.

Lignocaine was kindly donated by Astra Läkemedel AB, Södertälje, Sweden. 4-androstene-3,17-dione and streptozotocin were purchased from Sigma Chemical Co. Ltd., Poole, Dorset, U.K.

2.2. EXPERIMENTAL ANIMALS.

All rats used throughout the study were mature Wistar stock bred in the departmental animal house (males 300-350 g and females 225-250g body weight). They were kept in a controlled environment; temperature 19±1°C, 12 hours light (0700-1900), 12 hours dark (1900-0700), with free access to drink (tap water) and food (CRM Nuts, Labsure, Croydon, U.K.).

2.3. PREPARATION OF MICROSOMES.

2.3.1. Preparation of Post-Mitochondrial Supernatant.

Animals were killed by CO_2 asphyxiation followed by cervical dislocation. The livers were removed immediately and cooled in ice cold 0.25 M sucrose. The livers were rinsed in 0.25 M sucrose, dried with blotting paper and weighed. All subsequent steps were carried out at 4°C. The livers were then roughly chopped using scissors in four volumes of 0.25 M sucrose (4mls buffer to 1 gm wet weight of liver). The liver was homogenised in the same buffer using a Potter-Elvehjem homogeniser with loose-fitting Teflon pestle with a clearance of 0.40-0.45 mm and driven at 10, 000 rpm by a TRI-R STIR-R motor, model S63C (Rockville Centre, New York). 10 complete strokes were used to homogenize the liver by this method. The homogenate was centrifuged in an MSE HI-SPIN 21 centrifuge using a type 143 Rotor with an average centrifugal force of 13, 000 x g for 20 minutes. This step is considered to remove cell nuclei, mitochondria and large fragments of cellular debris. The supernatant of this spin was decanted and kept, the pellet being discarded. The preparation thus produced was the postmitochondrial supernatant.

2.3.2. Preparation of Microsomes by Ultracentrifugation.

The microsomal fraction of the liver was prepared by ultracentrifugation as described by Berg and Gustafsson, (1973). Postmitochondrial supernatant was dispensed into 5/8"x3" cellulose nitrate tubes (Beckman Instruments Inc., Palo Alto, California) which were then tightly capped. Twelve of these tubes were inserted into a Beckman 70Ti Rotor (in a Beckman Model L8-55M refrigerated ultracentrifuge) and spun at 105, 000 x g for 1 hour at 4°C. After the spin the supernatant (cytosol) was discarded, the pellet (microsomes) was treated as described below.

2.3.3. Removal of Haemoglobin From Microsomes.

Removal of haemoglobin from microsomes was by the method of Mihara and Sato (1978). One pellet was resuspended in 6 mls of buffer containing 10 mM EDTA and 1.15 % (w/v) KCl .

Resuspension was carried out using the Potter-Elvehjem homogenizer, three strokes were sufficient to return the microsomes to suspension. The suspension was then centrifuged at 105, 000 x g for 1 hour at 4°C as described above and the supernatant was discarded. The pellet containing the washed microsomes was subsequently used as detailed below.

2.3.4. Solubilisation of Microsomes.

Haemoglobin-free microsomes, were resuspended in 10 volumes of buffer (containing 20% glycerol, to prevent conversion to the inactive P-420 form, 1mM EDTA to inhibit lipid peroxidation, 1 mM dithiothreitol to keep sulphydryl groups in the reduced state in 0.1M potassium phosphate buffer at pH 7.25) at a protein concentration of approximately 10 mg/ml. The microsomal membrane proteins were solubilised by the careful addition of 10% (w/v) sodium cholate solution in a dropwise manner to the washed microsomal suspension while stirring the mixture on ice and under a constant

flow of oxygen-free nitrogen to prevent haem destruction arising from lipid peroxidation.

The sodium cholate was added to a final concentration of 1.8% (w/v) and the mixture was left stirring for a further 30 minutes. The solubilised preparation was centrifuged at 105,000 X g for 60 minutes at 4°C (in a Beckman Model L8-55M refrigerated ultracentrifuge) to remove unsolubilised material. The supernatant is referred to as the solubilised microsomes.

2.3.5. Concentration of Solubilised Microsomes.

Concentration of the mixed function oxidase enzymes in solubilised microsomes was carried out by the addition of solid ammonium sulphate to 80% saturation, the mixture was left on ice for 30 minutes and then centrifuged at 2, 500 x g for 10 minutes and the plug produced at the top was removed carefully and resuspended in 1 ml of 0.1M potassium phosphate buffer at pH 7.25 containing 20% glycerol, 1 mM EDTA, 1 mM dithiothreitol and 1.8% sodium cholate.

2.3.6. Delipidation of Concentrated Solubilised Microsomes.

5g of Bio-Rad P-30 was suspended in 200 ml of elution buffer containing 0.1M potassium phosphate pH 7.25, 20% glycerol, 1 mM EDTA, and 0.7% sodium cholate and left in a cold room for 24-48 h. The packing of the column was carried out in the cold room and equilibrated using the elution buffer. The solubilised preparation was delipidated by applying a 1 ml sample (to which sucrose had been added to increase the density) to the Bio-Gel P-30 column (1.5 x 50 cm) and eluting with 0.1M potassium phosphate buffer at pH 7.25 containing 20% glycerol, 1 mM EDTA, and 0.7% sodium cholate at a flow rate of 0.1 ml/min. Delipidated samples could be detected easily since they were yellowish in colour. Samples were normally combined and assayed for microsomal protein, lipid phosphorus and subsequently used directly for reconstitution.

2.4. CYTOCHROME P-450 ISOZYME 2C PREPARATION.

2.4.1. Preparation of DE52 Column.

DE52 (DEAE-cellulose) was purchased from Whatman international Ltd., Maidsone, Kent, England. The first stage in the preparation of the gel for ion- exchange chromatography purposes is to remove the fines. Fines are small fibres which, if not removed, block the flow of buffer through the column. The fines were removed by mixing approximately 53g of the cellulose gently in concentrated potassium phosphate buffer (0.2 M) and allowing the mixture to settle for 2-3 minutes About 15-30 ml of the buffer were used for every gram of dry cellulose powder and the pH of the buffer was adjusted to 7.4 using the acidic or basic components of the buffer mixture. The fines remaining in the supernatant above the slurry were carefully poured away and 600 ml of the same buffer was added to the cellulose. The suspension was transferred to a measuring cylinder, the volume of the gel was noted and the mixture was made up to 1.5 volume . The column was set up vertically in an area free of draughts, direct sunlight, and other heat sources and the stirred slurry was poured into the column. It is essential that, from the moment of pouring the slurry into the column to the stage of having a settled column bed of gel, the operations are carried out as quickly as possible otherwise convection currents will form in the slurry which cause a very slow flow rate, peaks overlap and incomplete separation.

The packing of the column was carried out at room temperature and equilibrated using the starting buffer (containing 10 mM potassium phosphate pH 7.4, 20% (v/v) glycerol, 0.5% sodium cholate, 0.2% Emulgen 913 and 0.1 mM EDTA).

2.4.2. Preparation of Solubilised Microsomes.

Male rats were killed and the microsomal fraction of the liver prepared by the ultracentrifugation method as described in the section 2.2.2. Haemoglobin was removed from the microsomes as described above in section 2.2.3. and the pellet was resuspended in buffer (containing 20% glycerol, 1mM EDTA in 0.13M potassium phosphate buffer at pH 7.4) at a protein concentration of approximately 10 mg/ml. The microsomal membrane proteins were solubilised by the careful addition of 10% (w/v) sodium cholate solution in a dropwise manner to the washed microsomal suspension while stirring the mixture on ice under a constant flow of oxygen-free nitrogen. The sodium cholate was added to a final concentration of 2.3 % (w/v) and the mixture was left stirring for a further 40 minutes. The solubilised preparation was centrifuged at 105,000 x g for 60 minutes at 4°C in a Beckman L8-55M ultracentrifuge to remove unsolubilised material. The supernatant is referred to as the solubilised preparation.

2.4.3. Polyethyleneglycol (PEG) 6000 Fractionation.

The solubilised preparation (168 ml) was fractionated by the careful addition of 30 ml 50% PEG 6000 in a dropwise manner while stirring the mixture on ice over a 10 minute period, and the mixture was centrifuged at 20,000 x g for 25 minute in a MSE HI-SPIN 21 centrifuge. The pellet was discarded and 11.5 ml of 50% PEG 6000 were added slowly to the supernatant, stirred for 10 min and centrifuged at 20,000 x g at 4°C for 12 minutes. The pellet was again discarded. Finally 61.5 ml of 50% PEG 6000 were added in a dropwise manner to the supernatant, the mixture was stirred for 10 minutes then centrifuged at 20,000 x g for 12 minutes. The pellets were resuspended to a volume of 60 ml in buffer A (containing 10 mM potassium phosphate pH 7.4, 20% glycerol, 0.5% sodium cholate, 0.2% Emulgen 913 and 0.1mM EDTA). The preparation was dialysed overnight at 4°C against 1 litre of 10mM potassium phosphate pH 7.4 buffer (containing, 20% glycerol, 0.5% sodium cholate, 0.2% Emulgen 913 and 0.1mM EDTA).

2.4.4. DE52 Column.

The male-specific isozyme of cytochrome P-450 was prepared from the PEG 6000 fraction by the method of Waxman, (1984). The fraction was applied to a column of Whatman DE52 resin (1.5 X 50 cm prepared as described in section 2.4.1.) at a flow rate of 30 ml/hr. The column was washed with 60 ml of buffer A (10mM potassium phosphate pH 7.4 containing, 20% glycerol, 0.5% sodium cholate, 0.2% Emulgen 913 and 0.1 mM EDTA) to ensure the quantitative transfer of the protein to the column and then eluted with 500 ml of buffer A + 20 mM KCl. At this point two yellow bands at 50 to 70 % of the way down the column and a red band (containing cytochrome b_5) at the top could be seen. Fractions of < 10 ml were collected and monitored at 417 and 295 nm. Fractions with an A417:295 $\geq$ 1 were collected.

2.4.5. Hydroxylapatite Column.

Fractions collected from the DE52 column were analysed by SDS-PAGE gel electrophoresis (see section 2.15) and homogeneous fractions were combined and dialysed overnight at 4°C against more than 10 volumes of buffer B, containing 10 mM potassium phosphate pH 7.4, 20% glycerol, 0.2% Emulgen 913 and 0.2 mM EDTA. The fractions were subsequently applied to a hydroxylapatite (Bio-Rad HTP purchased from Bio-Rad Laboratories California, USA) column (1.5x3 cm), equilibrated in the same buffer at a flow rate of 15 ml/hr and run at room temperature (Waxman and Walsh, 1983). The hydroxylapatite column was washed with 50 ml of buffer B+35 mM potassium phosphate followed by 40 ml buffer B+45 mM potassium phosphate and 40 ml of buffer B+70 mM potassium phosphate. Finally the isozyme was eluted with 120 ml buffer B+120 mM potassium phosphate.Pooled fractions of cytochrome P-450 were dialysed overnight against 1 litre of 50 mM potassium phosphate pH 7.4 buffer containing 20% glycerol and 0.1 mM EDTA and stored at -20°C in the dark under a nitrogen atmosphere.

2.4.6. Detergent Removal.

Detergent was removed from the male-specific cytochrome P-450 isozyme preparation by absorption onto a hyroxylapatite (1 x 1.5 cm) column and eluting with 0.2 M potassium phosphate buffer at pH 7.4 containing 20% glycerol, 1 mM EDTA, and 0.7% sodium cholate (buffer C) at a flow rate of 15 ml/hr as described by Cheng and Schenkman, (1982). Samples were dialysed against 2 x 20 volume of buffer C containing 10 mM potassium phosphate pH 7.4, 20% glycerol, 0.5% sodium cholate and then applied to a hydroxylapatite column (1 x 1.5 cm) which had been equilibrated in the same buffer. Removal of detergent (detected by monitoring the eluent at 280 nm) was accomplished by washing with buffer C until the absorption of the eluant at 280 nm was equivalent to that of buffer C. The cytochrome P-450 isozyme was eluted as a relatively sharp band using 0.5 M potassium phosphate buffer pH 7.4 containing 20% glycerol and 0.1 mM EDTA. The preparation was finally dialysed against 100 volume of 50 mM potassium phosphate buffer pH 7.4 containing 20% glycerol and 0.1 mM EDTA and stored in small fractions in the dark under nitrogen at -20^{o}C. Thawing and refreezing of the fractions was avoided as much as possible.

2.5. NADPH CYTOCHROME C(P-450) REDUCTASE PREPARATION.

NADPH Cytochrome C (P-450) reductase was prepared by the method described by Shephard et.al (1983). Male mature Wistar rats were fed a 0.1% (w/v) solution of sodium phenobarbital instead of tap water for 7 days. Total microsomal vesicles were isolated as described above (section 2.2.2.), the microsomal membrane pellet was resuspended at a final protein concentration of about 20 mg/ml in a 10 mM potassium phosphate buffer pH 7.25 (containing 20% glycerol, 1 mM dithiothreitol, 1 mM EDTA 2 μM flavin mononucleotide), and solubilised as described above (section 2.2.4.).

2.5.1. Purification Procedure.

Solubilised membrane vesicles (350 ml), at a protein concentration of 1.5 mg/ml, were loaded (at 15 ml/h) onto a column (1.5 X 3 cm) of 2`, 5`-ADP sepharose 4B (Pharmacia Fine Chemicals AB, Uppsala, Sweden) which had been equilibrated at 4°C with 100 ml of a buffer containing 10 mM potassium phosphate, pH 7.25, 20% glycerol, and 1 mM EDTA. After loading the sample the column was washed extensively with the same buffer and eluted by washing firstly with 250 ml of a solution containing 0.3 M potassium phosphate pH 7.7, 20% glycerol, 0.1 mM EDTA, 0.1% Lubrol PX, and then 350 ml of a solution containing 30 mM potassium phosphate, pH 7.7, 0.1 mM EDTA, 20% (v/v) glycerol, 0.15% (w/v) sodium deoxycholate. During this washing procedure, the colour of the column turned from dirty brown to bright yellow.The enzyme was subsequently eluted at a flow rate of 30 ml/hr with a solution containing 30 mM potassium phosphate pH 7.7, 0.1 mM EDTA, 20% (v/v) glycerol, 0.15% (w/v) sodium deoxycholate, 5 mM 2'-AMP, and 0.4 mM PMSF (phenylmethanesulfonyl fluoride). Fractions with an A455:380 ratio ≥ 1 were pooled together.

The enzyme eluted in a volume of approximately 15 ml and was dialysed for 48 hours against 100 volume of 30 mM potassium phosphate, pH 7.7, 0.1 mM EDTA, 20% glycerol. The dialysis buffer was changed once. Protein purity was analysed by SDS-PAGE as described by Laemmli (1970) and in section 2.15. The enzyme was stored in aliquots in the presence of 0.4 mM PMSF (phenylmethanesulfonyl fluoride) in small fractions in the dark under nitrogen at -20°C. Thawing and refreezing of the fractions was avoided as much as possible.

2.6. ULTRAFILTRATION OF THE PURIFIED MICROSOMAL PROTEIN.

Purified preparations were routinely concentrated to obtain the protein at useable stock concentrations. A clean membrane with a molecular weight exclusion cut off 20, 000, which had been moistened in water, was placed in an ultrafiltration cell (Micro Filtration System, California). The cell was attached to a cylinder of nitrogen via high pressure tubing. The protein solution was poured into the chamber and concentrated under 30PSI pressure.

2.7. EXTRACTION OF WHOLE MICROSOMAL LIPID.

Total microsomal lipid was extracted by a two step procedure (Folch et al. (1957); Bligh and Dyer, (1959) as detailed by Overturf and Dyer, (1969).

One gram of liver was homogenised with 10 ml of methanol for 1 minute, then 20 ml of chloroform was added and the process continued for a further 2 minutes. The mixture was filtered and the solid residue resuspended in chloroform-methanol (2:1 v/v, 30 ml) and homogenised for 3 minutes. After filtering, the solid was washed once more with chloroform (20 ml) and once with methanol (10 ml). The combined filtrates were transferred to a measuring cylinder and to the filtrate was added one quarter of the total volume of 0.88% potassium chloride in water . The mixture was shaken thoroughly and allowed to settle. The upper layer was removed by aspiration and to the lower layer was added one quarter of the volume of water-methanol (1:1) and the washing procedure repeated. The lower phase, containing the purified lipid, was transferred to a dry evaporating cylinder and connected to a Buchi Rotavapor-R. The solvent was partially evaporated off under vacuum in a water bath at 30°C. When the solution was concentrated to 10 ml, a 0.5 ml aliquot were removed and placed in a previously weighed test-tube. The solvent was completely evaporated off under nitrogen at 30°C. The lipid mixture was stored in the dark at -20°C in chloroform at a concentration of 10 mg/ml under a nitrogen atmosphere.

2.8. FRACTIONATION OF LIPIDS ON SILICA GEL.

The lipid mixture was fractionated as described by Rouser et al. (1970)

2.8.1. Column Preparation.

A slurry was prepared of 15-20 g silica gel 60 (70-200 mesh, Merck F 254) (preheated at 120°C for 2 h before use) in about 30-50 ml chloroform and poured into a 1x30 cm glass chromatography tube. The solvent level was allowed to drop to the top of the silica gel, and the bed was washed with 2 vols. of chloroform.

2.8.2. Application of the Sample and Elution of the Column.

A solution of 200-250 mg of total lipids in 5 ml of chloroform was placed gently on the top of the column and allowed to run into the column. Chloroform (1-2 ml) was then added to ensure quantitative transfer of the lipid mixture to the column. The elution of the column was carried out with chloroform (10 column volumes) to elute neutral lipids; and methanol (10 column volumes) to elute phospholipids. The total volume of each solvent was collected for monitoring by TLC and phosphorus content.

2.9. SEPARATION OF PHOSPHOLIPIDS BY THIN LAYER CHROMATOGRAPHY.

The phospholipid fractions prepared as in section 2.7 were separated by a modification of the method of Yavin and Zutra, (1977). For this technique three organic solvent mixture were prepared :

A) chloroform:methanol:40% aqueous methylamine (13:6:1.5) (v/v/v).

B) diethylether:acetic acid (glacial) (19:1) (v/v).

C) chloroform:acetone:methanol:acetic acid (glacial):distilled H_2O (10:4:2:3:1) (v/v/v/v/v).

All three organic solvent mixtures were freshly prepared.

Lipids were dissolved in 0.1 ml of chloroform:methanol (9:1, v/v) and spotted on silica-gel t.l.c. plates (10 cm x 10 cm) (Silica Gel 60, thin layer chromatography plate (Merck F254)) for two dimensional separation of phospholipids. The glass chromatographic chambers were equilibrated with the solvent mixtures (approximately 200 mls in total) 2 hrs before commencing the separation. Phospholipid samples were applied at the lower left hand corner (2 cm in), using a 10 µl disposable micro-pipette and the solvent dried in a stream of warm air. A total of 8 plates could be run in one batch. After a period of approximately 25 minutes in solvent A, by which time the solvent front was within 1 cm of the edge of the plate, the plates were removed and dried in a stream of warm air for 10-15 min. Each plate was then exposed to the fumes of a concentrated solution of HCl for approximately 5 min, then cool air for an additional 5 min. The plates were then placed in solvent B running in the second dimension (origin at lower corner), for a period of 10-15 min. The plates were subsequently dried for 5-10 min in a stream of cool air before placing in solvent C in the same dimension as that immediately above. A period of approximately 25 min was needed to allow the solvent front to reach within 1 cm of the edge of the plate. At this

point the plates were removed from the solvent and allowed to dry thoroughly in a stream of cool air. After drying the plates, the phospholipids were visualised by placing the plates in an iodine vapour tank. The spots corresponding to PE (phosphatidylethanolamine); PC (phosphatidylcholine); PI (phosphatidylinositol) and PS (phosphatidylserine) (see figure 2.1.) were scraped into test tubes and the phospholipids were extracted with 3-4 portions (5 ml) of chloroform. Water was removed by the use of anhydrous sodium sulphate. The extracts of phospholipids were evaporated to dryness in a stream of nitrogen in weighing flasks, and weighed. Phosphorus content was determined in each fraction as described in section 2.13.2. The purified, separated phospholipids were stored in the dark at -20^{o}C under a nitrogen atmosphere.

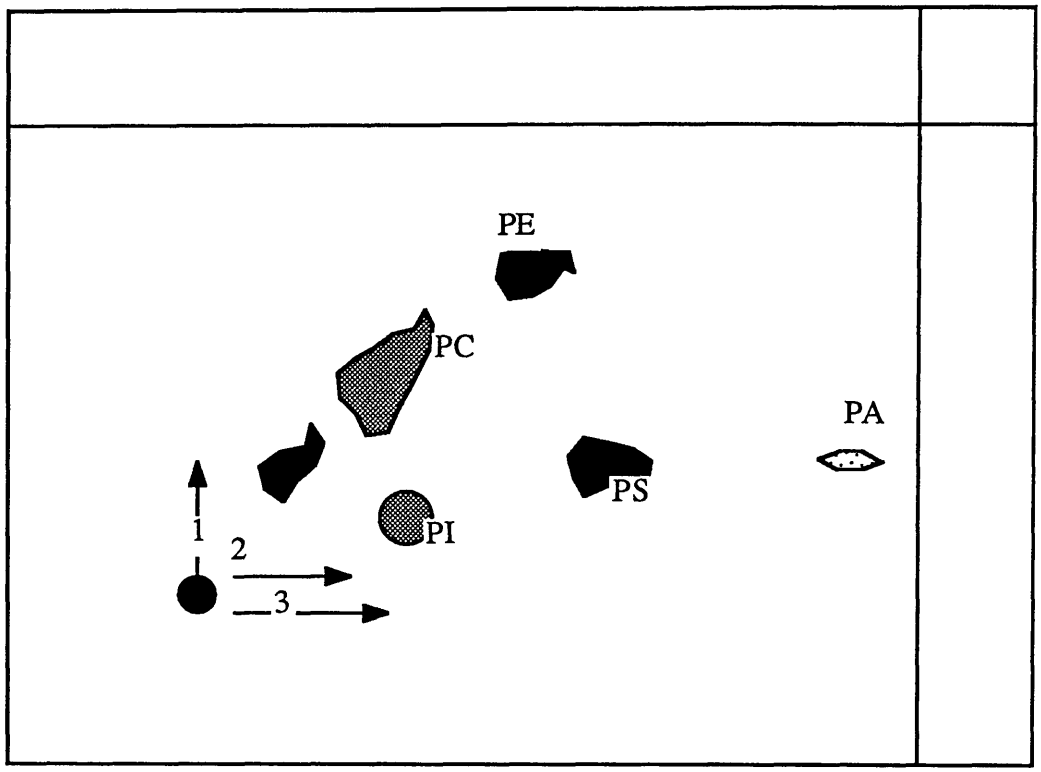


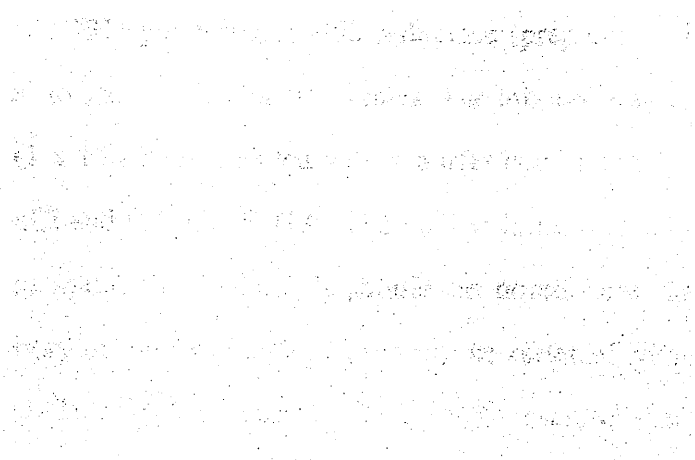
FIGURE 2.1.

Schematic representation of phospholipid separation by two-dimensional thin layer chromatography.

2.10. RECONSTITUTION OF MIXED FUNCTION OXIDASE.

2.10.1. Sephadex G-50 Column Preparation.

The reconstitution of a functional drug metabolising system was carried out by the method of Ingelman-Sundberg and Glaumann (1977). This technique of cholate-gel filtration makes use of a Sephadex G-50 (particle size 50-150μm) column. The column (1 x 15 cm) was equilibrated in 50 mM Tris HCl pH 7.4 containing 50 mM NaCl and 0.2 mM EDTA. Before being used, the column was precoated with a solution of protein and lipid as described below. The precoating solution was composed of 20 mg total microsomal phospholipid in 2 ml of equilibrating buffer (10% (w/v) cholate added till solution clear) and 10 mg of bovine serum albumin was added to the lipid solution. This solution was applied to the column and the eluate collected. The absorbance of the eluate at 280 nm was compared to a similar solution containing only lipids. The process was repeated until the absorbance of the lipid-only solution was greater than the absorbance of the lipid-protein eluate solution.



2.10.2. Reconstitution Using Delipidated Microsomes.

0.1 ml of the lipid solution, containing 1 mg of lipid, was taken and the chloroform was evaporated off at 45°C, under a stream of nitrogen. The lipid was resuspended by sonication in 0.1 ml of buffer containing 0.1M potassium phosphate at pH 7.25, 20% glycerol, 1 mM EDTA, and 1.8% sodium cholate. 0.5 ml of delipidated microsomes (prepared as in section 2.3.6.) contain 1 nmole cytochrome P-450 was added to the lipid suspension, mixed gently and left to stand on ice for 30 minutes. The delipidated microsomes/lipid mixture was applied to a Sephadex G-50 column (1 x 15 cm) and eluted with a buffer containing 10 mM Tris HCl (pH 7.4), 50 mM NaCl and 0.2 mM EDTA. The void volume was collected and used as the reconstituted metabolic system. [This technique allowed the small detergent molecules to be held back in the column while allowing incorporation of the enzymes into phospholipid vesicles which form as the cholate is removed].

2.10.3. Reconstitution Using Purified Enzymes.

0.1 ml of the lipid solution, containing 1 mg of lipid, was taken and the chloroform was evaporated off at 45°C, under a stream of nitrogen. The lipid was resuspended by sonication in 0.1 ml of buffer containing 50 mM potassium phosphate pH 7.4, 20% glycerol and 0.1 mM EDTA. A 0.5 ml aliquot contain 1nmole of purified cytochrome P-450 (prepared as in section 2.4.) was added to the lipid suspension, followed by 8 units of NADPH cytochrome P-450 reductase (prepared as in section 2.5.), mixed gently and left to stand on ice for 30 minutes. The mixture was applied to a Sephadex G-50 column (1 x 15 cm) and eluted with a buffer containing 10 mM Tris HCl (pH 7.4), 50 mM NaCl and 0.2 mM EDTA. The void volume was collected and used as the reconstituted metabolic system. It should be noted here that, in the determination of the activity of the reconstituted system, the order of reforming the vesicles is very important. To achieve maximal activity, it is essential that the three components (cytochrome P-450, reductase and phospholipid) are mixed before applying

to the Sephadex G50 column. The results are most consistent and maximal activity was achieved when the procedure was carried out in this manner. However we have tried to add the reductase after reforming the vesicles since it was free from detergent but the result obtained from this and the non-vesicle preparation was less than 50% of the microsomal activity. Poor performance of non-vesicle preparations has been reported by Haaparanta et al. (1980).

2.11. METABOLISM OF LIGNOCAINE BY THE RECONSTITUTED ENZYME SYSTEM.

2.11.1. Incubation Conditions.

The metabolising activity of the reconstituted system was measured by following the metabolism of ^{14}C-lignocaine. An appropriate volume of the reconstituted system, containing 0.1 nmole cytochrome P-450 was added to 30 µl of ^{14}C-lignocaine (containing 0.1µCi; 100 µg) and 1 mg of NADPH (at a concentration of 10 mg/ml) and the mixture was incubated at 37 °C in a shaking water bath for 60 minutes. The reaction was stopped by the addition of 4 drops of 10 M NaOH. The NaOH raised the pH of the reaction mixture to about 14 which not only denatured the enzymes present but caused the lignocaine to become unionised and, therefore, more readily extracted into chloroform.

2.11.2. Separation and Identification of the Lignocaine Metabolites.

After termination, 5 mls of chloroform was added to the mixture and shaken for 20 minutes in a rotary shaker to allow the metabolites to be extracted into the organic phase. The organic layer was removed and the procedure was repeated twice. The organic extracts were recombined and evaporated to dryness under a stream of nitrogen at 40°C. The metabolites and unchanged substrate were redissolved in 5 drops of chloroform by sonication for 30 seconds. The metabolites were spotted onto a thin layer

chromatography plate (silica gel, Merck F254) and run once in a solvent system consisting of chloroform/methanol (9:1). After separation, the radioactive bands were located by autoradiography in which X-ray film was cut to the same size as the TLC plate and placed on the top of the TLC plate and left in a dark box for 5-7 day after which the films were processed and developed and the radioactive metabolites were traced. The appropriate areas were scrapped into scintillation fluid (6 mls of Ecoscint from National Diagnostics, New Jersey) and the radioactivity quantitated by liquid scintillation counting using a Packard Tri-Carb liquid scintillation analyser (MODEL 2000 CA). Metabolites have been identified by their thin-layer and gas chromatographic behaviour and mass spectrographic profile in comparison to authentic standards, Skett et al. (1980).

CALCULATIONS OF RESULTS :-

$$S \times \frac{Cm}{Ct} \times \frac{1}{t} \times \frac{1}{n} \times \frac{1000}{MW} = \text{nmoles produced/min/nmole cytochrome P-450}$$

S = amount of substrate added (100 µg)

n = nmole of cytochrome P-450 (0.1 nmole)

t = incubation time in minutes (60 minutes)

Ct = total cpm

Cm = metabolites cpm

MW = substrate molecular weight (271)

2.12. METABOLISM OF 4-ANDROSTENE-3,17-DIONE BY THE RECONSTITUTED ENZYME SYSTEM.

2.12.1. Incubation Conditions.

The metabolising activity of the reconstituted system was measured by following the metabolism of ^{14}C-4-androstene-3,17-dione . An appropriate volume of the reconstituted system (containing 0.1 nmole cytochrome P-450) was added to 50 µl of ^{14}C-4-androstene-3,17-dione (containing 0.1µCi; 100 µg) and 0.1 mg of NADPH (at a concentration of 10 mg/ml) and the mixture was incubated at 37°C in a shaking water bath for 20 minutes. The reaction was stopped by the addition of 1 ml 0.9% NaCl and 5 ml chloroform : methanol (2:1).

2.12.2. Separation and Identification of the 4-Androstene-3,17-dione Metabolites.

After termination, the mixture was shaken for 20 minutes in a rotary shaker to allow the metabolites to be extracted into the organic phase. The organic (bottom) layer was removed. The extract was then evaporated to dryness at 30°C under a stream of nitrogen. The metabolites and unchanged substrate were redissolved in 5 drops of chloroform by sonication for 30 seconds. The metabolites were spotted onto a thin layer chromatography plate (silica gel, Merck F254) and run twice in a solvent system consisting of chloroform : ethylacetate (4:1) as a mobile phase. After separation, the radioactive bands were located by autoradiography as described in section 2.11.2. after which the radioactive metabolites were traced, the appropriate areas were scrapped into scintillation fluid (6 mls of Ecoscint from National Diagnostics, New Jersey) and the radioactivity quantitated by liquid scintillation counting using a Packard Tri-Carb liquid scintillation analyser (MODEL 2000 CA). Identification of the metabolites employed authentic standards as described by Berg and Gustafsson, (1973).

CALCULATIONS OF RESULTS :-

$$S \times \frac{Cm}{Ct} \times \frac{1}{t} \times \frac{1}{n} \times \frac{1000}{MW} = \text{nmoles produced/min/nmole cytochrome P-450}$$

S = amount of substrate added (100 μg)

n = nmole of cytochrome P-450 (0.1 nmole)

t = incubation time in minutes (20min

Ct = total cpm

Cm = metabolites cpm

MW = substrate molecular weight (280)

2.13. SPECTROPHOTOMETRIC TECHNIQUES.

All spectrophotometric techniques were performed on a Shimadzu UV/240 spectrophotometer.

2.13.1. Cytochrome P-450.

Cytochrome P-450 was routinely measured as the reduced carbon monoxide (CO) difference spectrum as described by Omura and Sato, (1964a,b). Microsomes were put into two plastic cuvettes (Elkay Products, Mass, USA), 1 ml aliquot in each. A base line spectrum was drawn between 400 nm and 500 nm. To each cuvette few crystals sodium dithionite were added and the content of the sample cuvette was gassed gently with CO for 30 seconds. The difference spectrum between the two cuvettes was then recorded between 400 nm and 500 nm. The change in absorbance at 450 nm, relative to baseline was then converted to a concentration of cytochrome P-450 using the extinction coefficient of 91 mM^{-1} cm^{-1}.

2.13.2. NADPH Cytochrome C (P-450) Reductase.

NADPH Cytochrome C (P-450) reductase activity was routinely measured by the method of Philips and Langdon (1962). This assay makes use of the increase in the absorbance at 550 nm of the artificial electron acceptor, cytochrome C, when reduced. 5 mls of 0.1M phosphate buffer (pH 7.4) and 0.5 ml of the sample solution was added to 0.5 ml of cytochrome C solution (3.7 mg/ml) in distilled water. The mixture was split into two 3 mls cuvettes and 10 µl of 10 mM NADPH solution was added to the test cuvette. The change of absorbance at 550 nm was recorded against a blank with the same contents as above except that distilled water was added instead of NADPH. The concentration of NADPH-Cytochrome C (P-450)-reductase was determined using the molar extinction coefficient of 19.1 mM^{-1} cm^{-1}. The activity of the reductase is expressed in units (a unit of reductase is define as the amount of enzyme which will reduce 1µmole of cytochrome C per minute at 20°C).

2.13.3. Cytochrome b_5.

Cytochrome b_5 was measured by the method of Estabrook and Werringloer(1978). Two cuvettes were set up containing identical microsomal or vesicle samples. The test cuvette was treated with 5 mg of sodium dithionite and the difference spectrum was recorded between 400 nm and 500 nm. The change in absorbance at 423 nm was used to determine the concentration of cytochrome b_5 using the extinction coefficient of 121 mM^{-1} cm^{-1}.

2.14. OTHER ASSAYS.

2.14.1. Protein Assay.

Protein concentrations were routinely measured by the method of Lowry et al. (1951). For this method three solutions were prepared.

A) 4% (w/v) Sodium carbonate in 0.1M NaOH.

B) 1% (w/v) Potassium disodium tartrate.

C) 0.5% (w/v) Hydrated copper sulphate.

A mixture of these three solutions was prepared freshly before every protein determination by mixing 50 parts of A. with 1 part of B. and 1 part of C. An aliquot of the protein suspension containing approximately 100 µg of protein was diluted to 1 ml with distilled water and added to 2 mls of the reagent mixture. The solution was mixed and left to stand for 10 minutes at room temperature. 0.3 ml of Folin Ciocalteu reagent (1M) was added, mixed thoroughly and left to stand at room temperature for 30 minutes before determining the optical density at 750 nm on a Shimadzu UV-240 spectrophotometer. Protein standards were prepared from a stock solution of bovine serum albumin (200 µg/ml).

2.14.2. Phosphorus Assay.

A phosphorus assay was used to determine the phospholipid content of the microsomal samples and the technique employed was a modification of the method of Rouser et al (1970). Extraction of phospholipid was carried out by the addition of 2 ml of chloroform/methanol (2:1) and 0.4 ml of 0.9% sodium chloride solution to 100 µl of the microsomal fraction. The mixture was centrifuged gently (1, 500 x g for 2 minutes) and the organic layer was removed. To 100 µl of the organic layer was added 0.65 ml perchloric acid and the mixture was heated in an oven at 200 °C for 20 minutes in sealed tubes. This allows the phosphorus in the phospholipids to be converted to phosphates.

The tubes were cooled and the following solutions were added rapidly, accurately and with mixing in the correct order :-

2.8 ml distilled water

1 ml of 1.25% ammonium molybdate

0.5 ml of 10% ascorbic acid

The tubes were then heated in a boiling water bath for 5 minutes after which time the absorbance was read at 810 nm on a Shimadzu UV-240 spectrophotometer. A standard curve was obtained using known phosphate concentrations. It should be noted that all glassware (pipettes, flasks, tubes, etc.) must be washed with chromic acid-sulphuric acid (Vogel, 1956) before use, then rinsed with distilled water followed by methanol and chloroform, and used immediately; test tubes, particularly, should not be stored before use. Failure to follow these instructions results in development of an anomalous deep blue colour.

2.15. SDS-PAGE GEL ELECTROPHORESIS.

2.15.1. Separation of Proteins by Gel Electrophoresis.

SDS gels were prepared and samples subjected to electrophoresis by the method of Laemmli (1970). The dimension of the gel plates was 16 x 16 cm to fit in the apparatus, and the thickness of the gels was 1.5 mm. The lower separation gels contained 10% acrylamide and the ratio of acrylamide to methylene-bis-acrylamide was 36.5 to 1. The concentration of the other components present in the separation gel was as follows:0.375M Tris-HCl (pH 8.8), 0.1% (w/v) SDS, 0.03 % (v/v)TEMED and 0.1% (w/v) ammonium persulphate. The upper stacking gels contained 5% (w/v) acrylamide, 0.13% (w/v) methylene-bis-acrylamide 0.12 M Tris-HCl (pH 6.8), 0.1% (w/v) SDS, 0.03 % (v/v)TEMED and 0.2% (w/v) ammonium persulphate. Samples were mixed with 0.5 volumes of buffer containing 0.025 M Tris-HCl (pH 6.8), 4.5 % (w/v) SDS, 45% (v/v) glycerol, 10 % (v/v) 2-mercaptoethanol and 0.002% (w/v)

bromophenol blue. The maximum amount of protein in each sample was 8 μg and the volume applied was never larger than 20 μl. The protein samples were completely reduced and denatured by heating at 100°C for 2 minutes. The electrophoresis buffer contained 0.025 M Tris, 0.192 M glycine and 0.1 % (w/v) SDS at pH 8.3. The samples were subjected to electrophoresis towards the anode at 200V for about 3 hours until the Bromophenol Blue dye marker was about 1 cm from the bottom of the gel. The gels were then processed as described below. Molecular weights of proteins were determined using standards supplied by Sigma Chemical Co. Ltd., Poole, Dorset, U.K. . Standard proteins used were, Albumin bovine 67000; Egg Albumin 45000; Pepsin 34700; Trypsin 24000.

2.15.2. Processing of Gels.

2.15.2.1. Staining of the Gels with Coomassie Brilliant Blue.

Gels were stained for 2-3 hrs at room temperature in 0.1% (w/v) Coomassie Brilliant Blue R-250 (purchased from BDH Chemicals, Poole, Dorest, U.K.) in methanol, acetic acid and water (5:1:1). Gels were destained for about 24 hrs at room temperature using a solution containing methanol, acetic acid and water (8:1:1) with several changes of solution. The gel was placed on a piece of absorbent paper slightly larger than the gel itself and covered with nonporous Cling Film plastic film, trapped air bubbles were released. The construction was placed on the gel dryer base and covered over with the sheet of silicon rubber and vacuum was applied for 10 minutes after which heat (60°C) was applied for 2h to draw all layers together. After the gel was completely dry it was photographed.

2.15.2.3. Silver Staining.

When the Coomassie Brilliant Blue staining method described above revealed that insufficient protein was present for adequate visualization in this way, the same gels were restained using the Bio-Rad silver stain (Bio-Rad Laboratories, California, USA). This method is said to be 10-50 fold more sensitive than staining with Coomassie Brilliant Blue R-250. The Bio-Rad silver stain is derived from the method of Oakley et al. (1980). A silver solution is prepared just prior to use by rapidly mixing 0.8 g silver nitrate in 4 ml water and 21 ml of 0.36% sodium hydroxide and 1.4 ml concentrated NH_3 solution. If any particles are left, concentrated NH_3 solution is added dropwise until they disappear. The developer solution is prepared by adding to 2.5 ml of 1% citric acid, 0.25 ml 37% formaldehyde and making the mixture up to 500 ml with distilled water. Staining was performed in a glass vessel. Gels, 1.5 cm thick, were rinsed quickly in water and kept in 50% (v/v) methanol for 20 minutes then washed with water for at least 10 minutes. Gels were incubated in the silver solution for 15 minutes, and then in the developer solution for about 10 minutes and then stopped with 25% photographic fixer (250 ml) for 1 minute. Between these steps the gels were washed with water. Following development the gels were dried as described in section 2.15.2.1. and were photographed.

2.16. PREPARATION OF PHOSPHOLIPID FATTY ACID METHYL ESTERS.

The fatty acids methyl esters were prepared by a modification of the method of Kates, (1964). For this technique four solutions were prepared :

A) Methanolic (90%) sodium hydroxide (0.3M), [dilute 10 ml of 3M aqueous NaOH to 100 ml methanol].

B) Potassium hydroxide (33%; 6M), [16.5 g KOH pellets dissolved to 50 ml in water].

C) Phenolphthalein indicator (1%), [dissolve 1g of phenolphthalein to 100 ml in 90% ethanol].

D) Hydrochloric acid (6M), [dilute 50 ml of concentrated. HCl with water to 100 ml].

A 0.5ml aliquot (containing 15-30 mg) of phospholipid as prepared by the method described in section 2.8., was placed in a side-arm flask, solvent was removed using a stream of nitrogen at 30°C. 5 ml of methanolic NaOH was added and the mixture was refluxed for 1-2 h. A few drops of phenolphthalein indicator were added and nonsaponifiables were extracted with 3-4 portions (5 ml) of petroleum ether (b.p. 30-60 °C). The alcoholic phase was acidifying with 0.3 ml of 6 M HCl to pH 2 and free fatty acids were extracted with 3-4 portions (5 ml) of petroleum ether (b.p. 30-60°C). The extracts of fatty acids were evaporated to dryness in a stream of nitrogen in weighing flasks, and weighed. The free fatty acids were refluxed for 1-2h in 4.5 ml of methanolic-HCl and extracted with 3-4 portions (5 ml) of petroleum ether (b.p. 30-60°C). Water was removed from the extracts by the addition of 2g anhydrous sodium sulphate, the extracts of fatty acid methyl esters were evaporated to dryness under a stream of nitrogen in weighing flasks, weighed and stored at -20°C in the dark under a nitrogen atmosphere.

2.17. GAS CHROMATOGRAPHY.

Samples of fatty acid methyl esters were prepared for GC analysis as described in section (2.6.). A solution of the sample (containing 1 mg of phospholipid) in chloroform was placed in a tube and the solvent was evaporated to dryness under a stream of nitrogen at 40°C. The residue was redissolved in toluene (100 µg/10µl) and 1 µl of sample was injected with a microsyringe onto the column. The methyl- esters were analysed on a 1% OV-1 (fused silica) column, film thickness 250 nm and 12 meter length, using a. PERKIN-ELMER 8420 CAPILLARY GAS CHROMATOGRAPH with FID detector and SGE unijector split mode at 190°C (isothermal) for 30 minutes.

We are grateful to Martin McAvoy, Department of Biochemistry, University of Glasgow for assistance with the GC analysis of fatty acid methyl esters.

2.18. STATISTICS.

Statistical significance was tested using Student's t-test for unpaired samples. Results were expressed as mean ± s.d. and the level of significance set at $p < 0.05$. Where appropriate Duncan's Multiple Range Test was used and the level of significance set at $p < 0.05$.

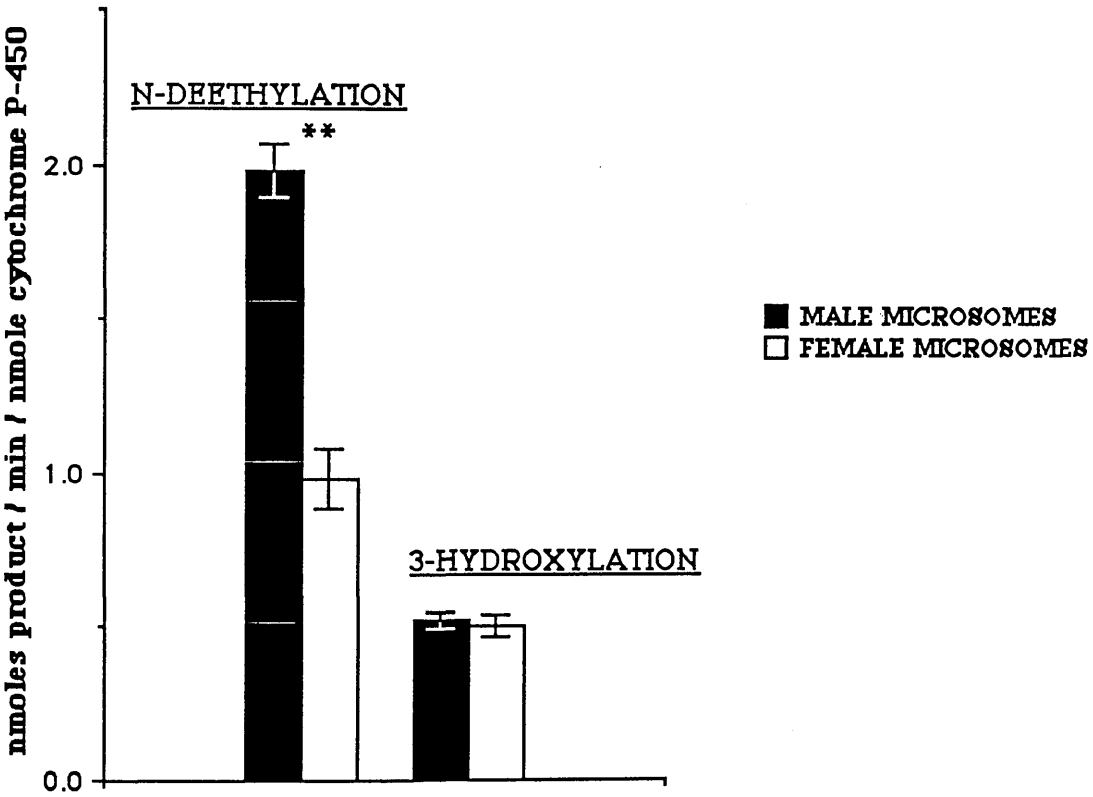
RESULTS

3.1. MICROSOMAL RESULTS.

The aim of this experiment was to verify the activity of the rat microsomes before the solubilisation stage and to confirm the previously reported existence of sex differences in microsomal metabolism of lignocaine (Skett and Gustafsson, 1979; Skett and Young, 1982; Skett and Weir, 1983; Skett et al., 1984; Skett and Joels, 1985). The microsomes used were prepared by ultracentrifugation (Materials and Methods 2.3.2.) and the incubation and detection of metabolites was performed as described in Materials and Methods 2.12.2. The results are expressed as the mean ± s.d. of six values. Figure 3.1. shows the production of the N-deethylated and 3-hydroxylated derivatives of lignocaine (figure 1.5.). As is seen N-deethylation rate is greater in the microsomes prepared from male animals than microsomes prepared from female animals. Statistical analysis of the results indicate that the N-deethylation route is significantly different ($p < 0.001$) whereas the 3-hydroxylation route is not found to exhibit a sex difference in metabolism.

FIGURE 3.1.

Sex differences in the metabolism of lignocaine using haemoglobin-free microsomes from mature male and female rat liver.



Results are expressed as mean ± s.d. of six values.

** = $p < 0.001$ compared to female values.

3.1.1. Measurements of the Components of Rat Liver Microsomes.

The components as prepared from male liver are shown in Table 3.1. and the same components isolated from female liver are shown in Table 3.2. It is seen from the two tables that the cytochrome P-450, NADPH cytochrome P-450 reductase and the microsomal lipid, were measured at each stage towards the reconstitution of the functional drug metabolising system. It can be seen from Table 3.1. and Table 3.2. that the ratios of cytochrome P-450 to NADPH cytochrome P-450 reductase vary slightly from step to step as the preparation procedure progresses. However the NADPH cytochrome P-450-reductase activity in the incubation was similar in comparison groups but varied somewhat between experiments. This applied to both male and female derived proteins. It should be noted that the cytochrome b_5 in the reconstituted hydroxylation system could not be determined due to high turbidity after the removal of the detergent and because of the small volume of the vesicle preparation. In order to measure the cytochrome P-450 content the assay procedure should be done as quickly as possible because of protein precipitation and thus the measurement of cytochrome b_5 in the vesicle preparation was ignored.

TABLE 3.1.

The contents of the drug metabolising system from mature male Wistar rats. These results are from a typical experiment.

Step	Lipid µmoles/ml	Cyt.P-450 nmoles/ml	Reductase unit/ml	Cyt.b_5 nmoles/ml	Cyt. P-450/ Reductase	Cyt.P-450/ Cyt.b_5
Microsomes	-	6.0	5.2	9.7	1.15	0.62
Hb-free Microsomes	-	5.0	5.1	10	1.08	0.50
Solubilised	-	4.8	5.2	7.5	0.92	0.64
Conc. Sol. Microsomes	750	15.0	16.0	24.0	0.94	0.63
Delipidated	15	3.0	3.7	4.9	0.81	0.61
Vesicles	-	0.38	0.53	N.D.*	0.72	-

* = Not Determined.

Step	Reductase nmoles/ml	Cyt.P-450/ Reductase
Microsomes	130	0.04
Hb-free Microsomes	127.5	0.04
Solubilised	130	0.04
Conc. Sol. Microsomes	400	0.04
Delipidated	92.5	0.03
Vesicles	13.25	0.03

TABLE 3.2.

The contents of the drug metabolising system from mature female Wistar rats. These results are from a typical experiment.

Step	Lipid µmoles/ml	Cyt. P-450 nmoles/ml	Reductase unit/ml	Cyt.b_5 nmoles/ml	Cyt.P-450/ reductase	Cyt.P-450/ Cyt.b_5
Microsomes	-	4.5	3.9	8.8	1.10	0.50
Hb-free Microsomes	-	4.0	4.1	8.5	0.97	0.49
Solubilised	-	3.8	4.0	8.0	0.95	0.47
Conc. Sol.Microsomes	540	11.1	14.2	21.0	0.90	0.51
Delipidated	9	1.5	2.8	2.9	0.86	0.51
Vesicles	-	0.25	0.33	N.D.*	0.75	-

* = Not Determined.

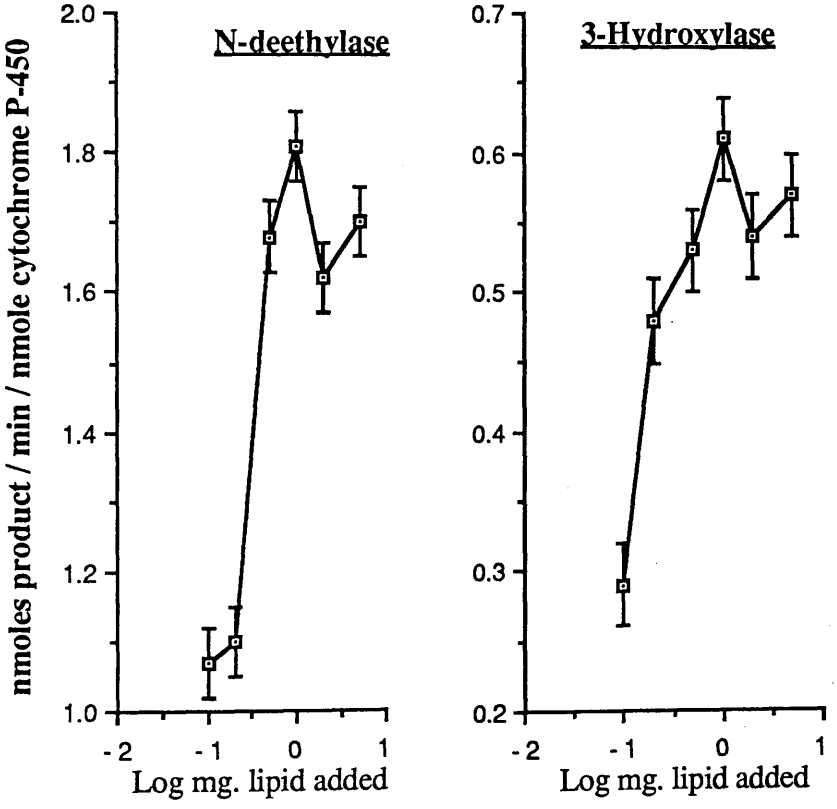
Step	Reductase nmoles/ml	Cyt.P-450/ Reductase
Microsomes	97.5	0.04
Hb-free Microsomes	102.5	0.04
Solubilised	100	0.04
Conc. Sol. Microsomes	355	0.03
Delipidated	70	0.02
Vesicles	8.25	0.03

3.1.2. Determination of Optimum Protein:Lipid Ratio for Reconstitution.

As is seen in figure 3.2., the addition of delipidated microsomes containing 0.1 nmole cytochrome P-450 to 100 nmoles of lipid (in this case microsomal lipid obtained from the male rat) gave the maximum enzyme activity, indicating that, this is the optimal amount of lipid to use for the reconstitution. A similar result was seen for all lipids used. The amount of lipid added is more than was removed by the delipidation procedure and accounts for over 97% of the lipid in the final preparation. The above ratio of protein to lipid was therefore used for the rest of the study.

FIGURE 3.2.

Determination of Optimum Protein:Lipid Ratio for Reconstitution. Using microsomal lipid and delipidated microsomes derived from male rat liver. 0.1nmole cytochrome P-450 was used in each reconstitution.



Based on a molecular weight of a typical phospholipid of 800, 1 mg of microsomal phospholipid equivelant to 1.25 nmole therefore the ratio of microsomal phosphlipid to microsomal phosphlipid is 12.5nmole

3.2. THE EFFECT OF ADDED LIPID COMPOSITION ON LIGNOCAINE METABOLISM BY DELIPIDATED MICROSOMES FROM MALE AND FEMALE LIVER.

These experiments were designed to investigate the influence of added lipid composition on the activity of cytochrome P-450 in a delipidated microsomal preparation to metabolise lignocaine. For this purpose the lipids were extracted from male and female rat liver microsomes using the method of Overturf & Dyer (Materials and Methods, 2.7.) and their effects compared to the effects of commonly used artificial lipids i.e. dilauroylphosphatidylcholine (DLPC) and dilauroylphosphatidylethanolamine (DLPE). The results obtained from the enzyme assays allowed the calculation of the lignocaine N-deethylase and 3-hydroxylase enzyme activities. The effects of incorporating the delipidated enzymes derived from the male and female liver into vesicles prepared from dilauroylphosphatidylcholine (DLPC) and dilauroylphosphatidylethanolamine (DLPE)/DLPC mixture (3:1 w/w) are shown in figures 3.3. and 3.4., respectively. It is seen that DLPE had no effect on the enzymes isolated from female liver (figure 3.3.) but significantly increased the N-deethylating activity of the enzymes isolated from the male animal (figure 3.4.), without having any effect on the 3-hydroxylating activity. Figure 3.5. and 3.6., show the effects of incorporating enzymes derived from male and female liver respectively into vesicles prepared from DLPC and male-derived microsomal lipids. It is seen that the N-deethylating activity of the enzymes is higher in microsomal lipids than in DLPC but the 3-hydroxylating activity is unaffected by the nature of the lipid. This is equally the case for enzymes derived from male and female animals.

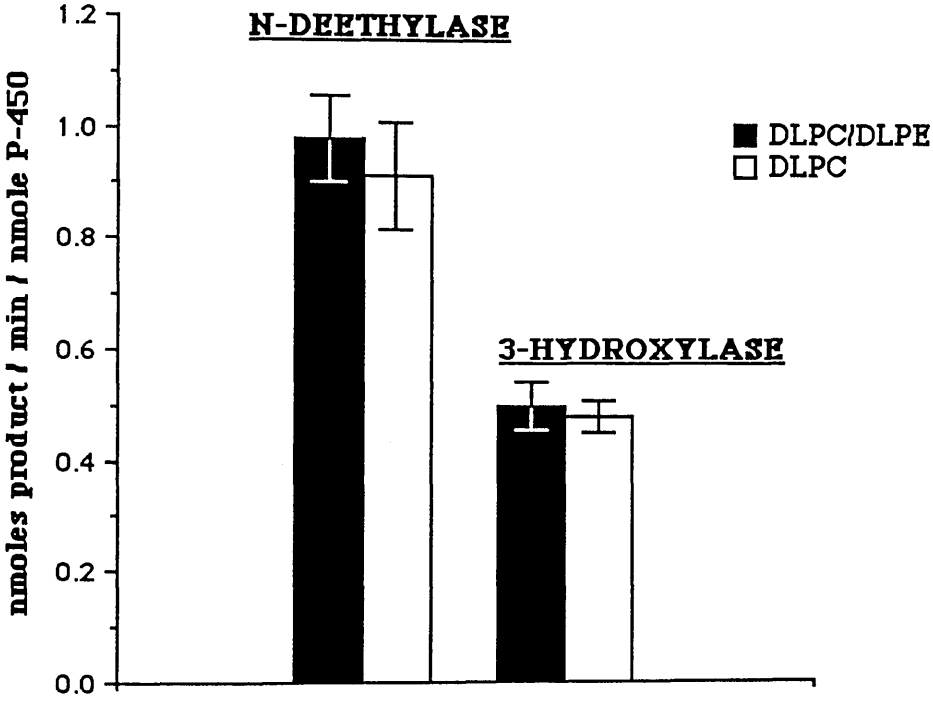
Figure 3.7. shows that the enzymes derived from male rats showed a lower N-deethylating activity in female-derived lipids while the 3-hydroxylating activity of the same preparation was not significantly affected. The enzymes derived from the female

showed a decreased N-deethylating activity in female derived lipids but 3-hydroxylating activity was unaffected by the female-microsomal lipid (figure 3.8.). It is also noted that N-deethylating activity was higher in the male-derived vesicles than the female derived vesicles whereas the 3-hydroxylating activity was similar. This result agrees with the sex differences seen in the rat (figure 3.1.).

A summary of the results obtained using the delipidated microsomal preparation is given in Table 3.3.

FIGURE 3.3.

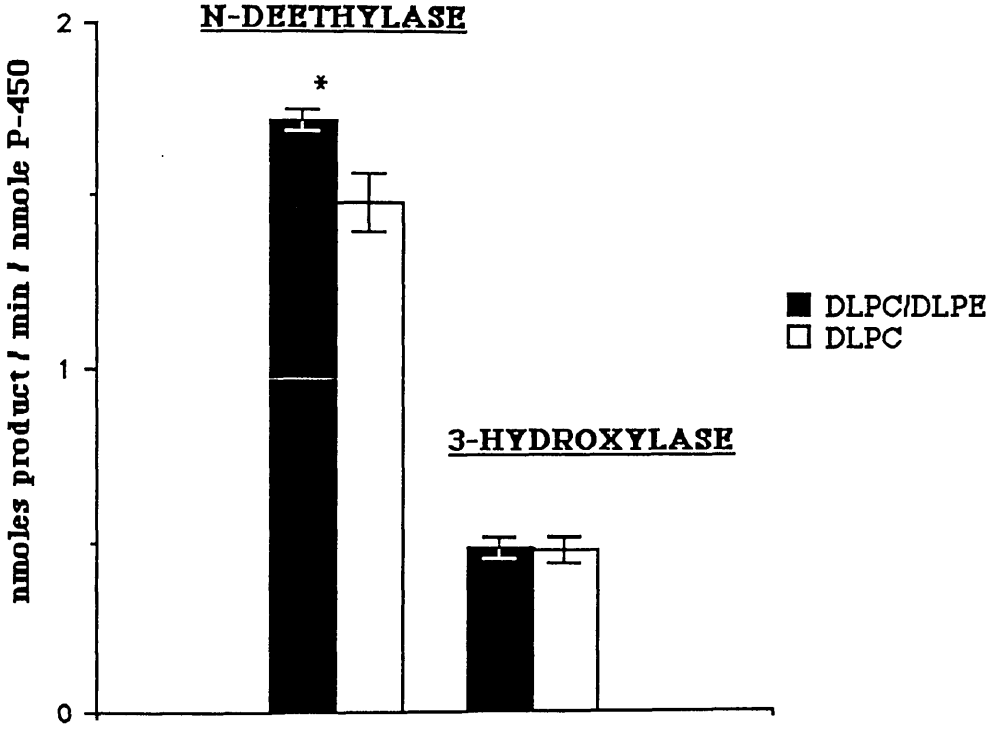
The effect of incorporating enzymes derived from female rat liver into vesicles prepared from dilauroylphosphatidylcholine (DLPC) and dilauroylphosphatidylethanolamine (DLPE)/ DLPC mixture (1:3 w/w) on metabolism of lignocaine.



Results are expressed as mean ± s.d. of six values.

FIGURE 3.4.

The effect of incorporating enzymes derived from male rat liver into vesicles prepared from dilauroylphosphatidylcholine (DLPC) and dilauroylphosphatidylethanolamine (DLPE)/ DLPC mixture (1:3 w/w) on metabolism of lignocaine.

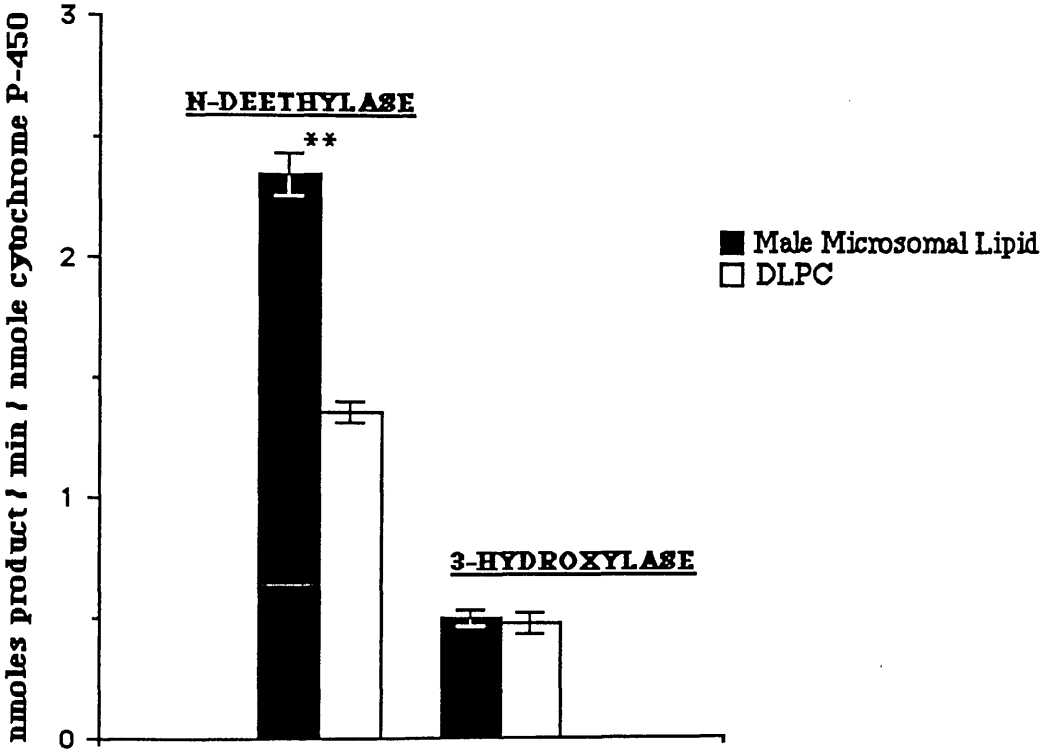


Results are expressed as mean ± s.d. of six values.

* = $p < 0.01$ compared to DLPC.

FIGURE 3.5.

The effect of incorporating enzymes derived from male rat liver into dilauroylphosphatidylcholine (DLPC) and male microsomal lipid on metabolism of lignocaine.

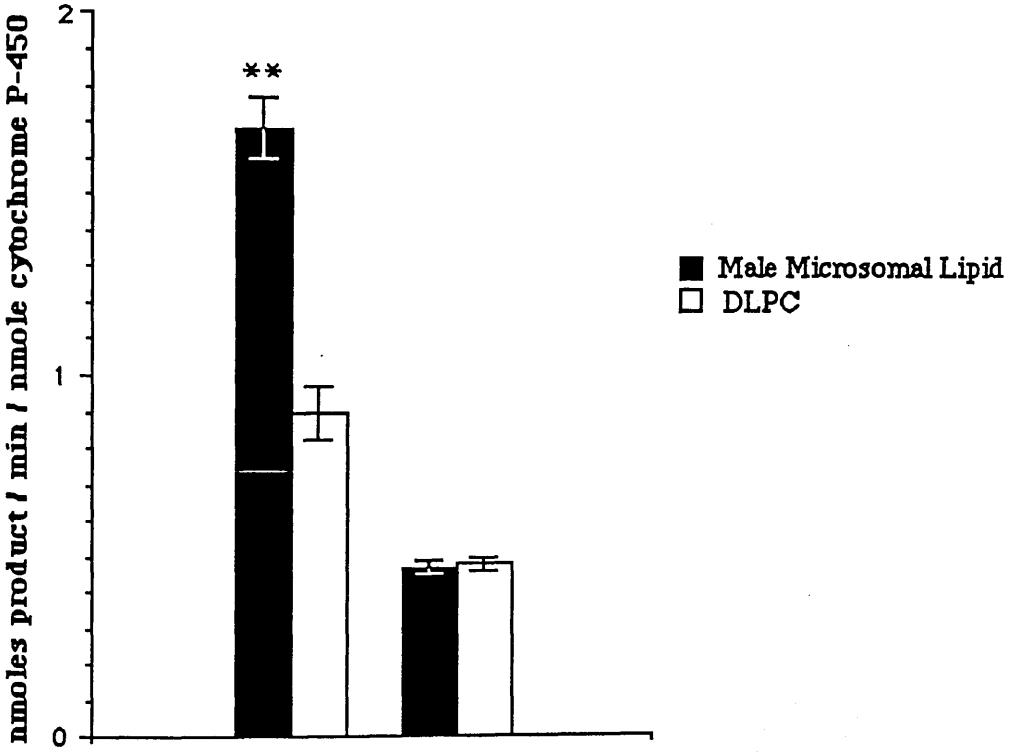


Results are expressed as mean ± s.d. of six values.

**= $p < 0.001$ compared to DLPC.

FIGURE 3.6.

The effect of incorporating enzymes derived from female rat liver into dilauroylphosphatidylcholine (DLPC) and male microsomal lipid on metabolism of lignocaine.

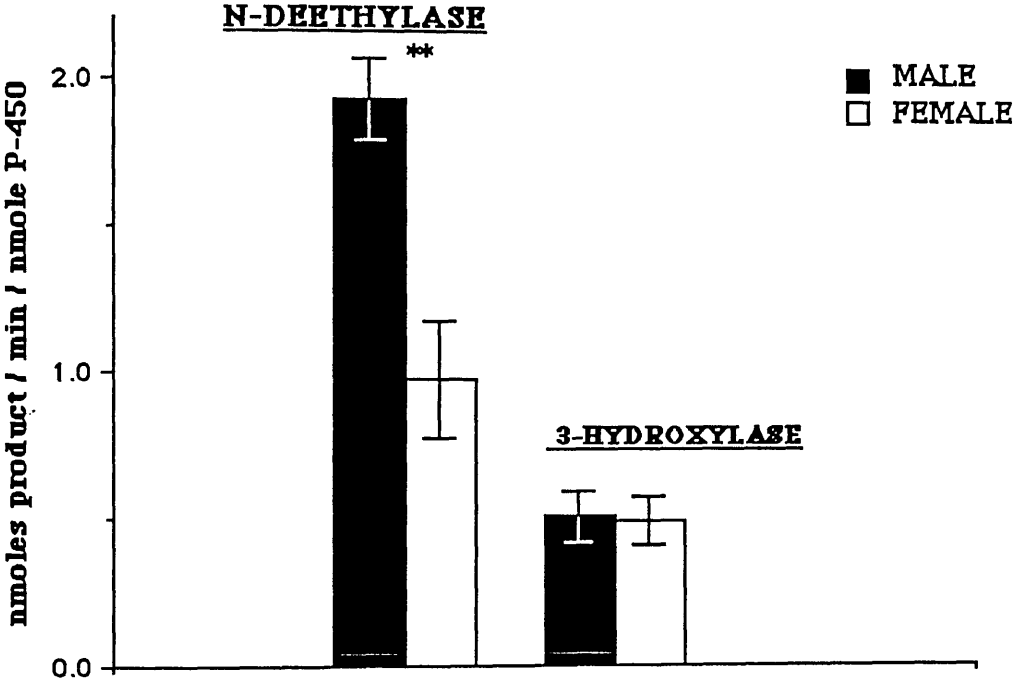


Results expressed as mean ± s.d. of six values.

**= $p < 0.001$ compared to DLPC.

FIGURE 3.7.

The effect of incorporating enzymes derived from male rat liver into microsomal lipids derived from male and female rat liver on metabolism of lignocaine.

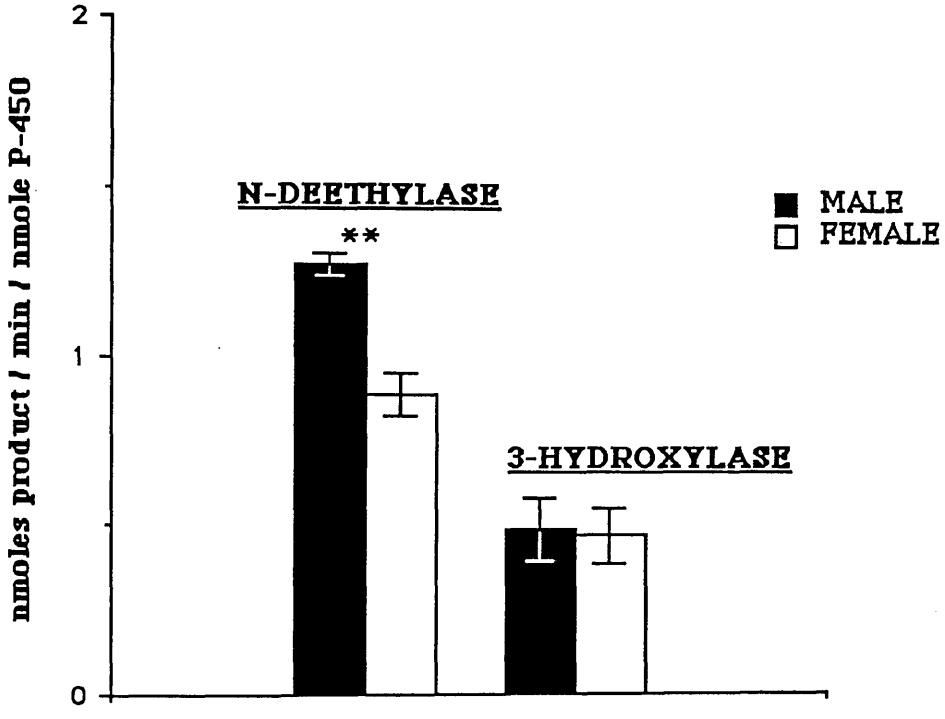


Results expressed as mean ± s.d. of six values.

**= $p < 0.001$ compared to female microsomal lipid.

FIGURE 3.8.

The effect of incorporating enzymes derived from female rat liver into microsomal lipids derived from male and female rat liver on metabolism of lignocaine.



Results expressed mean ± s.d. of six values.

**= $p < 0.001$ compared to female microsomal lipid.

Table 3.5.

A summary of the results obtained using the delipidated microsomal preparation.

Microsomes Derived From	N-Deethylase	3-Hydroxylase
MALE	1.98 ± 0.17**	0.49 ± 0.03
FEMALE	0.97 ± 0.21	0.48 ± 0.08

Enzyme derived from	Lipid	N-Deethylase	3-Hydroxylase
Female	DLPC	0.98 ± 0.09	0.48 ± 0.05
Female	DLPC/DLPE	0.92 ± 0.10	0.47 ± 0.04
Male	DLPC	1.48 ± 0.17*	0.48 ± 0.01
Male	DLPC/DLPE	1.72 ± 0.02	0.47 ± 0.03
Male	DLPC	1.45 ± 0.04**	0.48 ± 0.03
Male	male	2.31 ± 0.14	0.49 ± 0.01
Female	DLPC	0.90 ± 0.07**	0.48 ± 0.01
Female	male	1.68 ± 0.09	0.47 ± 0.06
Male	male	1.89 ± 0.03	0.50 ± 0.02
Male	female	0.97 ± 0.08**	0.48 ± 0.02
Female	male	1.22 ± 0.03	0.48 ± 0.05
Female	female	0.89 ± 0.08**	0.47 ± 0.04

Results expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± s.d. of at least six values.

**=p < 0.001; * = p < 0.01 compared to respective control.

DLPC=dilauroylphosphatidylcholine; DLPE = dilauroylphosphatidylethanolamine.

3.3. THE EFFECT OF ADDED LIPID COMPOSITION ON LIGNOCAINE METABOLISM BY THE MALE-SPECIFIC ISOZYME OF CYTOCHROME P-450, LIGNOCAINE N-DEETHYLASE.

3.3.1. Purification and Characterization of the Male-Specific Isozyme of Cytochrome P-450, Lignocaine N-deethylase.

A summary of the purification of the cytochrome P-450 isozyme lignocaine N-deethylase, from liver microsomes of untreated male rats is shown in Table 3.4. The procedure developed for the purification is based on the methods used by Waxman (1984). The fraction precipitated by the use of 10-13% polyethyleneglycol 6000 had a specific content of 3.3 nmoles cytochrome P-450/mg protein. However if, in the final precipitating stage, the polyethyleneglycol was increased to 16%, an enhanced specific content of cytochrome P-450 (up to 6 nmoles cytochrome P-450/mg protein) was seen and this represented 89.7% of the original microsomal cytochrome P-450. In contrast to the earlier study of Waxman (1984), cytochrome P-450 eluted from the DE52 column as two distinct peaks (figure 3.9.). Both peaks (F I, F II) were eluted by buffer A, containing 10 mM potassium phosphate pH 7.4, 20% glycerol, 0.5% sodium cholate, 0.2% Emulgen, 0.1 mM EDTA and 20 mM potassium chloride. Fraction FII showed a high contamination with other proteins as analysed by SDS-PAGE (figure 3.10.), whereas fraction F I was enriched in a single band corresponding to cytochrome P-450 (figure 3.10.).

Cytochrome P-450 was recovered from the DE52 column with a specific content of 9 nmoles cytochrome P-450/mg protein representing 10 fold purification at this stage. After dialysis the fraction (F I) was applied to a hydroxylapatite column and eluted in a stepwise manner with 35 mM-120 mM concentration of potassium phosphate buffer as described in Materials and Methods 2.4.5. The cytochrome P-450 eluted with the 120 mM fraction. The final recovery of the isozyme, after the separation and the removal of

the detergent using very small hydroxylapatite columns (1 ml bed volume) as described in Materials and Methods 2.4.6., was 0.32% of the initial microsomal cytochrome P-450. The specific content was 12.8 nmoles cytochrome P-450/mg protein. Figure 3.10. shows an SDS-PAGE of the electrophoretically homogeneous cytochrome P-450 isozyme produced, indicating a molecular weight of approximately 52000. The protein was homogeneous as assayed by SDS-PAGE. The isozyme gave an absorbance maximum for the reduced CO-difference spectrum at 448.8 nm with no traces of cytochrome P-420.

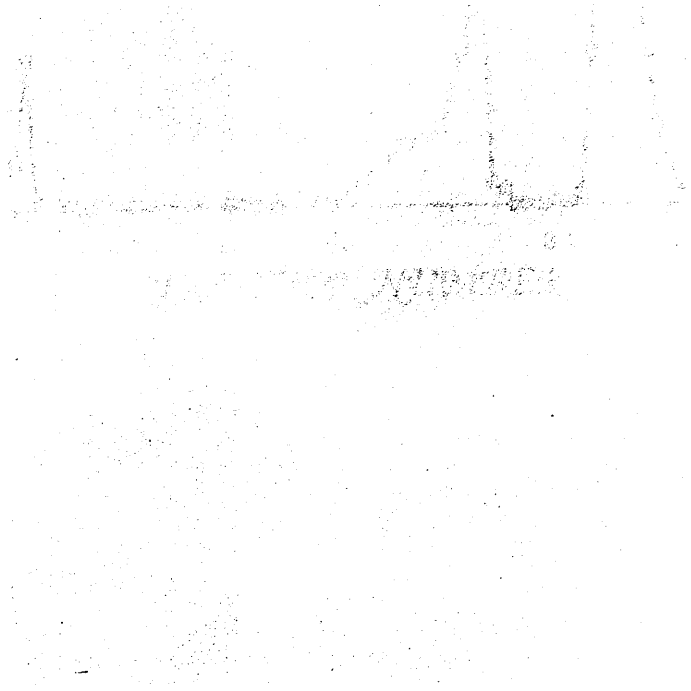


FIGURE 3.9.

DE52 column profiles of partially purified male-specific cytochrome P-450 isozyme from male rat. The P-450 peaks eluted from the DE52 column with 10 mM potassium phosphate buffer pH 7.4 containing 20% glycerol, 0.5% Na cholate, 0.2% Emulgen 913, 0.1 mM EDTA and 20 mM KCl. The fraction size was 8 ml.

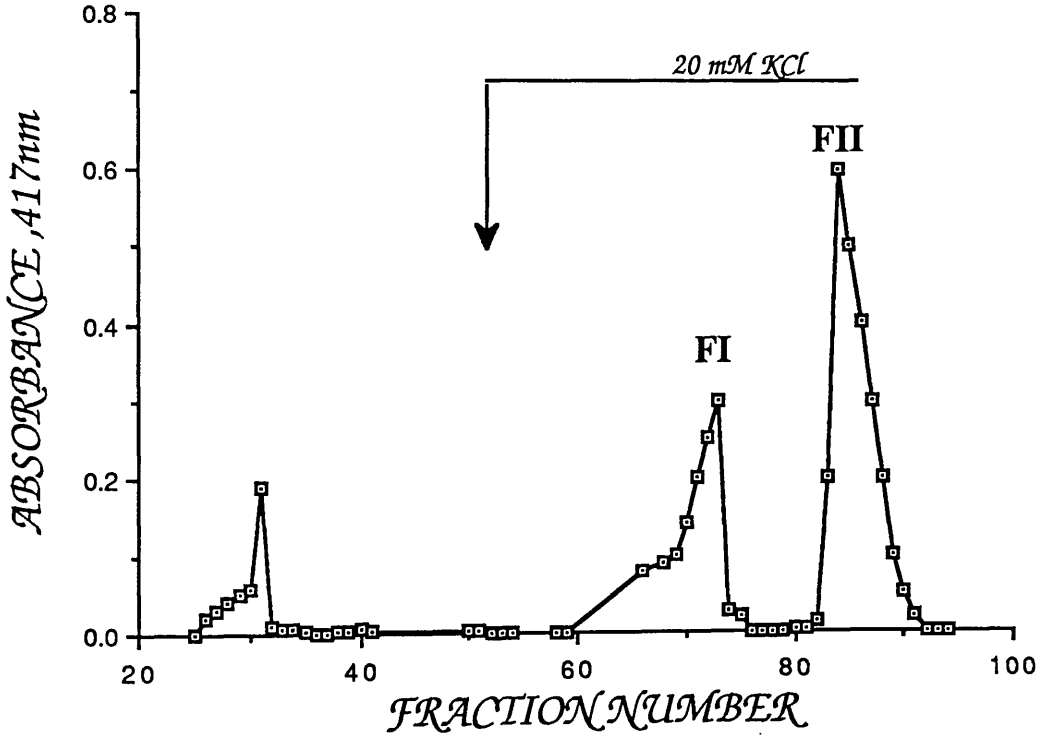


FIGURE 3.10.

SDS-PAGE of purified cytochrome P-450 isozyme from adult untreated male. The samples were analysed as described in Materials and Methods, 2.15., shown are: A, purified NADPH-cytochrome P-450-reductase; B, microsomes from untreated male rats; C, DE52 (FI) column eluate; D, HTP column eluate; E, standards (see Materials and Methods, 2.15.); . Gels were Stained with Coomassie Brilliant Blue.

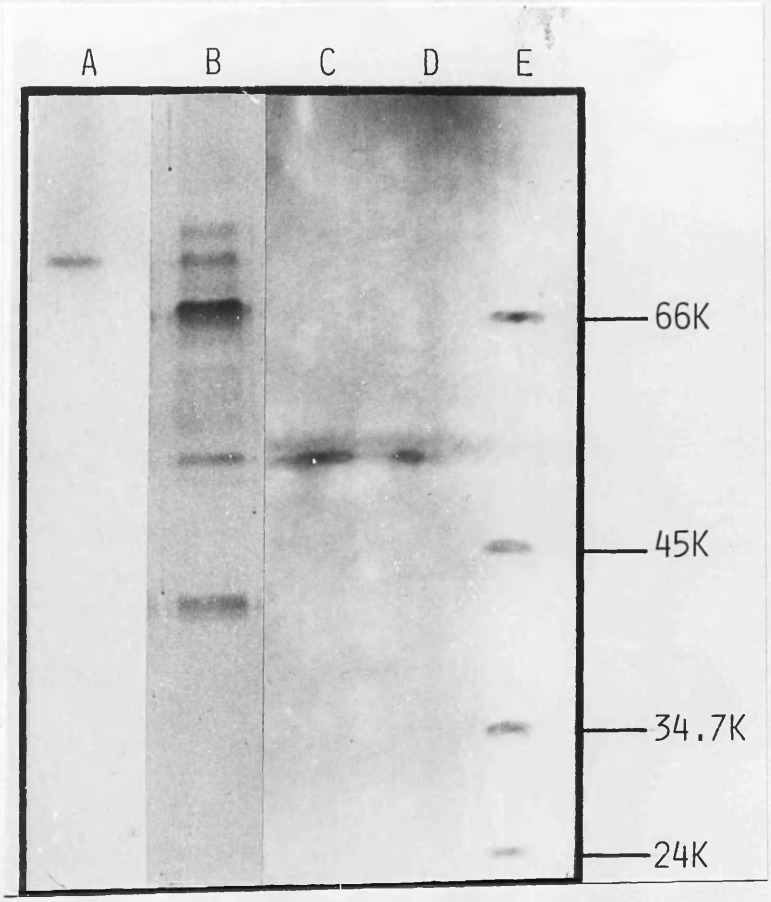


TABLE 3.4.

Purification of the male specific cytochrome P-450 isozyme, lignocaine N-deethylase from untreated male liver microsomes.

Step	Protein mg	P-450 nmoles	Specific Content nmoles/mg protein	Yield %
Microsomes	2951	2810	0.95	100
solubilised microsomes	2184	2520	1.15	89
PEG 6000 fractionation	360	2160	6.0	77
DE52 Column	4.2	40	9.4	1.42
HTP Column (1)	0.85	9.8	11.5	0.35
HTP Column (2)	0.71	9.1	12.8	0.32

3.3.2. Purification of NADPH-Cytochrome P-450-Reductase.

A summary of the purification of NADPH-cytochrome P-450-reductase is given in Table 3.4. The enzyme was purified using a 2'5'-ADP Sepharose 4B column as described in the Materials and Methods 2.5. The use of deoxycholate as the detergent in the buffer permitted subsequent removal of detergent by extensive dialysis. PMSF was used to elute the column and used in the storage buffer to prevent apparent proteolysis which occurred in its absence. The purified preparation of reductase was homogeneous as examined by SDS-PAGE gel electrophoresis and exhibited a subunit molecular weight of approximately 72000 (figure 3.10.). The preparation was detected spectrophotometrically and found to have an A455:A380 ratio of ≥ 1. The enzyme was recovered from the column with a yield of 70% and a catalysing activity of 104 units/mg protein (a unit of reductase is defined as the amount of enzyme which reduced 1μmole of cytochrome C per minute at 20 °C) and was free of other contaminants.

TABLE 3.5.

Purification of NADPH-cytochrome P-450-reductase from male sodium phenobarbital treated rats.

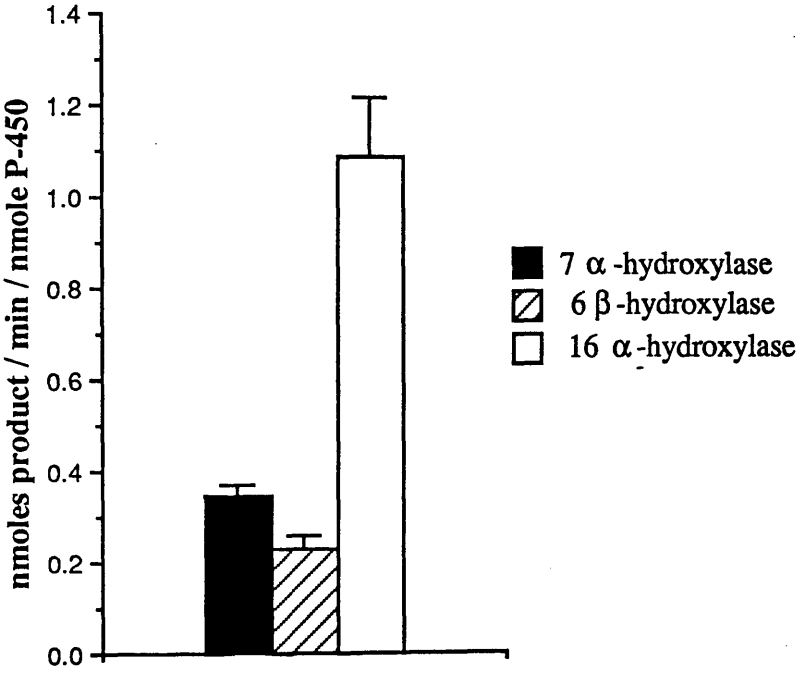
Step	Protein	Specific Content Reductase	Fold Purification	Yield
	mg	unit/mg protein		%
Microsomes	1250	0.064	1	100
Sol. Microsomes	1210	0.17	1.1	115
2'5' ADP Sepharose	2.3	104	80	70

3.3.3. The Metabolism of Lignocaine and Androst-4-ene-3,17-dione by the Purified Cytochrome P-450 Fractions.

Figure 3.11. shows the metabolism of androst-4-ene-3,17-dione in a reconstituted microsomal drug metabolising system containing NADPH cytochrome C (P-450) reductase, cytochrome P-450 (fraction F II) and microsomal lipid. It is seen that the androst-4-ene-3,17-dione was poorly metabolized to the 6β–, 7α– and 16α–hydroxylated derivatives by cytochrome P-450 (fraction F II) and thus fraction FII was both inefficient and nonselective in catalysing the metabolism of androst-4-ene-3,17-dione. Figure 3.12. shows the metabolism of lignocaine in a reconstituted microsomal drug metabolising system containing NADPH cytochrome C (P-450) reductase, cytochrome P-450 isozyme (fraction FII) and microsomal lipid. It is seen that fraction FII was very poor at catalysing the metabolism of lignocaine and produced both the N-deethylated and 3-hydroxylated metabolites. Due to the poor non-selective metabolism shown by fraction FII and its low purity no further purification was carried out and it was eliminated from the study. On the other hand the cytochrome P-450 FI fraction metabolises androst-4-ene-3,17-dione with a high effectiveness and regioselectivity (figure 3.13.). The catalytic activity of cytochrome P-450 (fraction FI) on androst-4-ene-3,17-dione agrees with those for the isozyme 2c of Waxman, (1984) and RLM5 of Morgan et al. (1985). However we have not detected any 6β-, and 7α-hydroxylase activity in the fraction as reported by Morgan et al. (1985). Figure 3.14. shows the activity of this isozyme (fraction FI) on N-deethylation of lignocaine. It is seen that the N-deethylase activity of fraction FI was the same as the unsolubilised microsomes but no 3-hydroxylase activity was detected. Accordingly a further purification on a hydroxylapatite column was carried out for this fraction, which apparently contains the purified lignocaine N-deethylase activity.

FIGURE 3.11.

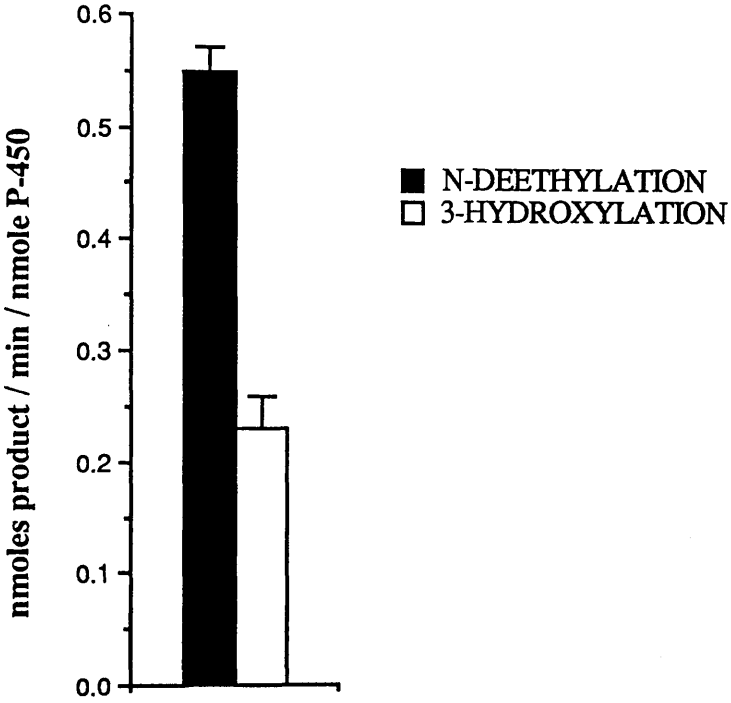
The metabolism of androst-4-ene-3,17-dione in a reconstituted system containing NADPH cytochrome C (P-450) reductase, cytochrome P-450 (fraction FII) and microsomal lipid. All components derived from male rat liver microsomes.



Results are expressed as mean ± s.d. of six values.

FIGURE 3.12.

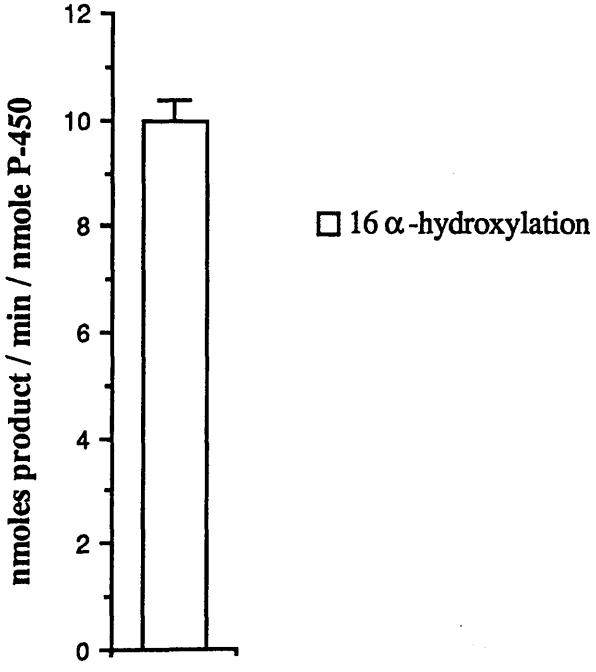
The metabolism of lignocaine in a reconstituted system containing NADPH cytochrome C (P-450) reductase, cytochrome P-450 (fraction FII) and microsomal lipid. All components derived from male rat liver microsomes.



Results are expressed as mean ± s.d. of six values.

FIGURE 3.13.

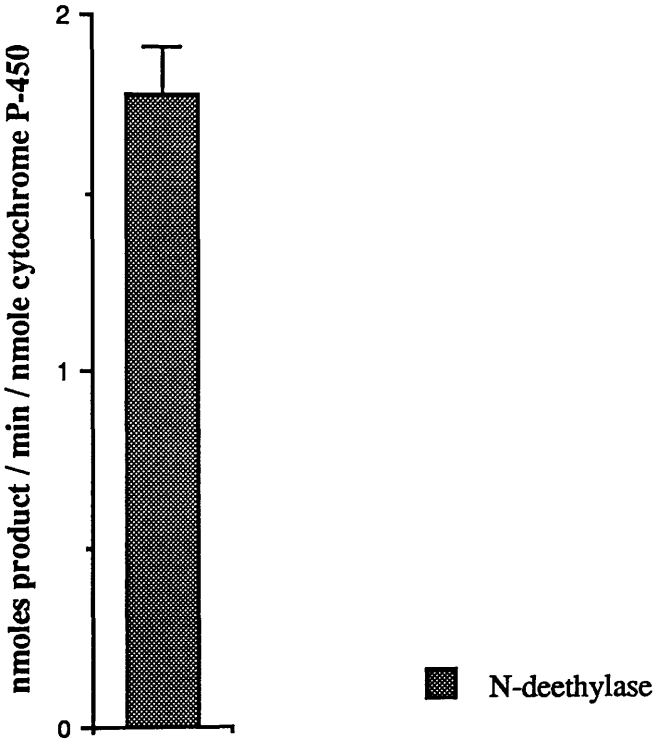
The metabolism of androst-4-ene-3,17-dione in a reconstituted system containing NADPH cytochrome C (P-450) reductase, cytochrome P-450 (fraction FI) and microsomal lipid. All components derived from male rat liver microsomes.



Results are expressed as mean ± s.d. of six values.

FIGURE 3.14.

The metabolism of lignocaine in a reconstituted system containing NADPH cytochrome C (P-450) reductase, cytochrome P-450 (fraction FI) and microsomal lipid. All components derived from male rat liver microsomes.



Results are expressed as mean ± s.d. of six values.

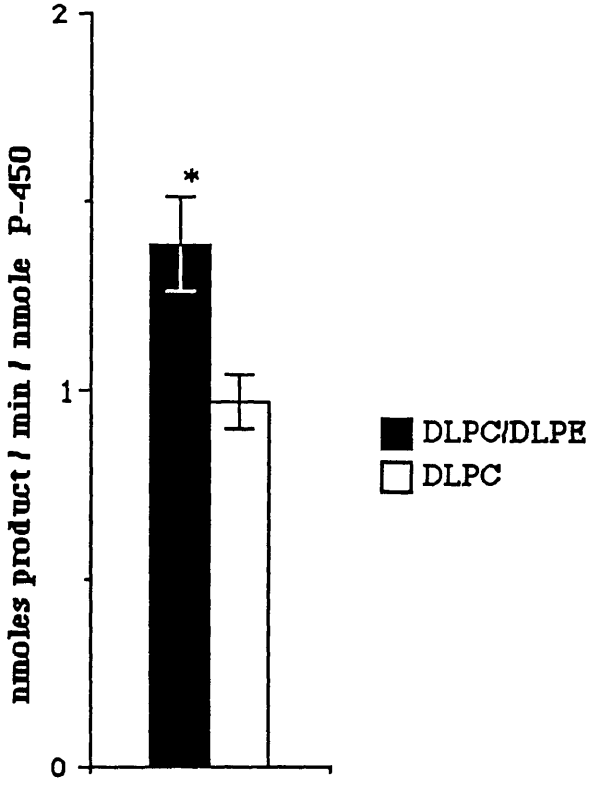
3.3.4. Reconstitution Using the Male-Specific Cytochrome P-450 Isozyme.

It is seen (figure 3.15.) that when the male-specific isozyme of cytochrome P-450 is incorporated into a membrane consisting of dilauroylphosphatidylcholine (DLPC) and dilauroylphosphatidylethanolamine (DLPE) mixture (3:1 w/w) there is an increase in the ability of the enzyme to N-deethylate lignocaine compared to when the isozyme is incorporated into DLPC. These data are consistent with those obtained using delipidated microsomes (figure 3.4.). It is not clear why the ability of the enzyme to N-deethylate lignocaine seen here is slightly less than the ability of the delipidated microsomes, but the 3-hydroxylase activity was not detected. Figure 3.16. shows the effect of incorporating the male-specific isozyme into vesicles prepared from hepatic microsomal lipids derived from male and female rats. The vesicles prepared from female-derived lipids gave a lower N-deethylase activity than those prepared from male-derived lipid. This is also similar to the results using delipidated microsomes (figure 3.7.). Figure 3.17. shows the effect of incorporating the enzymes into vesicles prepared from male- or female-microsomal lipid, DLPC, and DLPC/DLPE mixtures. In all experiments it was seen that male microsomal lipids are more efficient than dilauroylphosphatidylcholine (DLPC) in reconstituting N-deethylase activity and male-derived lipids gave a higher activity than dilauroylphosphatidylethanolamine (DLPE)/DLPC mixtures or female-derived lipids.

The results of the experiments incorporating the male-specific cytochrome P-450 isozyme into vesicles prepared from DLPC; DLPC/DLPE mixtures and total microsomal lipids are summarised in Table 3.6.

FIGURE 3.15.

The effect of incorporating the male-specific cytochrome P-450 isozyme into vesicles prepared from dilauroylphosphatidylcholine (DLPC) and dilauroylphosphatidylethanolamine (DLPE)/DLPC mixture (1:3 w/w) on metabolism of lignocaine.

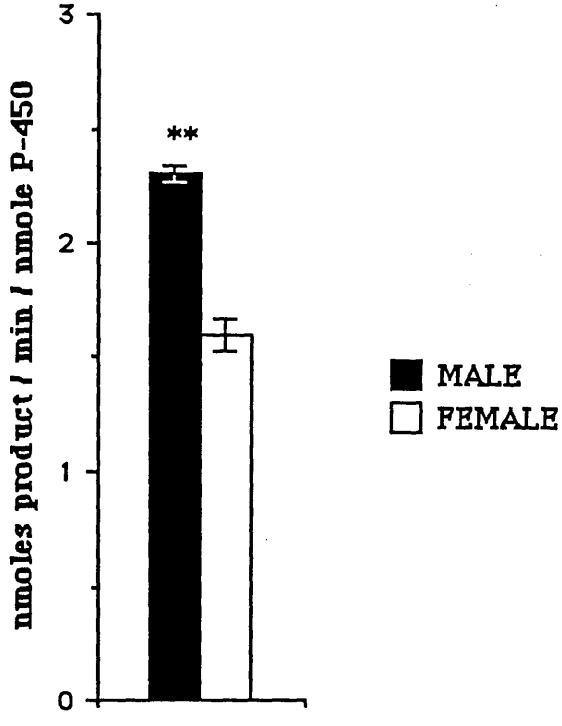


Results are expressed as mean ± s.d. of six values.

* = $p < 0.01$ compared to the DLPC.

FIGURE 3.16.

The effect of incorporating the male-specific cytochrome P-450 into vesicles prepared from microsomal lipids derived from male and female livers on the metabolism of lignocaine.

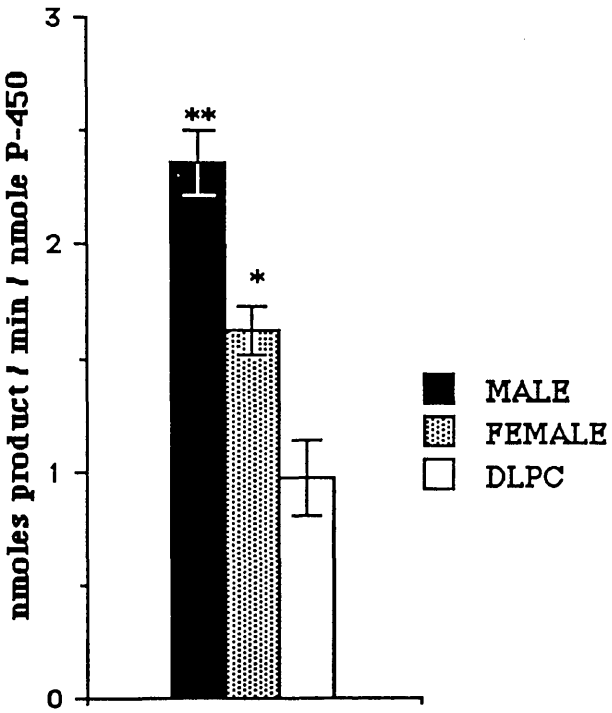


Results expressed mean ± s.d. of six values.

** = $p < 0.001$ compared to the female.

FIGURE 3.17.

The effect of incorporating the male-specific cytochrome P-450 isozyme into vesicles prepared from DLPC or male- and female-derived microsomal lipids.



Results are expressed as mean ± s.d. of six values.

** = $p < 0.001$; * = < 0.01 as compared to DLPC.

Table 3.6.

Summary of the results obtained from incorporating the male-specific cytochrome P-450 into vesicles prepared from DLPC; DLPE/DLPC mixtures and total microsomal lipids.

Lipid	N-deethylase
DLPC/DLPE	1.39 ± 0.17*
DLPC	0.97 ± 0.11

Male	2.31 ± 0.03**
Female	1.61 ± 0.07

Male	2.32 ± 0.15
Female	1.63 ± 0.13*
DLPC	0.99 ± 0.20**

Results are expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± s.d. of at six values.

** =$p < 0.001$; *=$p < 0.01$ compared to respective controls.

3.4. THE EFFECT OF DIFFERENT FRACTIONS OF LIPID EXTRACTED FROM MALE AND FEMALE RATS ON THE METABOLISM OF LIGNOCAINE BY A RECONSTITUTED ENZYME SYSTEM.

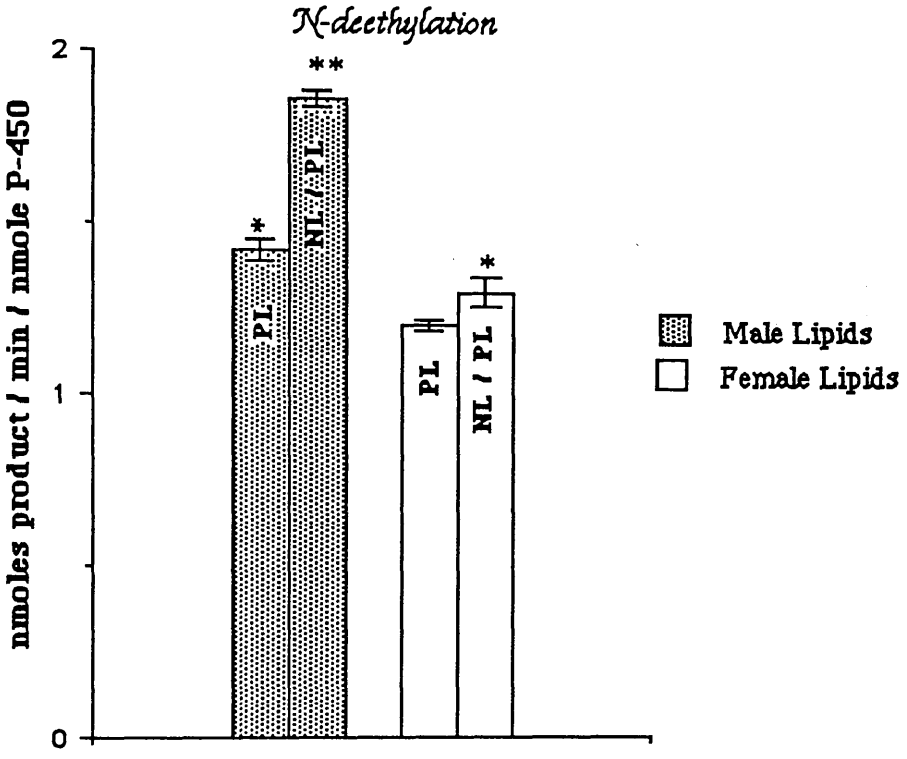
From the previous set of experiments, it seems possible that the sex differences seen in lignocaine-metabolising activity might be related to the microsomal lipid composition. We have demonstrated that changes in the drug metabolising activities can be brought about by alterations in the lipid composition. We decided, therefore, to investigate whether the activity of the microsomal lipid is related to certain fractions of the microsomal lipid extracted from male and female rats. Microsomal lipids were extracted from male and female rat livers as described in Materials and Methods (2.7.) and phospholipid and neutral lipid fractions separated using silica gel column chromatography as described in Materials and Methods (2.8.). The various lipid fractions were used to reconstitute the enzyme activities in delipidated microsomal preparations (section 2.3.6.). Figure 3.18. shows the effects of incorporating male-derived enzymes into vesicles prepared from phospholipid (PL) and neutral lipid (NL)/PL mixture derived from male and female livers. It is seen that the enzymes derived from male rats showed a lower N-deethylating activity in female-derived phospholipid and phospholipid/neutral lipid fraction than in the respective male-derived lipids. For the male-derived lipid fractions, the mixture of PL and NL gave a greater enzyme activity than PL alone but this was not the case for the female-derived lipids. Neutral lipids alone do not form vesicles and thus no result was obtained for NL alone. The 3-hydroxylating activity of both the male- and female-derived enzyme preparation was not significantly affected by any lipid fraction (figure 3.19. and 3.21.). Figure 3.20. shows the effect of incorporating female enzymes into vesicles prepared from phospholipid (PL) and phospholipid/neutral lipid (NL/PL) mixture derived from male and female livers. It is seen that the enzymes derived from the female showed a

decreased N-deethylating activity in female derived lipids when compared to the male derived lipid fractions. From the two experiments It is interesting to note that the N-deethylating activity was higher in the male-derived vesicles than the female derived vesicles whereas the 3-hydroxylating activity was similar in all cases.

A summary of the results obtained using different fractions of lipids extracted from male and female by reconstituted the delipidated microsomal preparation is given in Table 3.7.

FIGURE 3.18.

The effects on lignocaine N-deethylase activity of incorporating male protein into vesicles prepared from male- and female-derived microsomal lipid fractions.



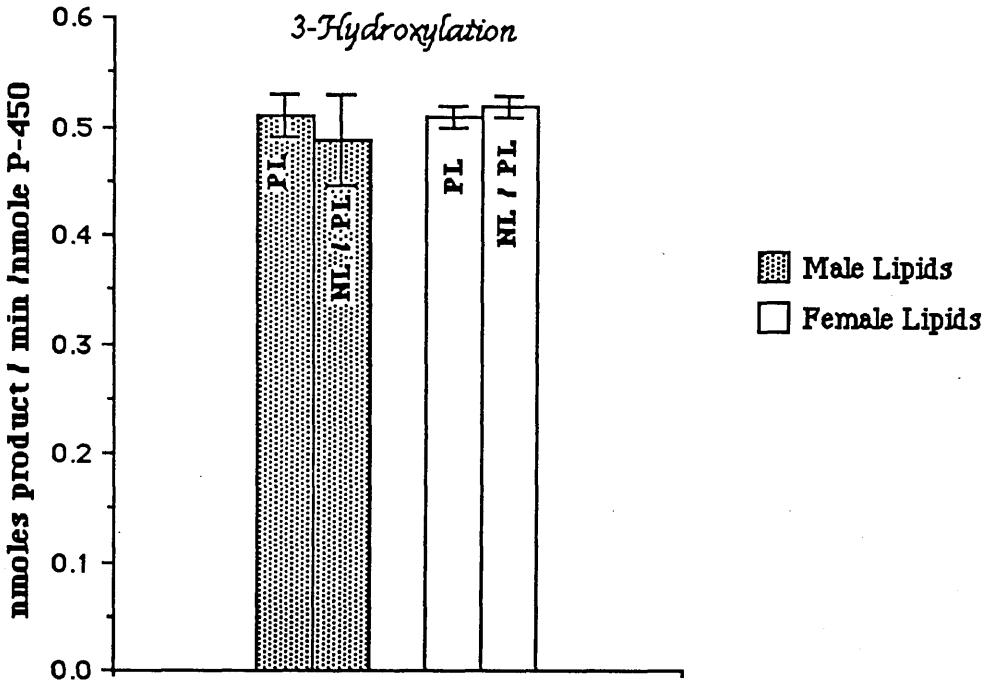
Results are expressed as mean ± s.d. of six values.

** = $p < 0.001$ compared to female (PL/NL) and female (PL); * = $p < 0.05$ compared to female (PL).

PL = phospholipid; NL = neutral lipid

FIGURE 3.19.

The effects on lignocaine 3-hydroxylase activity of incorporating male protein into vesicles prepared from male- and female-derived microsomal lipid fractions.

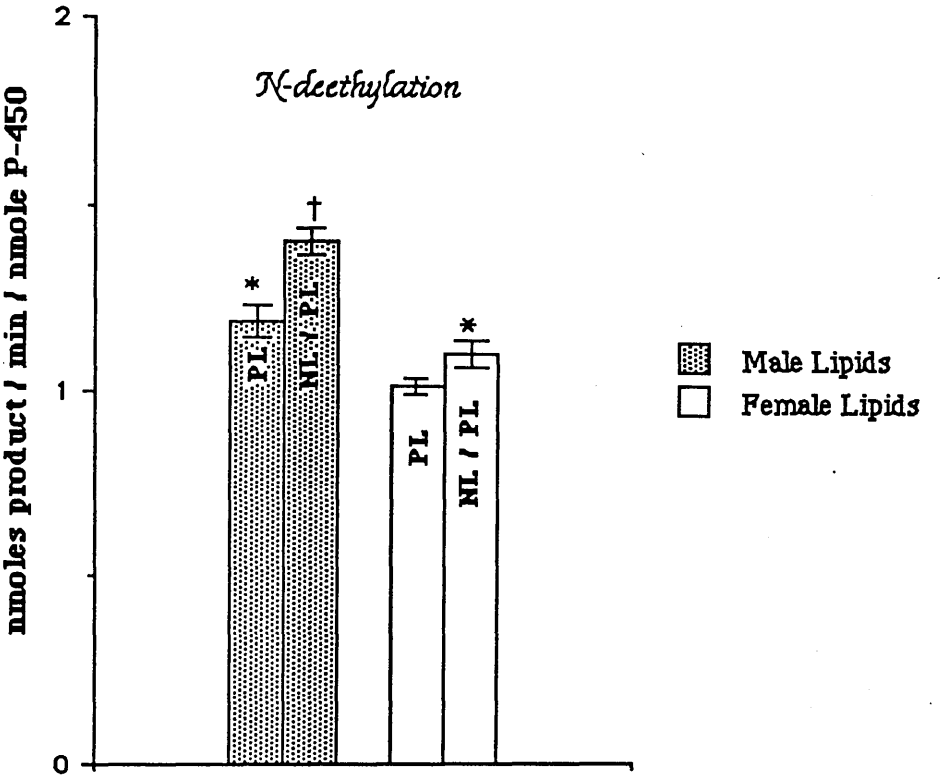


Results are expressed as mean ± s.d. of six values.

PL = phospholipid; NL = neutral lipid

FIGURE 3.20.

The effects on lignocaine N-deethylase activity of incorporating female protein into vesicles prepared from male- and female-derived microsomal lipid fractions.



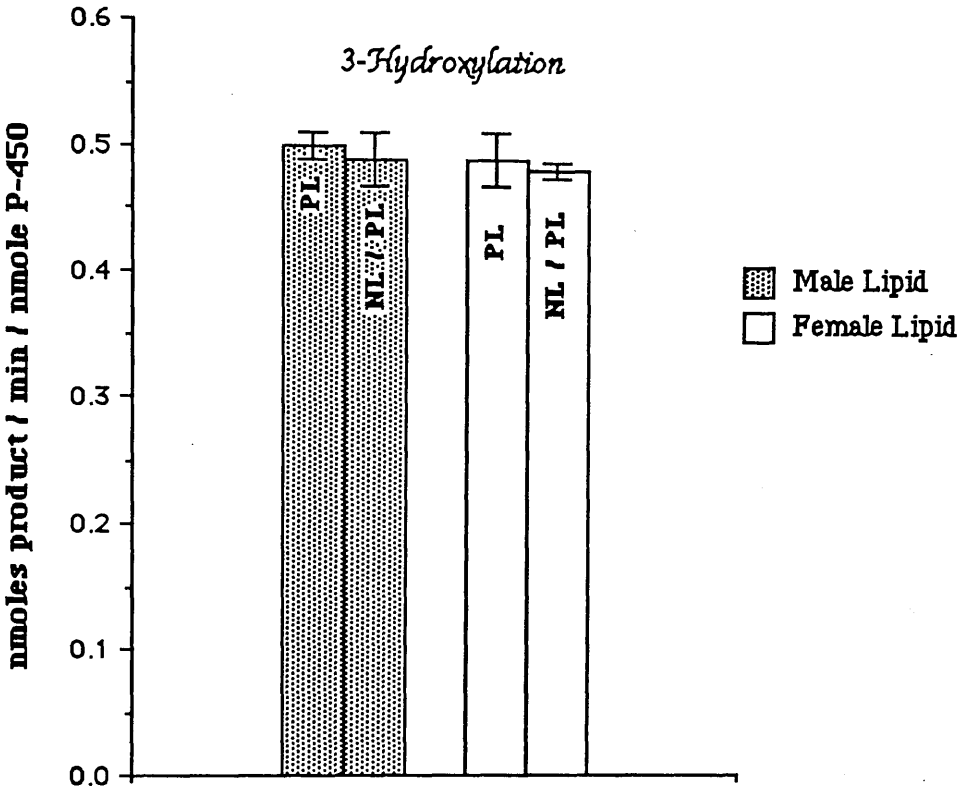
Results are expressed as mean ± s.d. of six values.

†= $p < 0.01$ compared to female (PL/NL) and male (PL); *= $p < 0.05$ compared to female (PL).

PL = phospholipid; NL = neutral lipid.

FIGURE 3.21.

The effects on lignocaine 3-hydroxylase activity of incorporating female protein into vesicles prepared from male- and female-derived microsomal lipid fractions.



Results are expressed as mean ± s.d. of six values.

PL = phospholipid; NL = neutral lipid

TABLE 3.7.

A summary of the results obtained from the incorporation of the delipidated microsomal preparation into vesicles prepared from lipid fractions extracted from male and female rat livers.

Lipid	Enzymes	N-deethylase	3-hydroxylase
Male (PL)	male	1.42 ± 0.02**	0.50 ± 0.03
Male (PL/NL)	male	1.86 ± 0.02	0.48 ± 0.05
Female (PL)	male	1.2 ± 0.01*	0.49 ± 0.02
Female (PL/NL)	male	1.3 ± 0.07	0.51 ± 0.02
Male (PL)	female	1.19 ± 0.04†	0.50 ± 0.02
Male (PL/NL)	female	1.4 ± 0.04	0.48 ± 0.05
Female (PL)	female	1.04 ± 0.02*	0.48 ± 0.06
Female (PL/NL)	female	1.1 ± 0.04	0.47 ± 0.01

Results expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± s.d. of at least six values.

**=$p < 0.001$; † = $p < 0.01$; *= $p < 0.05$ as compared to respective female values.

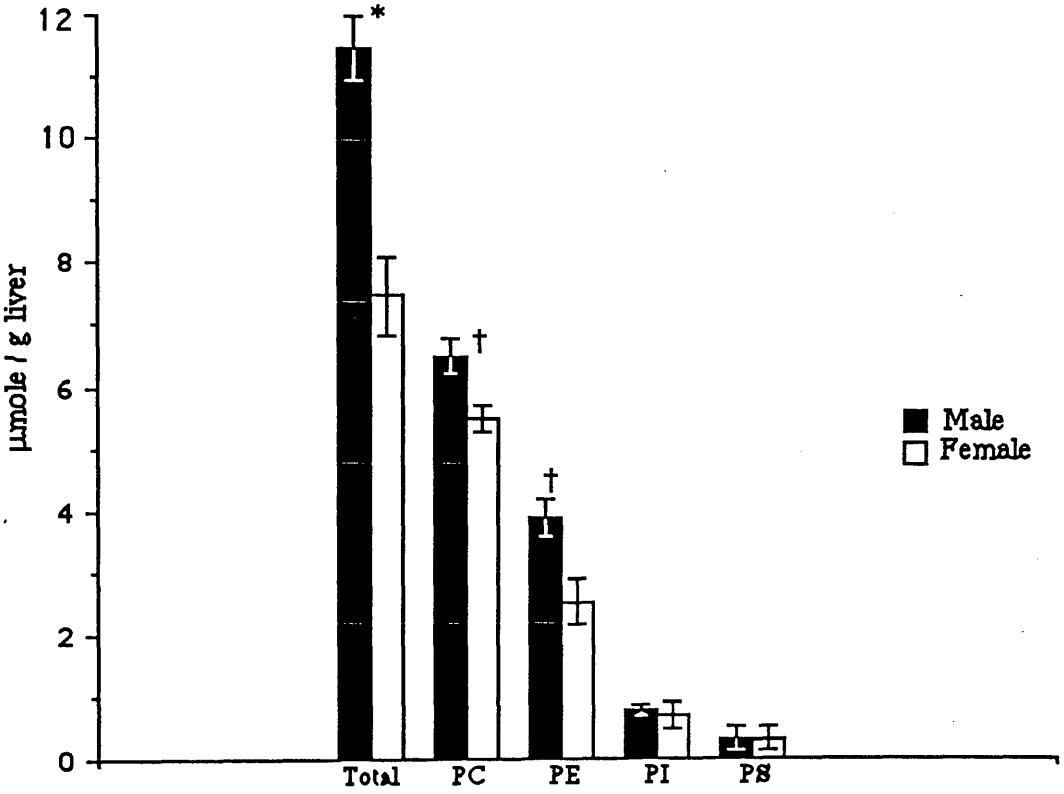
PL = phospholipid; NL = neutral lipid

3.4.1. Resolution of the Microsomal Phospholipid Composition in the Rat Liver.

Figure 3.22. shows the phospholipid composition of liver microsomes in male and female rats. The individual phospholipids from liver microsomes were separated by TLC as described in Materials and Methods (2.9.). The phospholipids were separated into their major individual fractions, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI). Phosphorus content was determined in all fractions as described in Materials and Methods, (2.15.2.). It is seen that the microsomal phospholipid phosphorus was significantly higher in the male than the female animals. Among individual microsomal phospholipids, phosphatidylcholine and phosphatidylethanolamine were significantly higher in the male than the female rats. However phosphatidylinositol and phosphatidylserine were similar in both sexes.

FIGURE 3.22.

Microsomal phospholipid composition in the liver of male and female rats. Fractions analysed were phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylinositol (PI) and phosphatidylserine (PS).



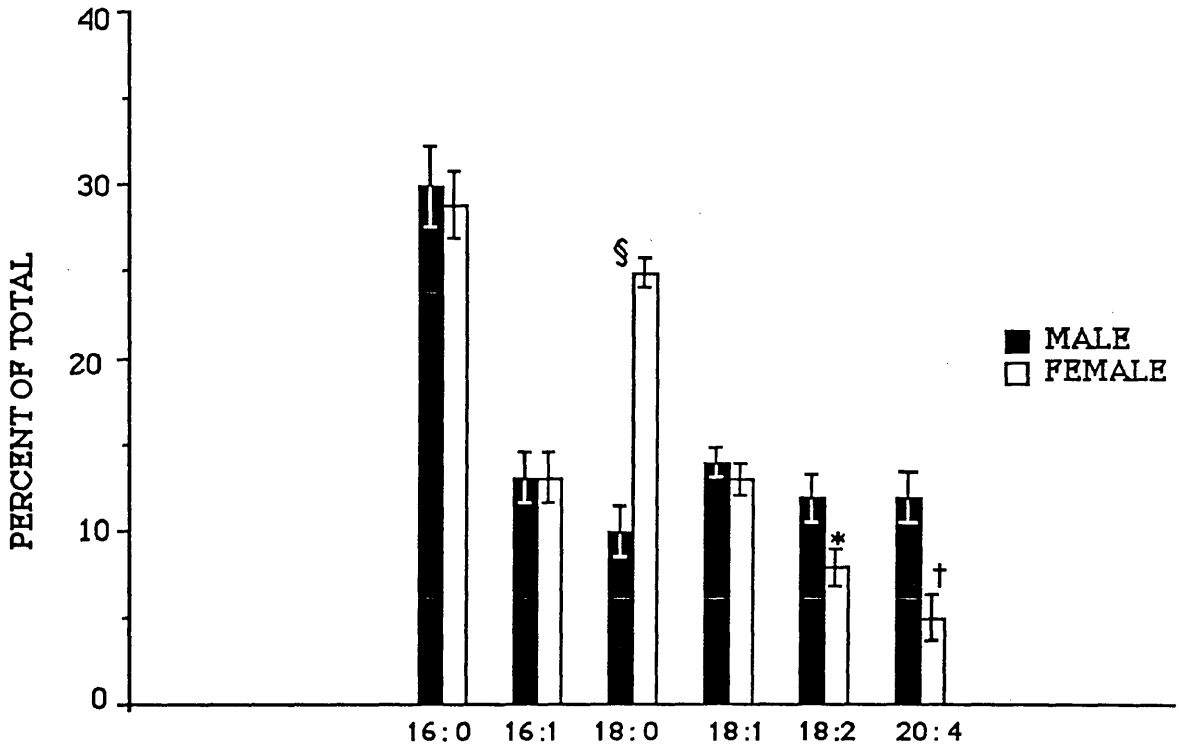
* = $p < 0.001$; † = $p < 0.05$ compared to female value.

3.4.2. Resolution of the Fatty Acid Composition of Microsomal Phospholipid in the Male and Female Rat Liver.

Microsomal phospholipid fatty acid composition assays were carried out as described previously in Materials and Methods (2.16.). Figure 3.23. shows the result of the fatty acid analysis of the microsomal phospholipid extracts. Of the fatty acids seen, there were no sex differences in palmitoleate ($C_{16:1}$) or palmitate ($C_{16:0}$). However the stearate ($C_{18:0}$) was significantly higher in female (25±1%) microsomal phospholipid fraction than the male (10±2%) microsomal phospholipid fraction. The livers of females had (6±2%) half as much arachidonate ($C_{20:4}$) as in the male (12±2%). Also linoleate ($C_{18:2}$)was considerably higher in the male than the female There was little difference in oleate ($C_{18:1}$) content. It should be noted that the total unsaturated fatty acid content of male (48%) livers was considerably higher than that of females (37%).

FIGURE 3.23.

Microsomal phospholipid fatty acid composition in male and female liver. Fatty acid composition was determined by gas chromatography as described in Materials and Methods section (2.18.). Male (n = 5) and female (n = 5).



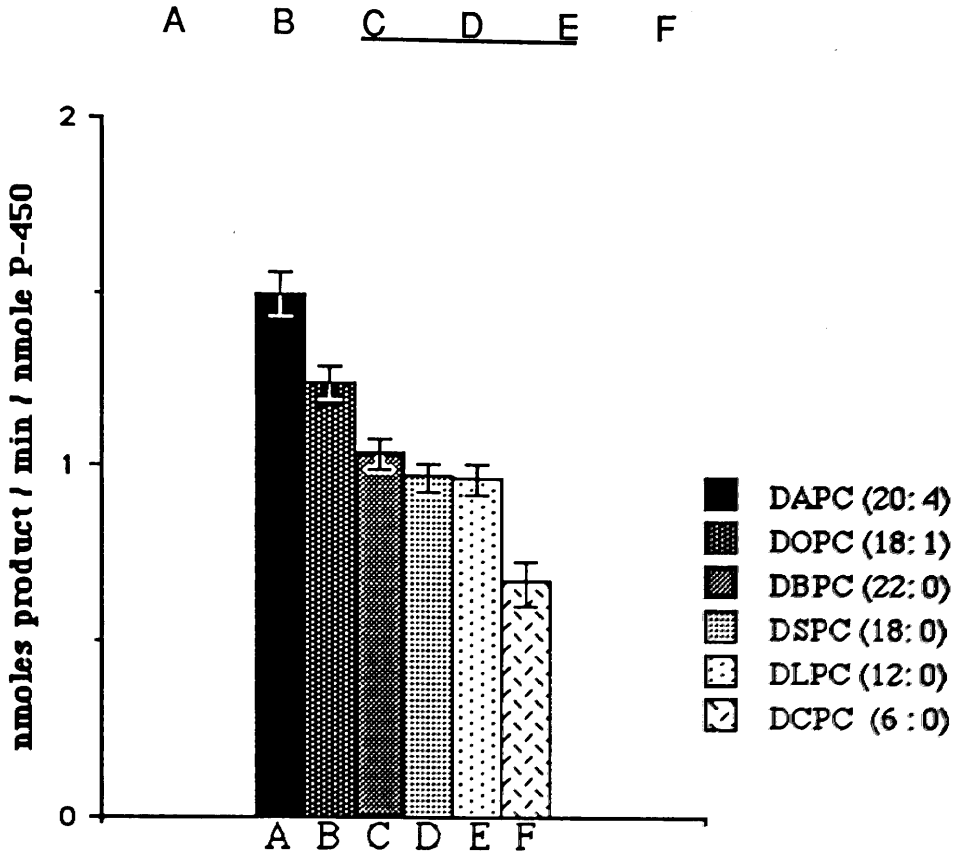
* = $p < 0.05$; § = $p < 0.001$; † = $p < 0.01$ compared to male value.

3.4.3. The Effect of the Fatty Acid Acyl Chain of Phosphatidylcholine on the Metabolism of Lignocaine by a Reconstituted Drug Metabolising Enzyme System.

Delipidated solubilised microsomes prepared from male mature Wistar rats were used in these studies and were incorporated into vesicles prepared from various PCs (phosphatidylcholine) containing fatty acids of differing acyl chain [dicaproylphosphatidylcholine (6:0); dilauroylphosphatidylcholine (12:0); distearoyl-phosphatidylcholine (18:0); dibehenoylphosphatidylcholine (22:0); dioleoylphosphatidylcholine (18:1) and diarachidoylphosphatidylcholine (20:4)]. All of the PCs were found to restore the N-deethylase activity of the cytochrome P-450 to differing extents (Figure 3.24.). Figure 3.24. shows the activity of different PCs (as a function of the fatty acid chain length and degree of unsaturation) in reconstituting male microsomes. Phosphatidylcholines having longer acyl chains were more efficient and the unsaturated PC (18:1) reconstituted the enzyme activity better than did the saturated PC of the same length (18:0). Diarachidoyl (20:4) PC gave the highest enzyme activity. However, figure 3.25. shows that the 3-hydroxylating activity is unaffected by the fatty acid chain length or the number of double bonds except when the chain length is reduced to 6 carbon atoms (DCPC).

FIGURE 3.24.

The effect on activity of lignocaine N-deethylase of incorporating enzymes derived from male rat liver into vesicles prepared from phosphatidylcholines having different acyl chains.



Results as mean ± s.d. of six values. Statistical analysis by Duncan's Multiple Range Test. Those group letters underline are not significantly different ($p > 0.05$).

Abbreviations:

DAPC.....L, α–diarachidoylphosphatidylcholine.

DOPC.....L, α–dioleoylphosphatidylcholine.

DBPC.....L, α–dibehenoylphosphatidylcholine.

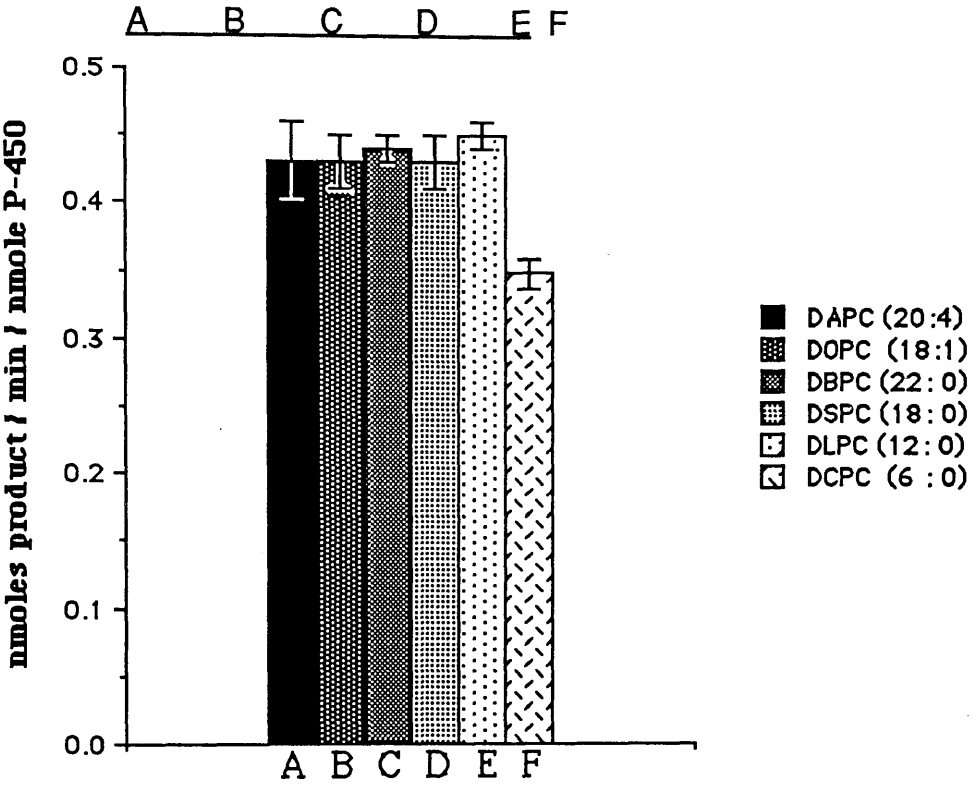
DSPC .....L, α–distearoylphosphatidylcholine.

DLPC .....L, α–dilauroylphosphatidylcholine.

DCPC.....L, α–dicaproylphosphatidylcholine.

FIGURE 3.25.

The effect on activity of lignocaine 3-hydroxylase of incorporating enzymes derived from male rat liver into vesicles prepared from phosphatidylcholines having different acyl chains.



Results are expressed as mean ± s.d. of six values. For statistical analysis see figure 3.24.

For DAPC, DOPC, DBPC, DSPC, DLPC, DCPC abbreviations see figure 3.24.

3.5. THE EFFECT OF DIABETES MELLITUS INDUCED BY STREPTOZOTOCIN ON THE METABOLISM OF LIGNOCAINE BY THE RAT LIVER.

3.5.1. Changes Produced by Streptozotocin-Induced Diabetes in Rats.

The injection of a single dose of 60 mg of streptozotocin/kg body weight to rats produced a constant hyperglycemia after 24 hrs. On the day of treatment the body weights of animals in the three experimental groups did not differ significantly but during the remainder of the experiment the diabetic rats lost weight compared to the controls (Table 3.8.) although they were exposed to the same amount of food and drink. Livers from diabetic rats were significantly lighter than those from controls when expressed as g/100 g body weight (Table 3.8.). The liver weights of the 21-day diabetic rats were lower than the livers of 3-day diabetic rats, but these values were about the same when expressed relative to the body weights (Table 3.8.).

TABLE 3.8.

Changes in body and liver weights in normal and streptozotocin-diabetic rats

Treatment	Body weight before treatment (g)	Body weight after treatment (g)	Liver weight (g)	Liver wt/ body wt x 100	Serum glucose (mM)
Control	340 ± 22	340 ± 22	15.8 ± 1.8	4.8 ± 0.74	5.9 ± 0.7
3-day diabetics	352 ± 25.8	330 ± 20	11.8 ± 2.4	3.5 ± 0.43	19 ± 2.5
21-day diabetics	344 ± 16	277 ± 26	10.1 ± 1.3	3.6 ± 0.8	16.3 ± 3.3

Results expressed as absolute weights or g/ 100 g body weight.

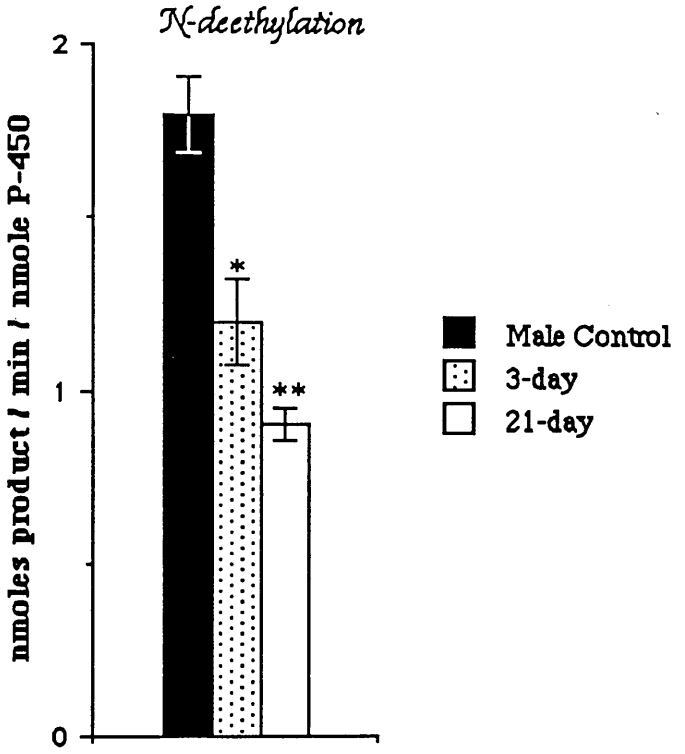
3.5.2. The Effect of Lipid Fractions Extracted from Streptozotocin-Diabetic Rat Liver on the Metabolism of Lignocaine by a Reconstituted Drug Metabolising System.

Figure 3.26. shows the effect of incorporating delipidated microsomal protein derived from male rat liver into vesicles prepared from microsomal lipid derived from control, 3-day and 21-day diabetic rats on the metabolism of lignocaine to its N-deethylated metabolite. It is seen that the microsomal lipid extracted from diabetic rats gave a significantly lower N-deethylase activity as compared to the control. It should be noted that the inhibition was greater in the microsomal lipid derived from 21-day diabetic rats. As is seen in figure 3.27. there is no effect of lipids extracted from diabetic rat livers on the 3-hydroxylation of lignocaine. Figure 3.28. shows the effect of preparing vesicles from the phospholipid fraction derived from control, 3-day and 21-day diabetic rats on the metabolism of lignocaine to its N-deethylated metabolite. It is seen that the phospholipids derived from diabetic rat liver were significantly worse in reconstituting the metabolising system. Again phospholipids derived from 21-day diabetic rats gave the lowest N-deethylase activity. Figure 3.29. shows there is no effect on lignocaine 3-hydroxylase of phospholipids derived from diabetic rats. Figure 3.30. shows the effect of incorporating male-derived, delipidated microsomal enzymes into vesicles prepared from a mixture of neutral lipid and phospholipid derived from control, 3-day and 21-day diabetic rats. It is seen that the N-deethylase activity was altered by the use of the lipid fractions derived from diabetic rats in the same manner as seen for the total microsomal lipid. The addition of the neutral lipid fraction to the phospholipid fraction gave a higher N-deethylase activity in all experiments performed. As with other fractions tested, no effect of the neutral lipid/phospholipid mixture was seen on lignocaine 3-hydroxylase activity (figure 3.31.).

A summary of the effect of lipid fractions from diabetic rat livers on lignocaine metabolism is given in Table 3.9.

FIGURE 3.26.

The effect on lignocaine N-deethylase activity of incorporating male-derived delipidated microsomal protein into vesicles prepared from the microsomal lipids derived from control; 3-day and 21-day diabetic rat livers.

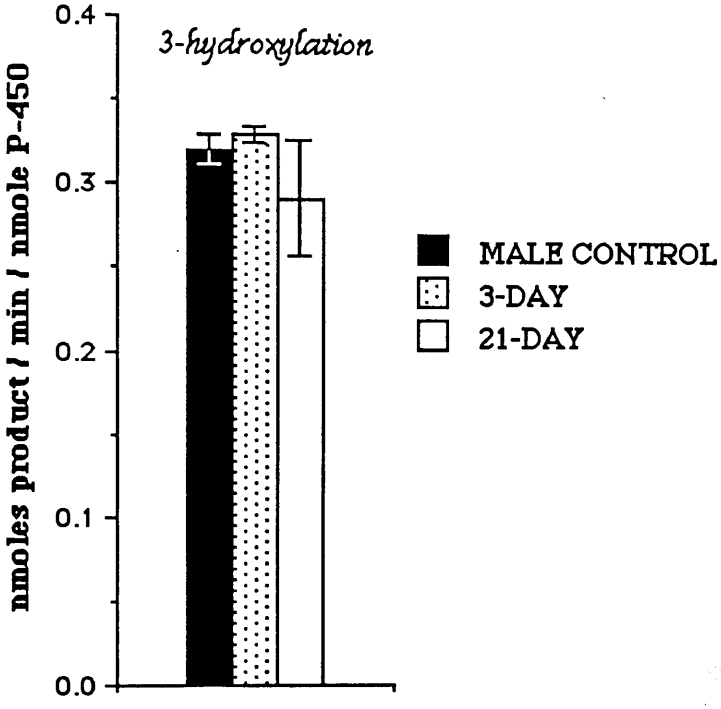


Results are expressed as mean ± s.d. of six values.

**= $p < 0.001$; * = $p < 0.01$ as compared to control.

FIGURE 3.27.

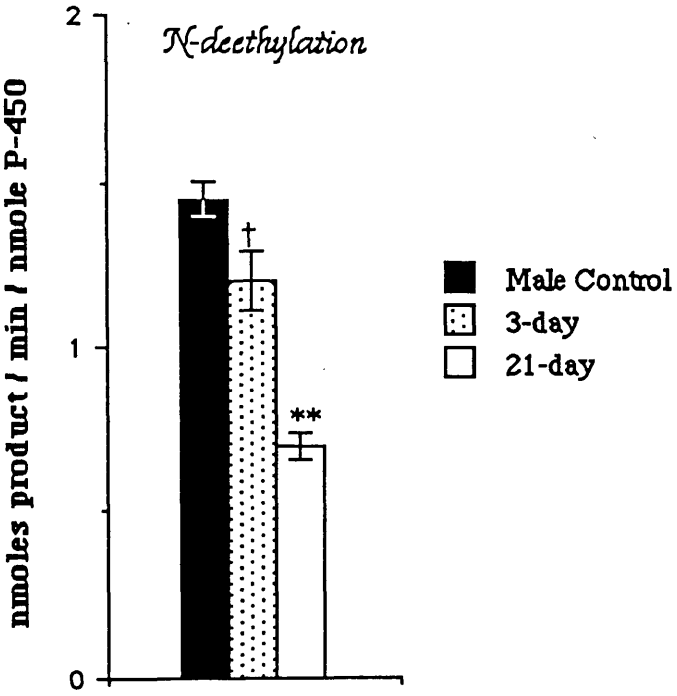
The effect on lignocaine 3-hydroxylase activity of incorporating male-derived delipidated microsomal protein into vesicles prepared from the microsomal lipids derived from control; 3-day and 21-day diabetic rat livers.



Results are expressed as mean ± s.d. of six values.

FIGURE 3.28.

The effect on lignocaine N-deethylase activity of incorporating male-derived delipidated microsomal protein into vesicles prepared from the microsomal phospholipid derived from control; 3-day and 21-day diabetic rat livers.

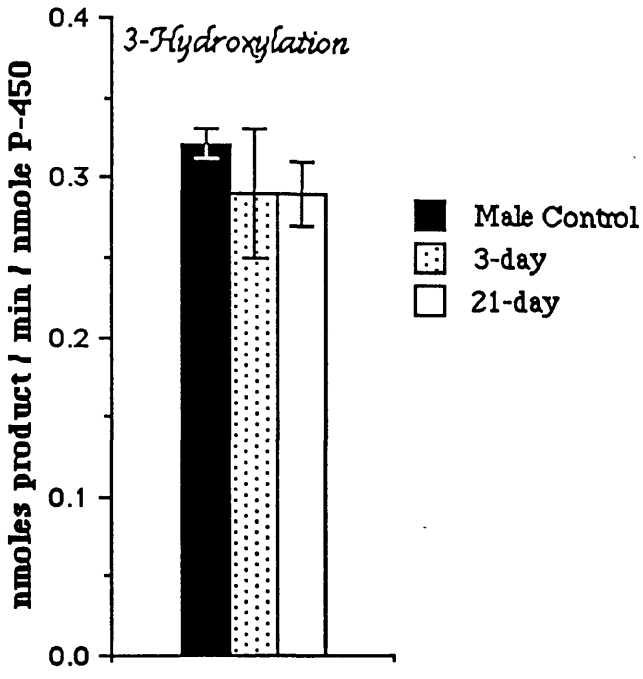


Results are expressed as mean ± s.d. of six values.

†= $p < 0.05$; ** = < 0.001 as compared to control.

FIGURE 3.29.

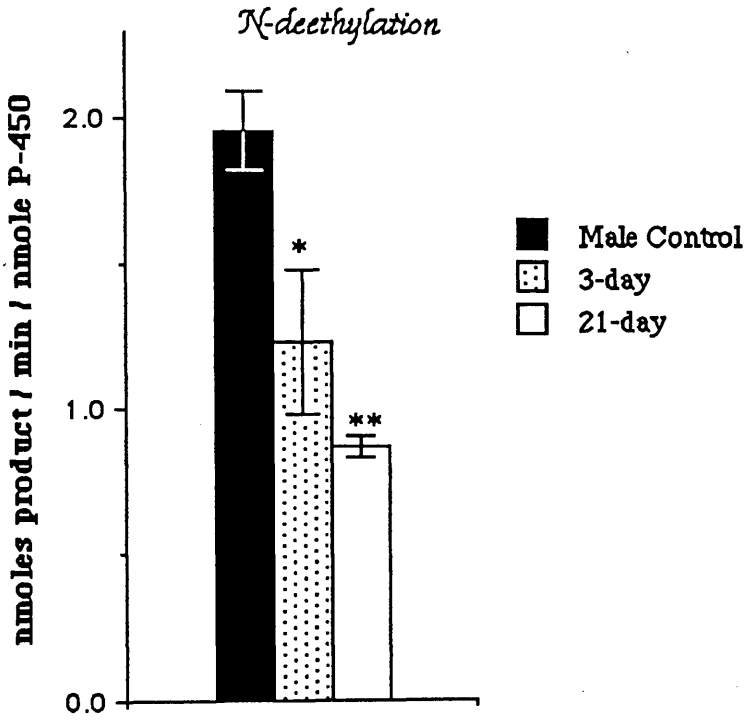
The effect on lignocaine 3-hydroxylase activity of incorporating male-derived delipidated microsomal protein into vesicles prepared from the microsomal phospholipid derived from control; 3-day and 21-day diabetic rat livers.



Results are expressed as mean ± s.d. of six values.

FIGURE 3.30.

The effect on lignocaine N-deethylase activity of incorporating male-derived delipidated microsomal protein into vesicles prepared from the mixture of phospholipid and neutral lipid derived from control; 3-day and 21-day diabetic rat livers.

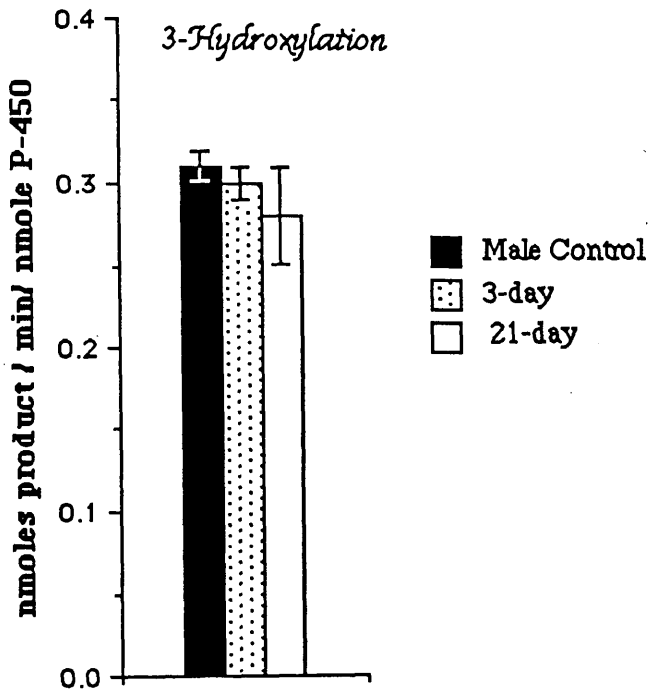


Results are expressed as mean ± s.d. of six values.

*= $p < 0.01$; **= $p < 0.001$ as compared to control.

FIGURE 3.31.

The effect on lignocaine 3-hydroxylase activity of incorporating male-derived delipidated microsomal protein into vesicles prepared from the mixture of phospholipid and neutral lipid derived from control; 3-day and 21-day diabetic rat livers.



Results are expressed as mean ± s.d. of six values.

TABLE 3.9.

The effects of incorporation of male delipidated proteins into vesicles prepared from control; 3-day and 21-day diabetic male microsomal lipid.

Lipid derived from	N-deethylase	3-hydroxylase
Whole lipid		
Control	1.8 ± 0.13	0.31 ± 0.02
3-day diabetic	1.2 ± 0.15*	0.33 ± 0.01
21-day diabetic	0.9 ± 0.06**	0.28 ± 0.05
Phospholipid		
Control	1.45 ± 0.05	0.32 ± 0.01
3-day diabetic	1.2 ± 0.08†	0.29 ± 0.04
21-day diabetic	0.7 ± 0.03**	0.29 ± 0.02
Neutral lipid/Phospholipid		
Control	1.8 ± 0.12	0.31 ± 0.01
3-day diabetic	1.2 ± 0.0.25*	0.30 ± 0.02
21-day diabetic	0.8 ± 0.03**	0.28 ± 0.03

Results are expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± s.d. of six values.

** = $p < 0.001$; * = < 0.01; † = $p < 0.05$ as compared to respective controls.

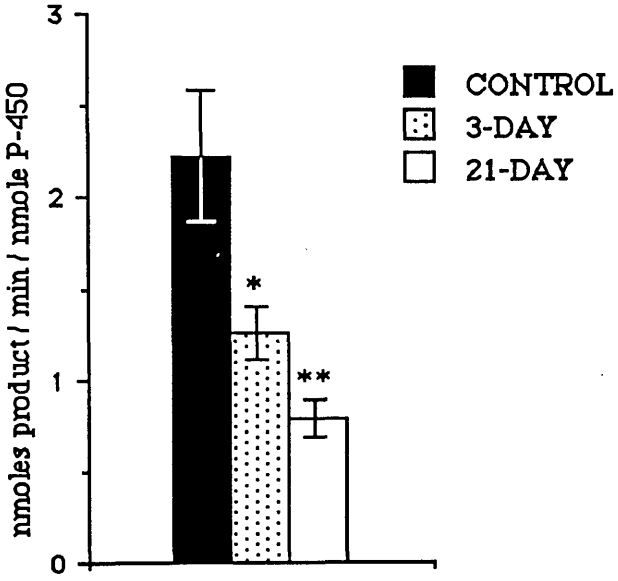
3.5.3. The Effect of Lipid Fractions Extracted from the Streptozotocin-Diabetic Rat on the Metabolism of Lignocaine by a Male-Specific Isozyme of Cytochrome P-450 in a Reconstituted Drug Metabolising System.

Figure 3.32. shows the effects of incorporating the male-specific cytochrome P-450 isozyme into vesicles prepared from control; 3-day and 21-day diabetic male-derived microsomal lipids. It is seen that there is an inhibition of the N-deethylation of lignocaine when the enzyme is incorporated into microsomal lipid derived from 3-day diabetic animals, the inhibition was even greater when the same enzyme was incorporated into 21-day diabetic microsomal lipid. Figure 3.33. shows the effect of incorporating the enzymes into vesicles prepared from hepatic microsomal phospholipid derived from male 3-day and 21-day diabetic animals. The vesicles prepared from the 3-day diabetic-derived phospholipid gave a lower N-deethylase activity than those prepared from the phospholipid derived from the control male. Phospholipid vesicles prepared from 21-day diabetic animals were very poor in reconstituting the N-deethylating activity of the isozyme. Figure 3.34. shows the effects of preparing vesicles from phospholipid/ neutral lipid mixtures from male, 3-day and 21 day diabetic rats. Both diabetic fractions gave lower N-deethylase activity when compared to the control activity. In all cases, the 3-hydroxylase activity of the isozyme could not be detected.

A summary of the effects of lipids derived from diabetic rat livers on the N-deethylation activity of a male-specific isozyme on cytochrome P-450 is given in Table 3.10.

FIGURE 3.32.

The effects of incorporating male-specific cytochrome P-450 isozyme into vesicles prepared from control; 3-day and 21-day diabetic male-derived microsomal lipids (N-deethylase activity).

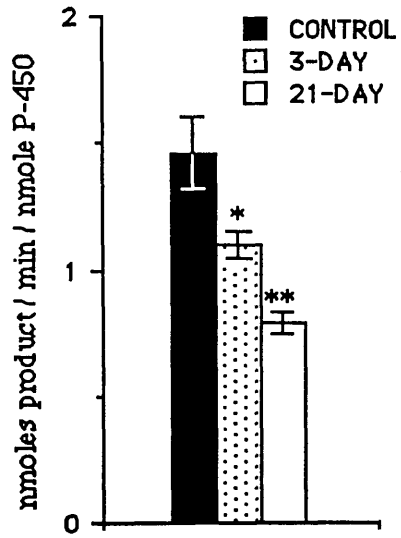


Results are expressed as mean ± s.d. of six values.

* = $p < 0.01$; ** = $P < 0.001$ as compared to control.

FIGURE 3.33.

The effects of incorporating male-specific cytochrome P-450 isozyme into vesicles prepared from phospholipid derived from control; 3- and 21-day diabetic rats.(N-deethylase activity).

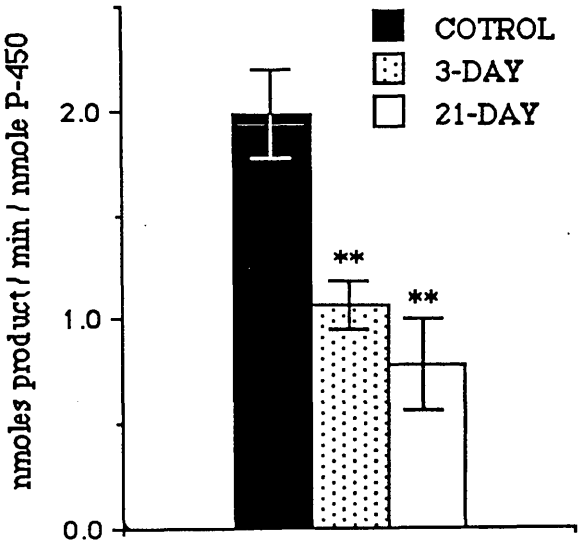


Results are expressed as mean ± s.d. of six values.

* = $p < 0.01$; ** = $P < 0.001$ as compared to control.

FIGURE 3.34.

The effects of incorporating male-specific cytochrome P-450 isozyme into vesicles prepared from mixture of phospholipid and neutral lipid derived from control; 3-day and 21-day diabetic male rats (N-deethylase activity).



Results are expressed as mean ± s.d. of six values.

** = $P < 0.001$ compared to respective control.

TABLE 3.10.

The effect of lipids derived from diabetic rat livers on the N-deethylating activity of a male-specific isozyme of cytochrome P-450.

Lipid derived from	N-deethylation
Whole lipid	
Control	2.3 ± 0.30
3-day diabetic	1.2 ± 0.13*
21-day diabetic	0.9 ± 0.1**
Phospholipid	
Control	1.45 ± 0.1
3-day diabetic	1.2 ± 0.06*
21-day diabetic	0.8 ± 0.04**
Neutral lipid/Phospholipid	
Control	1.8 ± 0.15
3-day diabetic	1.2 ± 0.08**
21-day diabetic	0.8 ± 0.15**

Results are expressed as nmoles product formed/min/nmole cytochrome P-450 and as mean ± s.d. of at least six values.

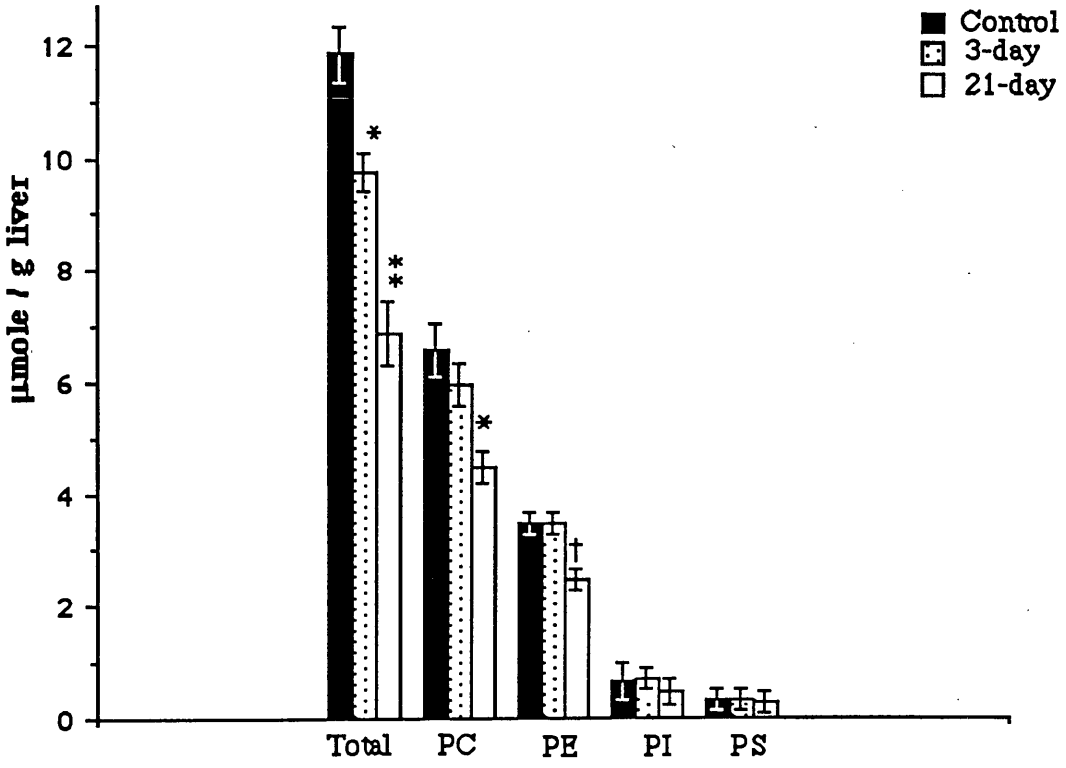
** = $p < 0.001$; * = $p < 0.01$ as compared to respective controls.

3.5.4. Resolution of the Microsomal Phospholipid Composition of Streptozotocin Treated Rats.

Figure 3.35. shows the phospholipid composition of liver microsomes in control and streptozotocin treated rats. The individual phospholipids from liver microsomes were separated by TLC and lipid phosphorus measured as described in the Materials and Methods 2.17. It is seen that the microsomal phospholipid phosphorus was 8% lower in the 3-day diabetic animals compared to controls. The phospholipids were separated into their major individual fractions i.e. phosphatidylcholine (PC), phosphatidylethanolamine (PE) phosphatidylserine (PS), and phosphatidylinositol (PI). Control microsomal phospholipid contained 57% phosphatidylcholine, 25% phosphatidylethanolamine, 10% phosphatidylinositol and 4% phosphatidylserine. There were significant changes in the amount of phosphatidylcholine, phosphatidylethanolamine in the 3-day diabetic rats. It is seen that the microsomal phospholipid phosphorus was decreased 40% in the 21-day diabetic animals compared to controls. There were significant changes in the 21-day diabetic microsomal phospholipid components appearing as a 34% decrease in phosphatidylcholine content, 25% decrease of the phosphatidylethanolamine content but no significant change in the phosphatidylinositol and phosphatidylserine content.

FIGURE 3.35.

Phospholipid composition of liver microsomes in control, 3-day and 21-day diabetic rats.



** = $p < 0.001$;* = $p < 0.01$; † = $p < 0.05$ as compared to respective controls.

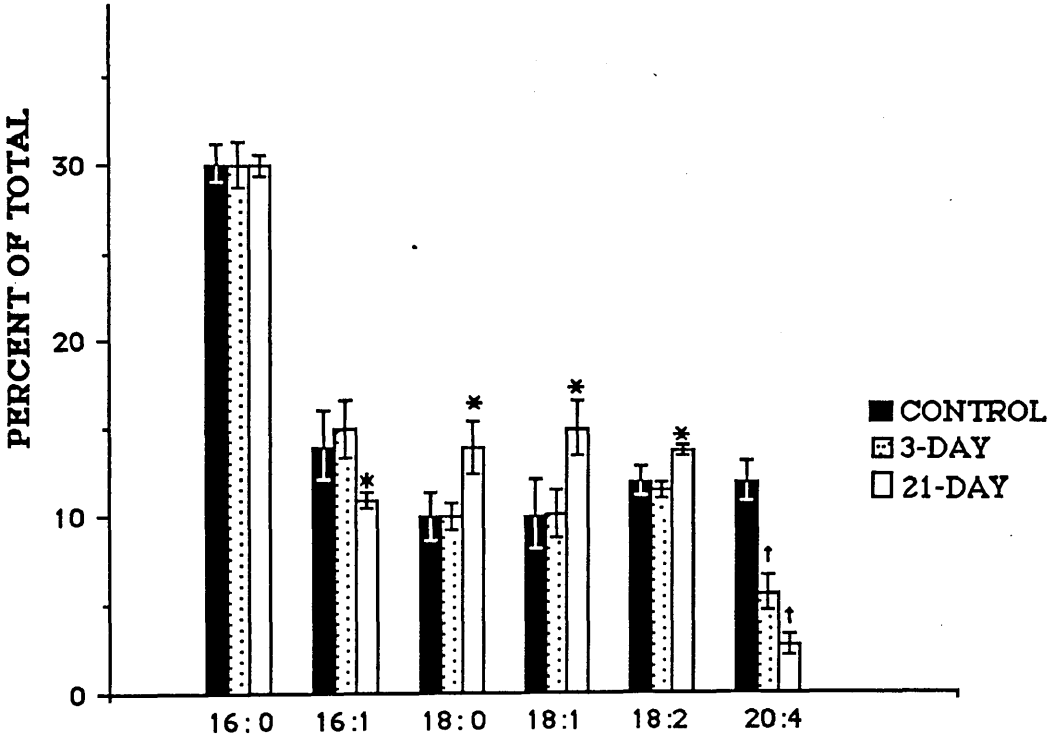
phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS) and phosphatidylinositol (PI).

3.5.5. Fatty Acid Composition of the Microsomal Phospholipid in Streptozotocin Treated Rats.

Figure 3.36. shows the fatty acid composition of microsomal phospholipid in the control; 3-day diabetic and 21-day diabetic rats. Liver microsomal phospholipid fatty acid composition is altered in the diabetic rats with significantly increased proportions of stearate ($C_{18:0}$) and linoleate ($C_{18:2}$) and dramatically decreased proportions of arachidonate ($C_{20:4}$), and palmitoleate ($C_{16:1}$), all of these changes are seen clearly in the 21-day diabetic animals. In 3-day diabetic animals there was no difference seen with respect to, stearate ($C_{18:0}$), palmitoleate ($C_{16:1}$) and oleate ($C_{18:1}$). No differences were seen in the palmitate ($C_{16:0}$) in all three fractions.

FIGURE 3.36.

Microsomal phospholipid fatty acid composition in control; 3-day- and 21-day diabetic rats. Fatty acid composition was determined by gas chromatography as described in Materials and Methods section (2.18.). Control (n = 3); 3-day (n = 3) and 21-day diabetic (n = 3).



* = $p < 0.05$; ** = $p < 0.01$; † = $p < 0.001$ as compared to respective controls.

DISCUSSION

4.1. THE SUBSTRATE.

The use of lignocaine as a substrate was suggested by the continuing research in this department into the hormonal control of drug metabolism, (Skett and Gustafsson 1979; Skett and Young 1982; Skett and Weir 1983; Skett et al., 1984; Skett and Joels 1985; Skett and Barr 1985). Lignocaine is a substrate which has been shown to possess sex differences in metabolism; it exhibits a male-specific N-deethylation and a sexually independent 3-hydroxylation (von Bahr et al., 1977). It, thus, is ideal for the study of the maintenance of sex differences in drug metabolism as there is an inbuilt control activity for direct comparison. To reinforce the use of lignocaine, the microsomal metabolism was determined using microsomes prepared as described in Materials and Methods 2.3.2. before the solubilisation process and with the concentrations of substrate and cofactors to be used in the later experiments. The incubation, separation and identification of metabolites was by the modified method of Skett et al., 1980 (Materials and Methods 2.12.). The results obtained are in agreement with the previously published reports on lignocaine (Skett and Young 1982). These results confirmed the finding that, the rate of N-deethylation was faster in the male microsomes than the female microsomes, whereas the rate of the 3-hydroxylation activity was similar in both sexes.

4.2. THE EFFECT OF ADDED LIPID COMPOSITION ON LIGNOCAINE METABOLISM BY DELIPIDATED MICROSOMES FROM MALE AND FEMALE LIVER.

The methods employed in this study i.e., the delipidation of solubilised microsomal proteins and subsequent reconstitution with lipids of known origin, gave a method for studying in isolation the role of lipids in the control of drug metabolism. After optimal reconstitution (1mg. lipid to 0.1 nmole cytochrome P-450) the added lipids accounted for over 97% of the total lipid fraction and the ratio of other

components was little changed from the original microsomal suspension (Table 3.1 and 3.2). In this study it is seen that, for the assay conditions used, a sex difference in metabolism is seen for the microsomal preparation (figure 3.1.) and that incorporation of microsomal enzymes into a membrane containing DLPC and DLPE in the proportions found in the microsomes (3:1(w/w) DLPC:DLPE), (DePierre and Ernster, 1975) gave an increased N-deethylating activity from the male derived enzymes (figure 3.4.) but no significant change in the female-derived enzymes (figure 3.3.) or the 3-hydroxylase activity measured in the male derived enzyme preparation (figure 3.3. and 3.4.). There is, thus, both a sex-and pathway-dependent effect of DLPE. An effect of incorporation of PE is well documented: Ingelman-Sundberg and Glaumann (1980) working with the rabbit cytochrome P-450 LM2, showed that PE was better than PC in maintaining 7-ethoxycoumarin O-dealkylation. These authors suggested that this effect was due to a charge difference between the different lipids (Haaparanta et al., 1980). It is interesting to note that the only effect seen of DLPE was in the male-derived system with the male-specific enzyme (the N-deethylase) (Skett et al., 1980). This indicates that the male-specific enzymes are more susceptible to alteration of lipid composition. This could be due to a sex-specific enzyme in the male being dependent on membrane charge or membrane fluidity, both of which are altered by the incorporation of DLPE (Ingelman-Sundberg et al., 1981; Strobel et al., 1970). The existence of a sex-specific cytochrome P-450 species in the male has been reported (Waxman 1984; Morgan et al., 1985).

Another possible explanation is an interaction with other proteins contained in the protein fraction of the microsomes. The most likely candidate is cytochrome b_5 which is present in the incubations and can influence metabolism catalysed by cytochrome P-450 (Ingelman-Sundberg and Glaumann, 1980). This possible influence of cytochrome b_5 would need to be followed up using purified preparations of cytochrome P-450 and cytochrome b_5.

In this study it was seen that the microsomal lipid was more effective than DLPC in reconstituting N-deethylase activity in both male- (figure 3.5.) and female-derived (figure 3.6.) enzyme preparations but, as before, 3-hydroxylase activity was unaffected. This agrees with earlier work with partially purified enzymes (J. Barr, Ph.D. Thesis, University of Glasgow, 1985) and with previous findings with rabbit cytochrome P-450 LM2 (Ingelman-Sundberg et al., 1981). Dilauroylphosphatidylcholine (DLPC), was found to stimulate drug metabolism in a reconstituted enzyme system (Miwa and Lu, 1981), although Parke (1981) suggested that this is an oversimplification of the in vivo situation as DLPC does not exist in microsomes and Taniguchi et al. (1984) have suggested that the enzyme insertion and interactions are affected differently when DLPC is used rather than vesicles prepared from natural phospholipid. The male-derived enzymes show a decreased N-deethylase activity in female-derived lipids but 3-hydroxylase activity is not significantly affected (figure 3.7.). This is similar to the effect seen by Barr and Skett (1984) using partially purified cytochrome P-450 and reductase preparations. As in the previous experiment, only the sex-dependent (male-specific) enzyme is affected using the female-derived lipids. It is noted that the sex difference seen for the N-deethylase in the microsomal preparation used (figure 3.7.) is only fully expressed if the correct lipid preparation is used i.e., male enzymes and male lipid mixture compared to the female reconstitution (figure 3.8.).

The microsomal lipids, as prepared, contain a complex mixture of phospholipids as well as triglycerides and cholesterol. Any of these components could account for the differences seen. Indeed, cholesterol has been reported to affect the metabolism of drugs when incorporated into DLPC vesicles (Skett and Cuthill 1986). Sex differences also exist in the proportions and fatty acid composition of the hepatic microsomal phospholipid (Belina et.al. 1975) and these differences could lead to the effects seen.

In summary, the N-deethylation of lignocaine catalysed by delipidated microsomal proteins from male and female rat liver is greater when reconstituted in microsomal lipid than in DLPC. The 3-hydroxylation of lignocaine is unaffected by this treatment. The above effect is mimicked by incorporation of DLPE into the DLPC vesicles with male- but not the female-derived enzymes. Microsomal lipids derived from the male were more effective than female-derived lipids in reconstituting enzyme activities with both male-and female-derived enzymes. In addition, the sex-dependence of the metabolism of lignocaine was maintained in the reconstituted system, indicating that this is a suitable system for investigating the role of lipids in maintaining sex-specific drug metabolism.

4.3. PURIFICATION AND CHARACTERIZATION OF THE MALE-SPECIFIC ISOZYME OF CYTOCHROME P-450, LIGNOCAINE N-DEETHYLASE.

Cytochrome P-450 in liver microsomes plays a major role in the oxidation of a wide variety of exogenous and endogenous compounds. The existence of two sex-dependent forms of cytochrome P-450 were first demonstrated by Kato and Kamataki (1982) and Kamataki et al. (1982) and they have been designated cytochrome P-450-male and cytochrome P-450-female. The male form was shown to metabolise 7-propoxycoumarin, aniline, aminopyrine and benzphetamine better than the female. The cytochrome P-450-male did not show any 6β–hydroxylase activity. Sex-related differences of cytochrome P-450-dependent oxidations of many drugs and steroid hormones in rat liver microsomes have been well documented (Kato and Onoda, 1970; Kato, 1974; Colby, 1980; Gustafsson et al., 1980) (see Introduction 1.3.) and several forms of cytochrome P-450 have been shown to participate in the hydroxylation of testosterone with different regio- and stereoselectivities (Ryan et al., 1984a; ; Cheng and Schenkman 1983; Waxman 1988).

In this study the purification of the male-specific cytochrome P-450 isozyme was performed by the technique suggested by Waxman and Walsh (1983) (Materials and Methods 2.4.5.). The percentage recovery of the cytochrome P-450 shown in Table 3.4. is in full agreement with other workers who suggest that the content of this isozyme in the microsomes is less than 1% (Morgan et al., 1985; Waxman 1984). The isozyme of cytochrome P-450 which has been isolated from male rat liver was homogeneous (at least 95% in a single protein band) as assayed by SDS-PAGE. The isozyme has a minimum molecular weight of approximately 52000 and a specific content of 12.8 nmoles cytochrome P-450/mg protein. The protein was found to have a specific activity for the 16 α–hydroxylation of 4-androstene-3,17-dione of 10 nmoles/min/nmole cytochrome P-450. These data suggest that the isozyme under study is the male specific

isozyme 2c of Waxman (1984) and RLM5 of Morgan et al. (1985). The results obtained by Waxman (1984) suggest that this isozyme can perform 6β–hydroxylation and 7α–hydroxylation which was not detected by us. A possible explanation for these differences lies in the difference in the reconstitution methods or the change of lipids from DLPC to total natural microsomal lipids. A change in the metabolic profile of steroid (testosterone) hydroxylation has been reported using partially purified cytochrome P-450, where DLPC was replaced by microsomal lipids but 6β–hydroxylation was unaffected (Shiverick and Neims 1979). Ryan et al. (1984b) suggested that it would seem that the metabolism of steroids by the cytochrome P-450 linked hydroxylation system is regiospecific, but this may also be regulated by the membrane used in reconstitution studies and not only by the species of cytochrome P-450 present. The isozyme was found to have the highest lignocaine N-deethylase activity. We, thus, consider that this enzyme is responsible for the N-deethylation of lignocaine seen in microsomes. However, it cannot be considered as the P-450 species involved in the 3-hydroxylation of lignocaine since this is not produced by this isozyme in these reconstitution experiments.

4.3.1. The Effect of Vesicle Lipid Composition on the Metabolism of Lignocaine by a Male-Specific Isozyme of Cytochrome P-450, Lignocaine N-Deethylase.

Kamataki et al. (1980) suggested that the terminal oxidase, cytochrome P-450, would be responsible for the expression of all sex differences. The data presented in this section does not agree with those workers (for review, see Lu and West, 1980; Skett, 1987) who attribute any sex difference exhibited in microsomal metabolism only to different forms of cytochrome P-450. Sex differences in cytochrome P-450 do exist (Waxman 1988; Morgan et al., 1985; McIntosh et al., 1980; Kato et al., 1986; Yamazoe et al., 1986a) but there are also differences in the lipid composition of the membrane (Belina et al., 1975) which are thought to be correlated to changes in drug metabolism (Cooper and Feuer 1972 a, b; Feuer, 1978). In an attempt to ascertain the respective roles of the cytochrome P-450 and lipid in control of sex differences in drug metabolism, we have isolated and reconstituted the various components into a functional enzyme system. As can be seen from figure 3.15., in the assay conditions used, an increase in the metabolism of lignocaine to its N-deethylated derivative is seen when the male-specific isozyme of cytochrome P-450 is incorporated into a membrane containing a mixture of DLPC and DLPE in the proportions found in the microsomes (3:1(w/w) DLPC:DLPE) (DePierre and Ernster 1975) rather than DLPC alone. An effect of incorporation of PE on cytochrome P-450-dependent metabolism is well documented as outlined earlier (Section 4.8.). This is similar to the effect of DLPE seen in our earlier work (section 4.2.) using delipidated microsomes. This indicates that the male-specific isozyme is susceptible to alteration of activity by lipid composition. This could be due to the enzyme (Morgan et al., 1985; Waxman 1984) being dependent on membrane charge or membrane fluidity, both of which are altered by the incorporation of DLPE (Strobel et al., 1970; Ingelman-Sundberg et al., 1980) or indeed it might be due to an alteration in the protein-protein interaction, (Taniguchi et al., 1984). These results would also

suggest that cytochrome b_5 is not needed for the effect of DLPE to be seen and thus, the lipid must interact with the cytochrome P-450 and/or the NADPH cytochrome P-450 reductase only.

In figure 3.16. the effect of incorporation of isozyme into microsomal lipids derived from male and female animals is shown. The female derived lipids gave a decrease in N-deethylase activity. This is similar to the effect seen by Barr and Skett (1984) using partially purified cytochrome P-450 and reductase preparation and our earlier work (section 3.2.) using delipidated microsomes. It is noted that the sex difference seen for the N-deethylase in the microsomal preparations is only fully expressed if the correct lipid preparation is used i.e., male-derived enzymes and male lipid mixture. In all experiments it was seen that the microsomal lipid was more effective than DLPC in reconstituting N-deethylase activity and male-derived lipids gave a higher enzyme activity than the DLPC/DLPE mixture (figure 3.17.). This agrees with earlier work with partially purified enzymes (Barr, 1985) and with previous findings with rabbit cytochrome P-450 LM2 (Ingelman-Sundberg et al., 1981). As discussed earlier (Section 4.8.) the microsomal lipids, as prepared, contain a complex mixture of phospholipid as well as triglycerides and cholesterol and any of these components could account for the differences seen. Sex differences exist in the proportions and fatty acid composition of the hepatic microsomal phospholipids (Belina, 1975; Rowe and Wills 1976; Lambert and Wills 1977a, b) and these differences could lead to the effects seen.

In summary, the N-deethylation of lignocaine catalysed by a purified male-specific cytochrome P-450 from rat liver is greater when reconstituted in microsomal lipid than in DLPC. Microsomal lipids derived from the male were more effective than female-derived lipids in reconstituting enzyme activity.

4.4. THE EFFECT OF DIFFERENT FRACTIONS OF LIPID EXTRACTED FROM MALE AND FEMALE RATS ON THE METABOLISM OF LIGNOCAINE BY A RECONSTITUTED ENZYME SYSTEM.

In the previous series of experiments it had been established that the male-specific lignocaine N-deethylase activity of delipidated microsomes and a purified cytochrome P-450 could be modulated by total microsomal lipids, with the male-derived lipids giving a higher activity. Thus, it seemed likely that part, if not all, of the sex differences in lignocaine N-deethylase was mediated by sex-dependent microsomal lipids. As explained in the Introduction (1.8) the total microsomal lipid is likely to contain, aside from phospholipid, differing quantities of cholesterol, fatty acids and triglycerides and this mixture is difficult to create artificially. The lipid membrane of the endoplasmic reticulum is known to be sexually differentiated (Belina et al., 1975) and the control of sex differences in metabolism seen previously may reside in any of the lipid fractions or in the fatty acid composition of the phospholipid. In order to investigate which fraction of the microsomal lipid is responsible for the activity seen in the total microsomal lipid, total lipids were extracted from the microsomes and fractionated into the two main classes, phospholipid and neutral lipids. Working on the assumption that a single fraction or a mixture of the different lipid fractions may be responsible for the sex differences seen in the rat liver microsomes, it was thought that delipidation of solubilised microsomal proteins and subsequent reconstitution with different fractions of lipids of known origin, would be a good method for studying, in isolation, the role of different lipid fractions in the control of sex differences in drug metabolism. Figure 3.18. shows the effects of incorporating male-derived enzyme into vesicles prepared from phospholipid (PL) and a neutral lipid (NL)/PL mixture derived from male and female livers. It is seen that the enzymes derived from male rats showed a lower N-deethylating activity in female-derived phospholipid and phospholipid/neutral

lipid fraction than in the respective male-derived lipids. For the male-derived lipid fractions, the mixture of PL and NL gave a greater N-deethylating activity than PL alone but this was not the case for the female-derived lipids. Neutral lipids alone do not form vesicles and thus no result was obtained for NL alone. The 3-hydroxylating activity of both the male- and female-derived enzyme preparation was not significantly affected by any lipid fraction (figure 3.19. and 3.21.). The effect of phospholipid on cytochrome P-450-dependent oxidation is well documented (Lu and Levin, 1974; Lambeth, 1985) and it was thought that the requirement for phospholipid was to provide a generalised hydrophobic environment (it can be replaced in part by detergent). Haaparanta et al. (1980) noted that, for the cytochrome P-450 LM2-catalysed O-deethylation of 7-ethoxycoumarin and oxygenation of benzopyrene, the existence of negatively charged phospholipid caused significant stimulation of activity in a reconstituted system. It is also thought that phospholipid composition or concentration could affect the functioning of the microsomal enzyme system by a variety of mechanisms (i.e. change in fatty acid side chain; unsaturation of the phospholipid fatty acyl side chain and change in the phospholipid/cholesterol ratio).(Wills 1980; Dhami et al., 1980). Figure 3.20. shows the effect of incorporating female enzymes into vesicles prepared from phospholipid (PL) and phospholipid/neutral lipid (NL/PL) mixture derived from male and female livers. It is seen that the enzymes derived from the female showed a decreased N-deethylating activity in female derived lipids when compared to the male derived lipid fractions. From the two experiments, it is interesting to note that the N-deethylating activity was higher in the male-derived vesicles than the female derived vesicles for both male- and female-derived enzymes. One possible reason for this enhancement is the presence of more cholesterol in the male-derived neutral lipid fraction. Indeed, cholesterol has been reported to affect the metabolism of drugs when incorporated into DLPC vesicles (Skett and Cuthill 1986) (see introduction 1.10.4.).

4.4.1. The Microsomal Phospholipid Composition in Male and Female Rat Liver.

Figure 3.22. shows the phospholipid composition of liver microsomes in male and female rats. The phospholipids were separated into their major individual fractions, phosphatidylcholine (PC), phosphatidylethanolamine (PE), phosphatidylserine (PS), and phosphatidylinositol (PI). It is seen that the total microsomal phospholipid phosphorus was significantly higher in the male than the female animals. This is similar to the finding of Belina et al. (1975). Among individual microsomal phospholipids, phosphatidylcholine was significantly higher in the male than the female rats (again in agreement with the report of Belina et al. (1975)). Phosphatidylethanolamine was significantly higher in the male than the female rats (not reported by Belina et al. (1975)) whereas phosphatidylinositol and phosphatidylserine were similar in both sexes. The higher concentration of PE in the male lipids fits in well with the fact that PE can stimulate lignocaine N-deethylase activity as does male-derived lipid. The ratio of PC/PE, however, was not sex differentiated and therefore of little or no significance. It seems unlikely that PS and PI are involved in maintaining sex differences in lignocaine metabolism. Fatty acid analysis of the microsomal phospholipid extracts showed that there was no sex differences in palmitoleate ($C_{16:1}$) or palmitate ($C_{16:0}$). However the stearate ($C_{18:0}$) was significantly higher in the female microsomal phospholipid fraction than in the male microsomal phospholipid fraction. The microsomal lipid of females had half as much arachidonate ($C_{20:4}$) as in the male and linoleate ($C_{18:2}$) was considerably higher in the male than the female There was little difference in oleate ($C_{18:1}$) content. It should be noted that the total unsaturated fatty acid content of male livers was considerably higher than the females. This sex difference in saturated/unsaturated ratio was similar to that reported by Feuer et al. (1980). There are thus, marked sex differences in some of the phospholipid classes and in the fatty acid composition of the microsomal phospholipids in the rat.

4.5. THE EFFECT OF THE FATTY ACID ACYL CHAIN OF PHOSPHATIDYLCHOLINE ON THE METABOLISM OF LIGNOCAINE BY A RECONSTITUTED DRUG METABOLISING ENZYME SYSTEM.

In 1968 Lu and Coon reported that one of the three components of their resolved and reconstituted fatty acid hydroxylation system was a heat stable, organic extractable factor. This same lipid fraction was found later to be essential for the reconstitution of a drug metabolising system. When this lipid fraction was fractionated by a silicic acid column (Lu et al., 1969a), the active fraction was found to have the same Rf value as the phosphatidylcholine standard. Furthermore, synthetic phosphatidylcholine was found to be as active as the crude lipid fraction in the reconstituted system. With this fact in mind and because of the data discussed in the previous section on the sex differences in fatty acyl side chain of the extracted phospholipid, we chose phosphatidylcholine having various acyl side chains to investigate the role of the fatty acyl chain in maintaining sex differences in lignocaine metabolism. Delipidated solubilised microsomes, prepared from male Wistar rats, were used in these studies and were incorporated into vesicles prepared from various PCs (phosphatidylcholine) containing fatty acids of differing chain length and degree of unsaturation [dicaproylphosphatidylcholine (6:0); dilauroylphosphatidylcholine (12:0); distearoylphosphatidylcholine (18:0); dibehenoylphosphatidylcholine (22:0); dioleoylphosphatidylcholine (18:1) and diarachidoylphosphatidylcholine (20:4)]. All of the PCs were found to restore the N-deethylase activity of the cytochrome P-450 but to differing extents (figure 3.24.). Phosphatidylcholines having longer acyl chains were more efficient and the unsaturated PC (18:1) reconstituted the enzyme activity better than did the saturated PC of the same chain length (18:0). Diarachidoyl (20:4) PC gave the highest enzyme activity. It seems therefore that the longer the chain length and the more unsaturated the acyl side chain is, the better it is at reconstituting lignocaine N-deethylase activity. These results would

seem to correlate well with earlier reports that feeding a lipid-free diet causes an inadequate synthesis of microsomal hydroxylating enzymes and cytochrome P-450 and that induction of cytochrome P-450 by phenobarbitone is much less efficient when saturated fatty acids are incorporated into the diet as the sole source of dietary lipid than when unsaturated fats are fed (Marshall and McLean 1971; Rowe and Wills 1976; Wills 1980). It has therefore been postulated that the polyunsaturated fatty acids might play an important role in the activity of the microsomal drug metabolising system (Marshall and McLean 1971). The fatty acids present in the microsomal lipid have also been seen to change the spin state of cytochrome P-450 and this has been related to the ability of the terminal oxidase to function (Gibson and Tamburini, 1984; Rispin et al., 1984; Gibson et al., 1980). Contrary to the results seen from the N-deethylase activity, the 3-hydroxylating activity is unaffected by the fatty acid chain length or the number of double bonds except when the chain length is reduced to 6 carbon atoms (DCPC). Short chain PC is known to prefer the micellar phase rather than a vesicle system which could affect the protein-lipid reconstitution system (Dencher 1986).

Lignocaine N-deethylase activity is, thus, activated preferentially by long chain, unsaturated fatty acyl side chains on the phospholipid. This would seem to correlate well with the higher content of linoleate (18:2) and arachidonate (20:4) and the lower content of stearate (18:0) in the male-derived lipid fraction, which also activates the N-deethylase activity.

4.6. THE EFFECT OF DIABETES MELLITUS INDUCED BY STREPTOZOTOCIN ON THE METABOLISM OF LIGNOCAINE BY THE RAT LIVER.

In order to further substantiate the apparent role of microsomal lipid composition and fatty acyl side chain on hepatic drug metabolism we decided to investigate this hypothesis in another (in this case pathological) condition in which drug metabolism is known to be affected. Diabetes mellitus, whether genetic or chemically-induced is known to markedly affected drug metabolism (Skett and Joels 1985; Skett 1987). The effect of diabetes mellitus are remarkably similar to the differences seen between male and female animals e.g. lignocaine N-deethylase is reduced in diabetic male animals but unaffected by diabetes in the female. The 3-hydroxylase is not effected by diabetes in either sex. Diabetes mellitus, thus affects the male-specific enzyme activity and only in the male. This is very similar to the effects of the lipids noted in the earlier part of the work described in this Thesis.

4.7. EFFECT OF LIPID EXTRACTED FROM THE DIABETIC RAT ON METABOLISM OF LIGNOCAINE BY A RECONSTITUTED ENZYME SYSTEM.

It was decided to test whether some or all of the effects of diabetes on lignocaine metabolism could be ascribed to changes in lipid composition of the microsomes. We investigated the effect of incorporating delipidated microsomal protein derived from male rat liver into vesicles prepared from microsomal lipid derived from control, 3-day and 21-day diabetic rats on the metabolism of lignocaine to its N-deethylated metabolite. The animals used were clearly diabetic as judged from serum glucose levels (> 16mM) and the fact that the livers from diabetic rats were significantly lighter than those from controls when expressed as g/100 g body weight. The liver weights of the 21-day diabetic rats were lower than the livers of 3-day diabetic rats, but these values were similar when expressed relative to the body weights. Similar result were obtained by Topping and Targ (1975). It is noted that the microsomal lipid extracted from diabetic rats gave a significantly lower N-deethylase activity as compared to the control. The inhibition was greater in the microsomal lipid derived from 21-day diabetic rats. There was no effect of lipids extracted from diabetic rat livers on the 3-hydroxylation of lignocaine. This would seem to indicate that the alteration of microsomal lipids does, indeed, play a role in the effect of diabetes mellitus on lignocaine metabolism in rat liver. If the microsomal lipids are separated into the neutral and phospholipid fraction and the fraction used to reconstitute enzyme activity as described previously, it is seen that the phospholipids derived from diabetic rat liver were significantly poorer than the control PL in reconstituting the metabolising system. Phospholipids derived from 21-day diabetic rats gave the lowest N-deethylase activity. There was no effect on lignocaine 3-hydroxylase of phospholipids derived from diabetic rats. The effects seen might be due to alteration in the phospholipid composition caused by the streptozotocin treatment such

as that demonstrated by Faas and Carter (1983) who suggested that the fatty acid composition in the total lipid extract was altered to decrease palmitoleic, oleic and arachidonic acids and increased linoleic and docosahexaenoic acids. It was further noted in this study that the N-deethylase activity was altered by the use of the lipid fractions derived from diabetic rats in the same manner as seen for the total microsomal lipid. The addition of the neutral lipid fraction to the phospholipid fraction gave a higher N-deethylase activity in all experiments performed. As with other fractions tested, no effect of the neutral lipid/phospholipid mixture was seen on lignocaine 3-hydroxylase activity. The reason for this enhancement is unclear, but one can only suggest that the neutral lipid fraction as prepared contain cholesterol whose effect on drug metabolism is well documented (see introduction 1.10.4.). It thus appears that the activity of the diabetic lipid resides in the phospholipid fraction but this effect can be enhanced by the neutral lipid fraction. Other workers (Past and Cook, 1983) have shown that the diabetes affects the microsomal profile differently in the male and female rat and these authors suggested that such differences might contribute to the well documented sex differences in the effect of diabetes on microsomal drug metabolism in rats (Kato and Gillette, 1965; Reinke et al., 1978; Faas and Carter, 1980). Most of the investigators have explained the changes in the activity in the hepatic drug metabolism in terms of changes in the hepatic-P-450-composition (Past and Cook, 1982) but the results in this study demonstrate that the changes in the lipid composition caused by the treatment alter the N-deethylase activity. It could be argued that the diabetic lipids are simply acting as an inhibitory factor in these reconstitutions. An independent set of experiments have been carried out, however, showing that the lipids isolated from diabetic rats do not act as inhibitory factors when added directly to microsomal incubations. (Dr. Skett personal communication).

It is of further interest that the microsomal lipids from the diabetic male animal gave enzyme activities similar to that of the control female, exactly as would be expected

in whole microsomal preparations. It would, thus, be of great interest to examine if the effect of the diabetic lipid was manifested in reconstitutions with the male-specific isozyme of cytochrome P-450 as was seen for the male and female microsomal lipids earlier in the study.

4.7.1. The Effect of Lipid Fractions Extracted from Streptozotocin-Diabetic Rat Liver on the Metabolism of Lignocaine by the Male-Specific Lignocaine N-deethylase.

The effects of incorporating the male-specific cytochrome P-450 isozyme into vesicles prepared from control; 3-day and 21-day diabetic male-derived microsomal lipids has been studied and it is seen that there is an inhibition of the N-deethylation of lignocaine when the enzyme is incorporated into microsomal lipid derived from 3-day diabetic animals, the inhibition was even greater when the same enzyme was incorporated into 21-day diabetic microsomal lipid. The same effects are seen if phospholipid/neutral lipid mixtures are used for the reconstitution. The same arguments could be put forward here as discussed previously (Section 4.4.) to rationise the effects seen, namely that alteration in phospholipid composition or concentration could affect the functioning of the microsomal enzyme system by a variety of mechanisms (i.e. change in fatty acid side chain; unsaturation of the phospholipid, change in the phospholipid/ cholesterol ratio) (Steir, 1976). Indeed the rat liver microsomal fatty acid composition and fatty acid desaturation have been studied in streptozotocin diabetic rats, (Faas and Carter 1980;1983; Holman et al., 1983) and it was found that streptozotocin induced diabetes changes the fatty acid composition of rat liver microsomal phospholipid. The authors demonstrated (Faas and Carter 1980;1983) that the fatty acids such as linoleic and docosahexaenoic acids were increased whereas palmitoleic, oleic and arachidonic acids were decreased in the major microsomal phospholipids. Furthermore it has been found that, in alloxan-induced diabetic rats, the conversion of phosphatidylethanolamine to phosphatidylcholine by stepwise methylation was decreased (Faas and Carter 1983). It was suggested that most of these changes in fatty acid composition in the diabetic rat are caused by the diminished fatty acid desaturase activities, since the fatty acid composition can be corrected by insulin therapy (Nelson 1980). Mathur et al. (1983) have demonstrated that the phospholipid fatty acid composition plays an important role in the membrane fluidity and certain enzyme

activities. The nature of the dietary lipid, and especially the content of polyunsaturated fatty acids regulates oxidative drug and carcinogen metabolism in the liver endoplasmic reticulum (Rowe and Wills, 1976; Lambert and Wills, 1977a, b; Wills, 1980).

Thus, as with the effects of the male and female microsomal lipids, the effect of the lipid derived from the diabetic male rat liver appears to derive from a direct lipid-cytochrome P-450 interaction specific for the male-specific lignocaine N-deethylase or an interference with the cytochrome P-450 and NADPH-cytochrome P-450 reductase interaction. In the case of the male/female difference the basis of the effect seemed to lie in the relative fatty acid composition of the phospholipids. We hoped to get confirmation of this postulated correlation by analysing the composition of the microsomal lipid derived from the diabetic animals.

4.8. CHANGES IN MICROSOMAL LIPID COMPOSITION PRODUCED BY STREPTOZOTOCIN-INDUCED DIABETES IN RATS.

The phospholipid composition of liver microsomes in control and streptozotocin treated rats was investigated and it is seen that the microsomal phospholipid phosphorus was 8% lower in the 3-day diabetic animals compared to controls whereas the microsomal phospholipid phosphorus was decreased 40% in the 21-day diabetic animals compared to controls. The phospholipids were separated into their major individual fractions i.e. phosphatidylcholine (PC), phosphatidylethanolamine (PE) phosphatidylserine (PS), and phosphatidylinositol (PI) and it was found that control microsomal phospholipid contained 57% phosphatidylcholine, 25% phosphatidylethanolamine, 10% phosphatidylinositol and 4% phosphatidylserine. This is consistent with the finding of DePierre and Dallner (1975) and Faas and Carter (1983). There were significant decreases in the content of phosphatidylcholine and phosphatidylethanolamine in the 3-day diabetic rats and in the 21-day diabetic microsomal phospholipid components appearing as a 34% decrease in phosphatidylcholine content, and a 25% decrease of the phosphatidylethanolamine content. Faas and Carter (1983), however, reported an increase in the total phospholipid in diabetic animals. Our study demonstrates that the fatty acid composition of microsomal phospholipid is altered by 3-day and 21-day diabetes in rats. Liver microsomal phospholipid fatty acid composition shows significantly increased proportions of stearate ($C_{18:0}$) and linoleate ($C_{18:2}$) and dramatically decreased proportions of arachidonate ($C_{20:4}$), and palmitoleate ($C_{16:1}$). All of these changes are seen clearly in the 21-day diabetic animals. Faas et al. (1980, 1983) suggested that the lower proportion of palmitoleate ($C_{16:1}$), is a result of diminished Δ^9 desaturase activity and the increase in linoleic acid and concomitant decrease in arachidonic acid may result from a diminished Δ^6 desaturase activity. This is also consistent with the finding of Topping and Targ (1975) and Takahashi et al. (1987). In 3-day diabetic animals there

was no difference seen with respect to; stearate ($C_{18:0}$), palmitoleate ($C_{16:1}$) and oleate ($C_{18:1}$). There were no differences seen in the palmitate ($C_{16:0}$) fraction in diabetes. These results agree with the finding of Mercuri et al. (1967).

If this data is compared to that for the sex differences in composition of microsomal phospholipid discussed in section 4.4.1., it is seen that there are marked similarities and differences. There is a similar decrease in PC and PE in both female and diabetic male lipids when compared to control males. stearate (18:0) is more prevalent in female and diabetic male phospholipid whereas arachidonate is lower in both as compared to the control male. On the other hand, palmitoleic acid (16:1) is decreased in diabetic phospholipids but does not exhibit a sex difference in distribution and linoleic acid (18:2) is lower in female phospholipids but higher than in the control male in diabetic male phospholipids.

As we know that the effect of the diabetic lipids is similar to female lipids when compared to control male lipids with respect to control of lignocaine N-deethylase activity and assuming that this is correlated to the composition of the lipid, we can rule out the content of palmitoleic, oleic and linoleic acids as of importance. this leaves the stearic and arachidonic acids and the increased amount of PC and PE as possible controlling influences. The ratio of PC/PE, however, changes little and is unlikely to influence activity (but cannot be entirely ruled out). Thus the content of stearate and arachidonate in microsomal phospholipids appears to be the most likely controlling influence for lignocaine N-deethylase in hepatic microsomes. Indeed, the content of arachidonic acid appears to correlate particularly well with lignocaine N-deethylase activity in all cases. The mechanism of this potential control is, at present, unknown.

4.9. GENERAL DISCUSSION.

The early work on the mixed function oxidase system in the liver indicated the requirement for three essential components, namely cytochrome P-450, NADPH-cytochrome P-450 reductase and a heat stable, organic solvent extractable factor (Lu and Coon, 1968) later found to be phospholipid. This same fraction was later found to also be required for the hydroxylation of steroids, carcinogens, and the oxidative metabolism of many drugs, (Lu and Coon, 1968; Strobel et al., 1970). The requirement for lipids was further refined when dilauroylphosphatidylcholine (DLPC) was found the most effective in reconstituting the enzyme activity (Miwa and Lu, 1981). It has therefore found widespread use as an artificial membrane to characterise partially pure and highly purified forms of cytochrome P-450 (Guengerich, 1977a; 1977b; Johnson et al., 1979; Koop et al., 1981; Ryan et al., 1984a; 1984b; Hirada and Negishi, 1984). The continued use of dilauroylphosphatidylcholine (DLPC) is, however, an oversimplification of the endoplasmic reticulum and misrepresents the physiological situation, as DLPC does not exist in vivo. Taniguchi et al. (1984) have suggested that protein insertion and interaction is affected by this artificial phospholipid as compared to the natural lipids. In an attempt to ascertain the role of membrane lipid in the regulation of sex differences in drug metabolism, and based on evidence by Barr and Skett (1984) who demonstrated that the male-specific lignocaine N-deethylase activity was markedly inhibited by the female-derived microsomal lipid whereas the non-sex dependent 3-hydroxylase was unaffected, we have shown that the lipid component of the microsomal membrane is very important for the maintenance of the sex differences in drug metabolism. Our results do not agree with the work of Kamataki et al. (1980;1981;1982;1983;1986) who attributed the sex differences seen in microsomal metabolism purely to the cytochrome P-450 isozymes. We think that the main reason for

this discrepancy is the continued use of the artificial dilauroylphosphatidylcholine (DLPC) by Kamataki and coworkers. As seen from the results, the use of the natural lipids give a totally different picture (Section 4.1.; 4.2.). Sex differences in cytochrome P-450 do exist (Introduction 1.3.1.) but there are also sex differences in microsomal lipid composition in the rat liver (Van Harken et al., 1969; Cooper and Feuer 1972a, b) and these have been correlated to altered drug metabolism in pregnancy (Neale and Parke 1973; Dhami et al., 1980; Turcan et al., 1981). Also Belina et al. (1975) indicated that changes in lipid composition follow sex differences in steroid metabolism. As seen from results with delipidated microsomes, the female-derived lipid was inhibitory to the N-deethylase but the 3-hydroxylase both in male- and female-derived microsomes was unaffected, also the activity of the N-deethyláse was much lower when dilauroylphosphatidylcholine (DLPC) was used rather than the microsomal lipid, but again the 3-hydroxylase activity was unaffected in all cases. The male-specific lignocaine N-deethylase, thus, would appear to have a very specific requirement for lipid whereas the non-sex dependent 3-hydroxylase is less selective. We have extended this work with the isolation of the isozyme of cytochrome P-450 responsible for the N-deethylation of lignocaine. It turns out that this is most probably isozyme RLM5 of Morgan et al. (1985) and 2c of Waxman (1984). When this isozyme is incorporated into male-derived microsomal lipid, it shows the highest activity. The female-derived microsomal lipid gave a much lower activity. Again the microsomal lipids derived from male and female were more effective than the dilauroylphosphatidylcholine (DLPC). This data indicated that it is a direct interaction of the lipid with the enzyme (s) or an alteration of the protein-protein interactions caused by the lipid which leads to the change in enzyme activity and that it is the isozyme responsible for the N-deethylation that is particularly affected in this way. In order to assess which portion of the microsomal lipid causes these changes, the microsomal lipid has been fractionated into phospholipid and neutral lipid. Using delipidation and subsequent relipidation of microsomal lipid

preparation, we have confirmed that the phospholipid is the most effective portion for the maintenance of the drug metabolism and the sex differences in drug metabolism. It is clear that the male-derived phospholipid was more efficient than the female-derived phospholipid when incorporated into both male- and female derived delipidated microsomes. The mixture of phospholipid and neutral lipid fractions was very effective, indeed it restored the N-deethylase activity to the level of the microsomes. A possible explanation for these effects is the presence of cholesterol in the neutral lipid fraction. Indeed, cholesterol has been reported to affected the metabolism of drugs when incorporated into DLPC vesicles (Skett & Cuthill 1986). Changes in the phospholipid/cholesterol ratio may cause a similar effect (Stier, 1976). Experiments were carried out using the male-specific isozyme responsible for the N-deethylation of lignocaine and again it was shown that the phospholipid fraction was the most effective fraction when reconstituted with this isozyme, but it was less effective than the whole microsomal lipid. It is only when the combination of phospholipid and neutral lipid was used the control activity was achieved. Again the male derived phospholipid was more effective than the female-derived phospholipid. This effect may be due to differences in the phospholipid composition as indicated by Belina et al. (1975) who showed that sex differences in steroid metabolism follow changes in microsomal lipid composition. We analysed the phospholipid composition and our analysis shows sex differences in microsomal phospholipid composition and in fatty acid acyl chain of the microsomal phospholipid. Also the analysis of the phospholipid from the diabetic animals showed that the streptozotocin treatment does alter the phospholipid composition and the ratio of acyl chains found. All these changes might be associated with differences in enzyme activity, which is in full agreement with the finding of Belina et al. (1975) and Feuer et al. (1980). The nature of the fatty acids has previously been shown to regulate oxidative drug metabolism in the liver endoplasmic reticulum (Rowe and Wills 1976; Lambert and Wills 1977a, b). In 1980 Wills demonstrated that the dietary lipid alters the rate of

oxidative metabolism due to alterations of fatty acid composition of the endoplasmic reticulum, he suggested that the polyunsaturated fatty acids are important, as a constituent of membrane phospholipids, in holding the cytochrome P-450 in an active conformation in the membrane thus enhancing its efficiency. A diet of purified synthetic components which alter the fatty acid composition could markedly lower cytochrome P-450 concentration and hydroxylating enzyme activity in the liver microsomal fraction (Marshall and McLean 1971). Our results indicated clearly that the arachidonic acid is the most likely candidate for further investigation to ascertain its role in maintaining the drug metabolising activity.

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