

FACTORS AFFECTING HEPATIC DRUG METABOLISM IN THE PREGNANT RAT

by

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To Kathy

SUMMARY

Changes in hepatic drug metabolism during pregnancy have been characterised and the role of steroids and other hormonal factors investigated. Studies with human placental lactogen and rat placental homogenate suggest that during gestation elevated levels of circulating placental lactogen cause a significant decrease in maternal hepatic mixed-function oxidase activity when expressed per gram liver. Drug enzyme activity, determined with the substrates aniline (p-hydroxylation), ethylmorphine (N-demethylation) and p-nitrobenzoic acid (reduction), decreased progressively during gestation to 53-73% of non-pregnant control levels by day 20 of gestation. Enzyme activity remained low 1 day post-partum but had returned to control non-pregnant levels by 5 days post-partum. The total capacity of the liver to metabolise drugs remained unchanged or increased because liver weight was elevated by up to 40% during pregnancy.

Alterations in hepatic drug metabolism are possibly mediated via changes in microsomal phospholipids and/or the cytochrome P450 spin state equilibrium. since pregnancy was associated with a significant decrease in (i) microsomal total phospholipids, (ii) the phosphatidylcholine to phosphatidylethanolamine ratio and (iii) the high-spin form of ferricytochrome P450.

Changes in drug metabolism were not related to alterations in the concentration, substrate induced binding affinity (K_s) or maximal spectral change (ΔA_{Max}) of cytochrome P450 or to the activity of NADPH-cytochrome c reductase. The rise in circulating steroids, particularly progesterone, during gestation does not appear to be an important modulator of mixed-function oxidase activity.

The effect of phenobarbitone and 3-methylcholanthrene and of a choline supplemented diet on mixed-function oxidase activity during gestation has been investigated.

The significance of changes in the haemoprotein spin state and microsomal phospholipids to hepatic drug metabolism has been discussed. Possible mechanisms of action of placental lactogen have been discussed with respect to the related polypeptide, growth hormone.

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CHAPTER ONE GENERAL INTRODUCTION

1.1 DRUG METABOLISM AND THE MICROSOMAL MONOOXYGENASE SYSTEM

1.1.1 The importance of the hepatic microsomal monooxygenase system

Drugs form only a relatively small percentage of the organic chemicals to which living organisms in our biosphere are increasingly exposed. The U.S. Environmental Protection Agency has estimated that there may be as many as 50,000 commonly used chemicals, not including pesticides, pharmaceuticals and food additives and as many as 1,500 different active ingredients in pesticides (Blumberg, 1978). The U.S. Food and Drug Administration estimates that there are about 4,000 active ingredients in drugs and about 2,000 other ancillary compounds used in the drug industry. In addition there are in the order of 2,500 additives used for nutritional value and flavouring and 3,000 chemicals used to preserve food. All these compounds, collectively called "xenobiotics", do not enter normal metabolic routes and must be excreted. Numerous xenobiotics, including many chemical carcinogens, are so hydrophobic that they would remain in the body indefinitely were it not for their metabolism to more polar compounds.

Drugs are metabolised in two phases; 'Phase 1' metabolism in which one or more polar groups (such as hydroxyl) are introduced into the parent molecule, thereby presenting the 'Phase 2' conjugating enzymes (eg. UDP

glucuronyl-transferase) with a substrate. The conjugates are sufficiently polar to be excreted from the cell and the body. During 'Phase 1' metabolism drugs generally lose their pharmacological activity, though examples of drug activation are well documented.

The hepatic microsomal cytochrome P450 mediated monooxygenase system is the most important example of 'Phase 1' enzymes. In addition to its role in xenobiotic metabolism the monooxygenase (or mixed-function oxidase) system also metabolises endogenous substrates such as steroids, fatty acids, and prostaglandins (Brenner,1977).

A discussion of 'Phase 1' and 'Phase 2' metabolism is presented by Goldstein et al. (1974).

1.1.2 Cytochrome P450: Historical Aspects and Distribution

Oxygen is a ubiquitous and essential commodity of life and plays a central role in metabolism and energy generation in all aerobic organisms. The most familiar role of oxygen is as a terminal oxidant in the respiratory chain where a four-electron reduction generates two molecules of water. An equally important role for oxygen in metabolism was demonstrated in 1953 when Mason and Hayaishi independently demonstrated that oxygen atoms from atmospheric O₂ could be enzymatically incorporated into the carbon chains of intermediate metabolites without equilibration with water (Mason et al.,1953; Hayaishi et al.,1953). Enzymes of this class were designated monooxygenases or dioxygenases depending on whether one or both atoms of oxygen were inserted.

In 1955 it was reported that a rabbit liver microsomal fraction contained an enzyme system which could hydroxylate aromatic compounds in the presence of NADPH and molecular oxygen (Brodie et al, 1955) and in 1957 Mason proposed that certain biological oxidations were catalysed by a class of enzyme he called 'mixed-function oxidases' (Mason, 1957). The mixed-function oxidase system performs a monooxygenation in which one atom of atmospheric oxygen is inserted into the substrate while the second atom is reduced to form water.

Klingenberg (1958) and Garfinkel (1958) discovered a carbon monoxide binding pigment in liver microsomes. Omura and Sato (1963) called this pigment 'P450' because it had an optical absorption peak in the Soret region, at about 450nm, when it was poisoned by carbon monoxide in the reduced state. They determined that it was a type of cytochrome or haem containing protein which they called cytochrome P450.

The subsequent involvement of cytochrome P450 with enzymic hydroxylation was demonstrated when it was shown that light of 450nm was optimal for the reversal of carbon monoxide inhibition of steroid C-21 hydroxylation (Estabrook et al, 1963).

Much of the early work was hampered by the difficulty experienced in purifying the mammalian membrane-bound cytochrome P450 system. Initial attempts to solubilize mammalian cytochrome P450 resulted in its conversion to inactive cytochrome P420 (Omura and Sato, 1964a). The properties of cytochrome P450 were therefore studied in the NADH-dependent soluble cytochrome P-450cam isolated from Pseudomonas putida (Katagiri et al, 1968). Mammalian cytochrome P450 was finally solubilized in 1967 with the use of glycerol (Ichikawa and Yamano, 1967). Shortly thereafter Lu and Coon (1968)

successfully solubilized rabbit liver microsomes with deoxycholate in the presence of glycerol and obtained cytochrome P450, NADPH-cytochrome P450 reductase and phosphatidylcholine fractions using DEAE-cellulose chromatography. When these three fractions were recombined under certain conditions, laurate hydroxylase activity could be reconstituted. Subsequently the mixed-function oxidase activity of such reconstituted systems was demonstrated using steroids, drugs and alkanes as substrates (Lu et al, 1969a,b; Lu, 1976; Lu and West, 1978).

The mixed-function oxidase system is now recognised to be a complex multi- component system comprising NADPH, a phospholipid-protohaem-sulphide-protein complex known as cytochrome P450 and a linking electron transport system of NADPH-cytochrome P450 reductase and possibly cytochrome b5 (Coon et al, 1973; Estabrook et al, 1973). Among the reactions catalysed by this system are N-dealkylations (eg. aminopyrine), O-dealkylations (eg. codeine), side chain oxidations (eg. barbiturates), aromatic ring hydroxylations (eg. aniline) and sulfoxidation (eg. chlorpromazine).

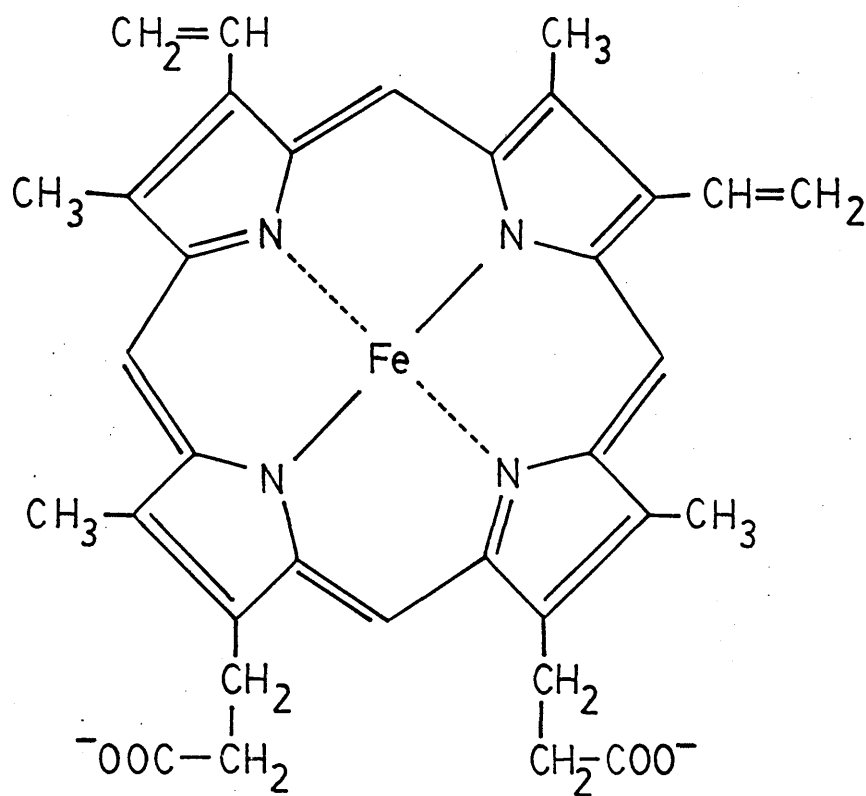
Cytochrome P450 is widely distributed in nature. It has been found in a large number of mammals, various birds, fish, insects, plants, yeasts and bacteria. In mammals cytochrome P450 has been found in liver, kidney, lung, placenta, testis, adrenal gland, skin, brain, corpus luteum, intestinal mucosa, spleen, heart, aorta, lymphocytes and platelets. It is mainly found in the endoplasmic reticulum but it is also found in mitochondria of steroidogenic tissues such as the adrenal gland and corpus luteum. A review of the distribution of cytochrome P450 has been written by Wickramasinghe (1975).

1.1.3 The structure of cytochrome P450

In 1930 Keilin classified cytochromes into three spectroscopically distinct groups which he called 'a', 'b', and 'c' in order of their absorption maxima: 'a' absorbing at the longest wavelength and 'c' at the shortest. Cytochrome P450 is classified as a 'b' cytochrome (systematic name cytochrome B420) and contains an iron protoporphyrin IX group (Figure 1). Cytochrome P450 differs from other 'b' type cytochromes in that it can react with oxygen, cyanide and carbon monoxide. One of the most characteristic and distinguishing optical features of cytochrome P450 is that the Soret maximum of the reduced haemoprotein when complexed with CO occurs at a wavelength 30nm longer than the usual CO-haem complex. This unusual property is highly dependent on the nature of the protein structure and lipid environment. Treatment of microsomes with agents that alter the lipid structure of the membrane, such as detergents and lipases, and with agents that denature the haemoprotein, such as urea and proteases, cause a conversion of cytochrome P450 to cytochrome P420 (Omura and Sato, 1964a; Imai and Sato, 1967b; Mason *et al.*, 1965). Cytochrome P420 is an inactive form of cytochrome P450 and has a spectrum typical of a type 'b' cytochrome with a Soret maximum at 420nm.

The iron atom of cytochrome P450 haem has four planar ligand interactions with the four nitrogen atoms of the protoporphyrin ring system and has two ligand positions available for further binding. The nature of the two axial ligands has been the subject of intense investigation. Based on model compound studies, it was first suggested by Stern and Peisach (1974) and subsequently confirmed by others (Collman and Sorell, 1975; Chang and Dolphin, 1975), that the red shifted Soret maximum of cytochrome

Figure 1 Iron Protoporphyrin IX



Prosthetic group of 'b' class cytochromes, P450,
haemoglobin, myoglobin erythrocytorin, catalase and peroxidase.

P450 arose from a chromophore which consisted of haem, carbon monoxide and a mercaptide (RS-) sulphur atom believed to be bound to the haem trans to the CO. It was thought that the presence of this axial sulphur ligand differentiated cytochrome P450 from other CO binding haemoproteins which absorbed at or near 420nm when CO bound. Furthermore, model compounds containing the mercaptan (RSH) in place of the mercaptide showed Soret bands at "normal" wavelengths (413-422nm).

When isolated or in situ, cytochrome P450 rests in the ferric oxidation state and is thus amenable to study by electron spin (paramagnetic) resonance (EPR) which detects unpaired electrons. The ferric form of cytochrome P450 has 5 electrons in the iron d-orbital and depending on the spin pairing of these electrons the haemoprotein can exist in the high-spin ($S=5/2$, 5 unpaired electrons) or low-spin configuration ($S=1/2$, 1 unpaired electron). Cytochrome P450 has been shown to exist in both high-spin and low-spin states (Jefcoate and Gaylor, 1969; Hill et al., 1970). In the absence of substrates, the EPR spectrum of cytochrome P450 is that of a low-spin ferric haem compound (Mason et al., 1965; Blumberg and Peisach, 1971; Tsai et al., 1970). Studies using model systems with thiol agents and haemoglobin or metmyoglobin showed similar low-spin EPR spectra (Jefcoate and Gaylor, 1969; Peisach et al., 1973; Hill et al., 1970; Blumberg and Peisach, 1971; Tang et al., 1976) to those obtained with cytochrome P450 from mammalian (Jefcoate and Gaylor, 1969; Peisach et al., 1973; Stern et al., 1973) and bacterial (Tsai et al., 1970) sources.

The nature of the sixth ligand to haem is still equivocal, though cytochrome P450 has been shown to have a heterogenous ligand field in the vicinity of haem (Peisach et al., 1973). Various proposals have been made

which include water (Peterson and Griffin,1973; Griffin and Peterson,1975), the imidazole nitrogen of a histidine residue (Jefcoate and Gaylor,1969; Tang et al.,1976; Chevion et al., 1977; Peisach et al.,1979) and a hydroxyl group (Nebert et al.,1976; Kumaki and Nebert,1978).

1.1.4 The mechanism of cytochrome P450 mediated monooxygenations

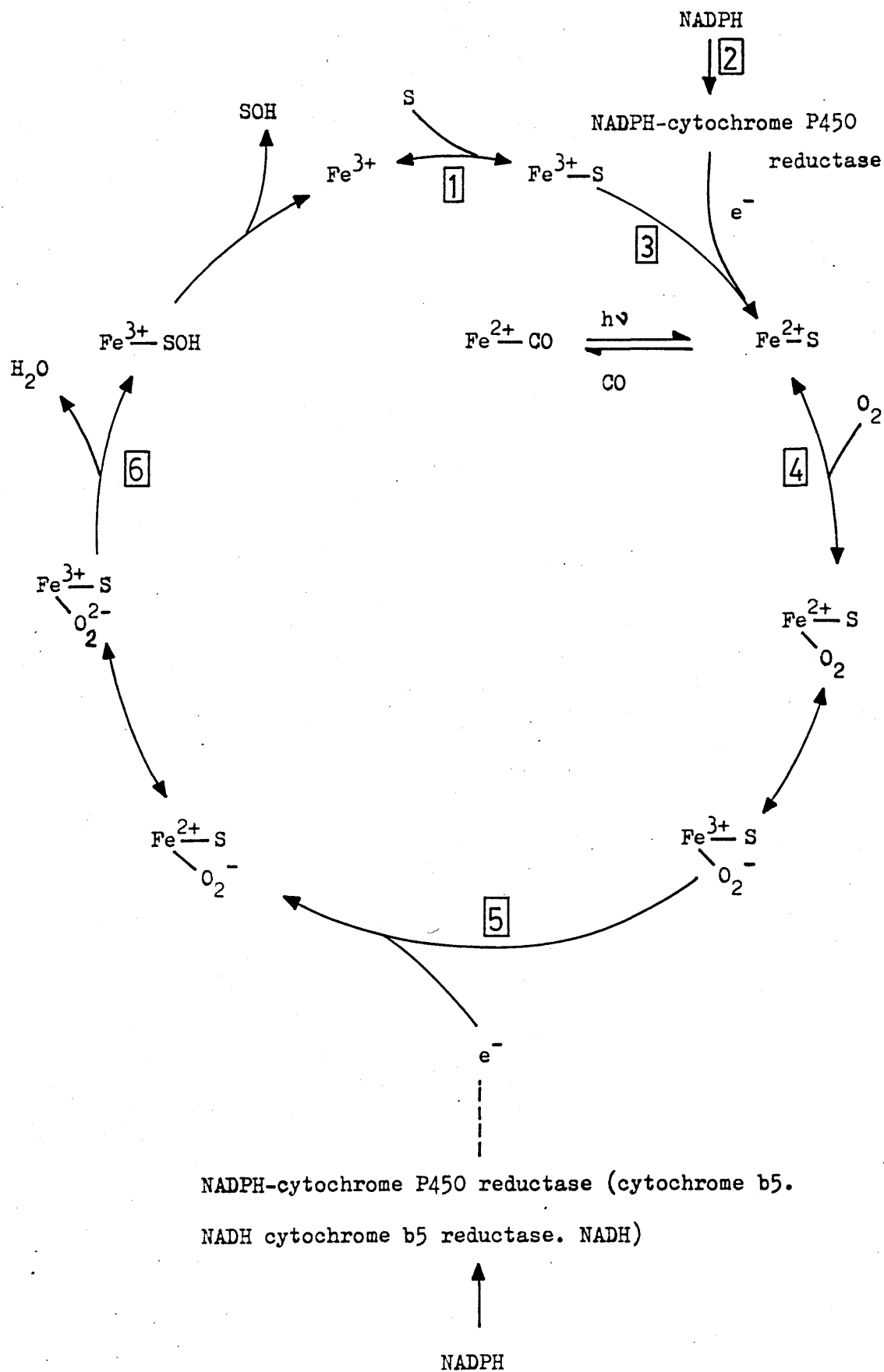
Estabrook et al. (1971) proposed the following mechanism for the cytochrome P450 mediated mixed-function oxidation of xenobiotics and endogenous substrates (Figure 2)

- (a) Association of substrate to oxidised cytochrome P450.
- (b) Reduction of NADPH-cytochrome P450 reductase by NADPH.
- (c) Reduction of cytochrome P450-substrate complex by reduced NADPH-cytochrome P450 reductase.
- (d) Addition of oxygen to reduced cytochrome P450-substrate complex.
- (e) Reduction of oxygenated reduced cytochrome P450-substrate complex by another electron probably from reduced NADPH-cytochrome P450 reductase.
- (f) Decomposition of oxygenated reduced cytochrome P450-substrate complex to yield hydroxylated substrate, oxidised cytochrome P450 and water.

The nature of the rate limiting step in this scheme has been the subject of intense research in recent years. It has been complicated by a number of factors such as the heterogeneity of the system with respect to cytochrome P450, the presence of significant amounts of endogenous substrates even in partially purified systems and the broad substrate specificity of the system whereby the rate limiting step may be different for different substrates. Each of the reaction steps outlined above will

Figure 2 The mechanism of microsomal cytochrome P450 mediated

monooxygenations (after Estabrook et al., 1971)



be discussed in greater detail.

(1) Association of substrate to oxidised cytochrome P450

The association of substrate with oxidised cytochrome P450 can be followed spectrophotometrically (Remmer et al, 1966; Imai and Sato, 1966; Schenkman et al, 1967). These changes, monitored by difference spectroscopy, are of three types (Schenkman et al, 1972). Type '1' spectral changes are characterised by an absorption maximum at 385-390nm and a minimum at 415-425nm. Type '2' changes show an absorption maximum at 425-435nm and minimum at 390-400nm. The third type, known as 'reverse type 1' (type R1) is essentially a mirror image of the type '1' change (absorption maximum of 415-425nm, minimum 385-390nm).

The spectral dissociation constant (K_s), analogous to the Michaelis constant (K_m), and the maximum absorbance change (ΔA_{max}), analogous to the maximal velocity (V_{max}) of an enzyme reaction, can be determined by plotting the spectral change against substrate concentration as a double reciprocal Lineweaver-Burk plot. The constant K_s is defined as the concentration of substrate giving a half maximal spectral change and is independent of protein concentration. The ΔA_{max} is dependent on substrate and cytochrome P450 concentration (Estabrook et al, 1972b).

The type '1' spectral change is the most common substrate-cytochrome P450 interaction and is produced by a wide range of endogenous and exogenous compounds such as steroids, fatty acids, barbiturates and polycyclic aromatic hydrocarbons. The type '1' spectral change is elicited, in general, by lipophilic compounds and a correlation between K_s

values and lipid solubility for some type '1' compounds has been demonstrated (Kitigawa et al., 1972; Al-Gailany et al., 1975). The interaction of a type '1' compound with cytochrome P450 has been shown to cause an increase in the polarity of the sixth haem ligand though type '1' compounds interact with the apoprotein and not the haem portion of cytochrome P450 (Schenkman, 1968). This interaction is associated with the conversion of the haemoprotein from low-spin to high-spin (Whysner et al., 1970; Waterman et al., 1973; Tsai et al., 1970). The increase in high-spin form of cytochrome P450 shifts the redox potential of the cytochrome to a less negative value which results in an increased rate of electron flow from NADPH-cytochrome P450 reductase to cytochrome P450 (Sligar et al., 1979; Ristau et al., 1979; Sligar, 1976). Thus a common characteristic of type '1' compounds is that they accelerate the reduction of cytochrome P450 and it seems that the haem iron spin equilibrium could be of importance in the regulation of monooxygenase activity. In some cases the rate of metabolism has been shown to be proportional to the binding of the substrate to microsomes (Schenkman, 1970) and with many type '1' compounds, similarity between the spectral dissociation constant and the K_m of the hydroxylation has been found (Schenkman et al., 1967; Jansson et al., 1972; Schenkman et al., 1973; Ulrich, 1969; Orrenius et al., 1970).

The type '2' spectral change is caused by the interaction of amines or compounds containing nitrogen, with a lone pair of electrons (eg. pyridine and imidazole) with cytochrome P450 producing a ferrihaemochrome (Schenkman et al., 1967; Jefcoate et al., 1969; Schenkman, 1970; Temple, 1971). This involves an electron transfer between the nitrogen atom of the added compound and the sixth ligand position of the haem iron (Schenkman et al., 1967). Few compounds which cause a type '2' spectrum are metabolised by cytochrome P450; aniline, however, is an exception. Schenkman (1970)

showed that the interaction of aniline with cytochrome P450 has a type '1' component and it is this that is related to aniline metabolism. The type '2' spectrum of aniline is due to an interaction at the haem iron since it competes with CO for reduced cytochrome P450 (Schenkman et al., 1967). This is associated with the production of a low-spin iron which has an absorbance peak with a longer wavelength (red shift). This is thought to be due to an increase in ligand field strength.

The reverse type '1' (type R1) spectral change is produced by a wide range of compounds, including acetanilide, short chain monohydric alcohols and Warfarin. It is associated with a high-spin to low-spin conversion of cytochrome P450 in which the sixth ligand position of the iron becomes occupied by a weak ligand (Estabrook et al., 1973). The nature of the type 'R1' spectrum has yet to be elucidated. There are three current hypotheses.

(a) It has been suggested that the type R1 spectrum is similar to the type '2' interaction (Whysner et al., 1970). This appears to be unlikely since type R1 compounds cannot displace CO or alter the binding of aniline to the oxidised haemoprotein (Schenkman et al., 1973).

(b) The type R1 spectrum is possibly caused by a reversal of the type '1' spectrum due to a displacement of endogenous substrates already bound to cytochrome P450 (Schenkman et al., 1973; Diehl et al., 1970). When butanol was added to microsomes in which the type '1' binding sites had been saturated with cyclohexane, a type R1 spectrum was produced (Diehl et al., 1970). Furthermore Schenkman et al. (1973) found that solvent extraction of microsomes reduced the type R1 spectrum induced by phenacetin. Vore et al. (1974) found similar results using microsomes from 3-methylcholanthrene treated rats and Powis et al. (1977) found that the addition of bovine serum

albumin to microsomal suspensions caused a type R1 spectrum which the authors concluded was due to the displacement of endogenous substrates from cytochrome P450. In contrast to these results it has been suggested that the type R1 spectrum is due to substrate binding at a specific site of cytochrome P450 rather than the displacement of endogenous substrates. In addition type '1', type '2' and type R1 binding sites were contrasted to be in different environments depending on the interaction of cytochrome P450 with phospholipids in the endoplasmic reticulum (Al-Gailany, 1975).

(c) Since many of the compounds causing a type R1 spectrum contain oxygen it has been suggested that the oxygen atoms interact with the haem to displace the sixth ligand in a similar way to the nitrogen atoms that cause type '2' spectral changes (Mailman et al., 1974). The lower nucleophilicity of the type R1 groups is responsible for the hypsochromic shift from the type '2' spectrum. Furthermore it has been suggested that type R1 binding is due to interaction between the type R1 compound and high-spin cytochrome P450 (Yoshida and Kumaoka, 1975; Kumaki et al., 1978).

(2) Reduction of NADPH-cytochrome P450 Reductase with NADPH

The mechanism of reduction in microsomal hydroxylations can be understood more clearly after considering the properties of the NADPH-dependent flavoprotein: NADPH-cytochrome P450 reductase. NADPH-cytochrome P450 reductase has been purified to apparent homogeneity and consists of a single polypeptide chain of molecular weight 78,000 as determined by sodium dodecylsulphate gel electrophoresis (Dignan and Strobel, 1975, 1977; Yasukochi and Masters, 1976; Vermilion and Coon, 1978). It is unusual among flavoproteins in that it contains two different flavin groups, FAD and FMN, per molecule (Iyanagi and Mason, 1973; Yasukochi and

Masters,1976). It has been suggested that one flavin group may be specifically concerned with electron transfer from NADPH and the other with electron transfer to the terminal acceptor cytochrome P450 (Iyanagi and Mason,1973; Iyanagi et al.,1974). Masters and colleagues have proposed that the aerobically stable form of the enzyme obtained by reduction with NADPH contains two electron equivalents (Masters et al.,1965a.b.; Yasukochi and Masters,1976), in which both flavin groups are probably in the semiquinone form. Other workers (Iyanagi and Mason,1973; Iyanagi et al.,1974; Vermilion and Coon,1978) have suggested that the air stable form of the enzyme is in a one electron reduced state in which one of the two flavins exist in the semiquinone form and the other in the oxidised form.

From the finding that the reduction of cytochrome c by microsomal NADPH-cytochrome P450 reductase is at least one order of magnitude more rapid than the reduction of cytochrome P450 in the microsomal fraction the conclusion has been drawn that penetration of the microsomes by NADPH and subsequent reduction of the flavoprotein is not the rate limiting step (Holtzman,1970). This has been confirmed by Björkhem (1972) using deuterated NADPH.

(3) Reduction of cytochrome P450-substrate complex by reduced cytochrome P450 reductase

NADPH-cytochrome P450 reductase can transfer electrons directly to three types of acceptor, cytochrome c and P450 (Masters et al.,1971; Glazer et al.,1971), oxygen (Aust et al.,1972), and artificial acceptors such as ferricyanide, dichlorophenolindophenol, azo compounds and a variety of quinones (Masters et al.,1971; Iyanagi and Yamazaki,1969; Nishibayashi et al.,1967). It appears that the enzyme functions as a single electron

transferring system and all of the artificial acceptors undergo single electron reduction. Molecular oxygen similarly undergoes a one electron reduction to form superoxide. Cytochrome c is a single electron acceptor and by analogy it might be expected that the natural acceptor cytochrome P450 would also undergo single electron reduction by NADPH-cytochrome P450 reductase. This has subsequently been confirmed by an anaerobic titration of purified and microsomal cytochrome P450 (Ullrich et al, 1968; Peterson et al, 1977; Cooper et al, 1977) and potentiometric studies (Waterman and Mason, 1970; Guengerich et al, 1975).

Gigon et al. (1969) have shown that ethylmorphine, hexobarbital, aminopyrine and imipramine, all of which cause type '1' spectral changes, markedly enhance the rate of reduction of cytochrome P450 by NADPH-cytochrome P450 reductase. In contrast, type '2' compounds such as aniline and nicotinamide decreased the rate of reduction. This substrate induced enhancement of cytochrome P450 reduction was found to be closely related to the rate of hydroxylation and a number of subsequent studies have confirmed it to be rate limiting for a number of type '1' compounds (Diehl et al, 1970; Holtzman and Rumack, 1973; Gillette and Sasame, 1970; Gillette, 1971). Substrate binding to cytochrome P450 is associated with an increase in the high-spin character; this shifts the redox potential of the cytochrome by 70mv to a less negative value (Sligar, 1976; Sligar et al, 1979). This enables the terminal cytochrome acceptor of the mixed function oxidase to be more readily reduced by NADPH-cytochrome P450 reductase. The nature of the endoplasmic reticulum and its lipid content has been shown to be important in the rate of drug metabolism via an effect on the spin state of cytochrome P450 (Gibson et al, 1980a.b)

Thus it appears that formation of the enzyme-substrate complex 'triggers' the hydroxylation of the substrate providing an efficient regulation mechanism for the monooxygenase system. Diehl et al. (1970) pointed out that this mechanism prevents a futile cycle that reduces oxygen by NADPH without subsequent substrate hydroxylation.

(4) Addition of oxygen to the reduced cytochrome P450-substrate complex.

The addition of oxygen to reduced cytochrome P450 or reduced cytochrome P450-substrate complex is a very fast reaction and can only be rate limiting in the overall reaction under specific conditions. Gunsalus and Lipscomb (1972) reported that in a purified bacterial cytochrome P450 system the rate of binding of oxygen to cytochrome P450cam was more than 20 times faster than the rate limiting step. Carbon monoxide, however, was competitive with oxygen for the terminal oxidase and was thus able to inhibit hydroxylation. In the case of aniline hydroxylation, it has been suggested that the addition of oxygen to the reduced cytochrome P450-substrate complex is the rate limiting step (Schenkman, 1971, 1972). Addition of aniline decreases both NADPH oxidation and oxygen consumption by the microsomal fraction and it appeared that aniline interfered with oxygen activation, thereby slowing down its own hydroxylation.

(5) Reduction of the oxygenated reduced cytochrome P450-substrate complex

The reduced cytochrome P450-substrate complex undergoes one further electron reduction. This can be donated by NADPH-cytochrome P450 reductase, however a number of studies have shown that cytochrome b5 and

NADH-cytochrome b5 reductase can also mediate in the reduction (Hilderbrandt and Estabrook, 1971; Estabrook et al., 1971; Sasame et al., 1973). This observation explains the synergistic effect of NADH on microsomal monooxygenation (Conney et al., 1957; Cohen and Estabrook, 1971a) in which, in the presence of high concentrations of NADPH, NAD had a greater than additive effect on microsomal drug metabolism.

Estabrook and his colleagues (Cohen and Estabrook, 1971b; Hildebrandt and Estabrook, 1971) suggested that the ferrous cytochrome P450-substrate complex can be reduced by an electron supplied by either NADPH via NADPH-cytochrome P450 reductase or NADH via cytochrome b5. Staudt et al. (1974) have proposed that cytochrome b5 exerts a sparing effect on the mixed-function oxidase. This was based on the observation that the extent of NADH synergism was higher with compounds such as hexane which produced uncoupling of the microsomal mixed-function oxidase. They suggested that cytochrome b5 discharged activated oxygen which would otherwise be released to the medium from cytochrome P450. This was developed by Schenkman et al. (1976) who envisaged uncoupling as being due to a slower reaction between substrate and activated oxygen than between activated oxygen and oxygenated cytochrome P450 with release of H_2O_2 . Electrons from cytochrome b5 were suggested to reduce the uncoupled cytochrome P450 bound active oxygen at a rate faster than it could be released to the medium to react with another cytochrome P450 bound active oxygen. Estabrook et al. (1971, 1973) found that the effect of phenobarbital induction was to shift the rate limiting step from the conversion of ferric cytochrome P450 to ferrous cytochrome P450 to the transfer of the second electron to oxycytochrome P450.

(6) The decomposition of the oxygenated reduced cytochrome

P450-substrate complex

The oxygenated-reduced cytochrome P450-substrate complex dissociates to give oxidised cytochrome P450, water and hydroxylated substrate. A variety of reactive oxygen or oxygen-containing species have been reported to be formed at this stage. These include hydrogen peroxide (Hilderbrandt et al., 1973; Schenkman et al., 1979), the superoxide anion radical (Aust et al., 1972; Bartoli et al., 1977; Schenkman et al., 1979) and singlet oxygen (Sugioka and Nakano, 1976).

Hamilton (1964) suggested the involvement of an oxene mechanism involving an active oxygen species analogous to a carbene or nitrene. Support for this mechanism has been provided by Guroff et al. (1967) who observed the 'NIH' shift in which an existing ring substituent was displaced to an adjacent group by the incoming hydroxyl group. Rahimtula and O'Brien (1974, 1975) showed that cumene hydroperoxide and other hydroperoxides could sustain the hydroxylation and O-dealkylation of a variety of substrates in rabbit liver microsomes in the absence of NADPH and molecular oxygen. Similarly other 'active oxygen' containing compounds such as sodium chlorite and sodium periodite have been shown to support hydroxylation of steroids and fatty acids in the absence of NADPH and molecular oxygen (Hrycay et al., 1975a, 1976).

Studies with model systems by Ullrich et al. (1972) led them to the conclusion that the active oxygen species might be an iron bound oxygen in the oxene form($:\ddot{O}$) which electrophilically attacks the substrate. The ferryl complex $Fe^{4+}O^-$ (in resonance with a ferric oxene complex $Fe^{3+}O^- \rightleftharpoons Fe^{3+}-O$) has been suggested as the most likely candidate for the 'active oxygen' of the mixed-function oxidase (Rahimtula et al., 1974; Gustafsson et al., 1976).

The microsomal reactions of oxygen have been reviewed by O'Brien.

1.1.5 Cytochrome P450 dependent reductions

The hepatic microsomal fraction can catalyse the reductive metabolism of a variety of compounds (Gillette, 1966, 1969; Mitchard, 1971). Many of these reactions are catalysed by microsomal flavoproteins; cytochrome P450, however, appears to be involved in the NADPH-dependent reduction of azo- and nitro-compounds and these reactions can be partially inhibited by carbon monoxide (Gillette et al., 1968; Hernandez et al., 1967). More recent studies have shown that cytochrome P450 catalyses the reduction of tertiary amine N-oxides under anaerobic conditions (Sugiura et al., 1976; Iwasaki et al., 1977; Kato et al., 1978) and it has been suggested that the N-oxide coordinates at the sixth position of the haem iron in the same position as oxygen and carbon monoxide. The mechanism for the NADPH dependent reduction of tertiary amine oxides is thought to involve a two electron reduction of cytochrome P450 in a similar manner to cytochrome P450's action as a terminal oxidase (Sugiura et al., 1976). Cytochrome b5 has also been found to be involved in N-hydroxylamine reduction in an NADH dependent reaction (Kadlubar and Ziegler, 1974).

1.1.6 The structure of the endoplasmic reticulum and the molecular organisation of the mixed-function oxidase system.

The hepatic endoplasmic reticulum consists of a complex membrane network comprising tubules, vesicles and lamellae, on which are located a large number of enzymes and enzyme systems. The endoplasmic reticulum is divided into rough and smooth components; the former (60%) is associated with ribosomes on the cytoplasmic surface. Compositional studies of isolated rat liver microsomes revealed that the membrane is about 70% protein and 30% lipid (of which 85% is phospholipid) by weight (Glaumann and Dallner, 1968; Eriksson, 1973). This represents approximately 23 molecules of phospholipid per protein molecule. The phospholipid composition is 55-60% phosphatidylcholine, 20-25% phosphatidylethanolamine, 5-10% phosphatidylserine, 5-10% phosphatidylinositol and 4-7% sphingomyelin. The fatty acid moieties of the phospholipids consist mainly of 16-22 carbon atoms. The relative amount and degree of unsaturation depends to some extent on the diet of the animal. The structure of the endoplasmic reticulum has been reviewed by Depierre and Dallner (1975). The organisation of the mixed-function oxidase within the endoplasmic reticulum has been the subject of much research in recent years. Consideration of the molecular arrangement of this enzyme system must bear in mind the following points:

- (1) The microsomal membrane is a single membrane 70-80Å thick (Claude, 1969). This suggests a limit of 2 or 3 protein molecules, of molecular weight 50,000, spanning the membrane.
- (2) Cytochrome P450 can account for as much as 15% of liver microsomal protein and the number of molecules of the haemoprotein can be 20-30 times

greater than that of NADPH-cytochrome P450 reductase in the membrane (Estabrook et al.,1976)

(3) The reductase has a molecular weight of about 78,000 daltons and is bound to the membrane through its hydrophobic portion (Vermilion and Coon,1974; Dignum and Strobel,1975; Yasukochi and Masters,1976). The hydrophilic and catalytic portion of the reductase probably protrudes from the membrane since it can be cleaved by trypsin digestion (Estabrook et al.,1976).

(4) Cytochrome P450 is relatively resistant to proteolytic digestion, in particular in the presence of a substrate to be hydroxylated, suggesting that it is deeply buried in the membrane.

Two organisational models have been developed to account for these and other observations: the "rigid" and "non rigid" system (Franklin and Estabrook,1971). In the "rigid" system the reductase molecule is surrounded by many cytochrome P450 molecules in a complex; the structural organisation precludes any interaction between electron carriers from different complexes. According to this model, only 50% of cytochrome P450 can be reduced enzymatically when 50% of the reductase is inactivated. Mersalyl has been used to inactivate the reductase and the results were reported to be consistent with the rigid system (Franklin and Estabrook,1971). Yang (1975) however, observed that when the reductase activity in the microsomes was inactivated to different extents (up to 80% inhibition) by mersalyl almost all of the cytochrome P450 could still be reduced enzymatically, although the rate of reduction was significantly retarded. These results suggested that cytochrome P450 and the reductase were not rigidly associated and that these enzymes possessed lateral mobility in the membrane. These observations are consistent with the "non rigid" model. This model does not imply that the monooxygenase enzymes are

randomly distributed in the membrane. Certain domains of the membrane may have high local concentrations of these enzymes (Depierre and Dallner, 1975; Schultz and Staudinger, 1971; Winkvist and Dallner, 1976). Matsubara et al. (1976) and Peterson et al. (1976) have studied the rate of NADPH-dependent reduction of cytochrome P450 in microsomes. Biphasic reduction kinetics have been observed. Upon studying the effect of temperature on the reaction a break in the Arrhenius plot was observed for the slow phase but not for the fast phase of the reaction. In the light of this finding Peterson et al. (1976) have suggested that the monooxygenase system exists as "clusters" with 8-12 cytochrome P450 molecules arranged around a central reductase molecule. This central reductase is able to reduce randomly the cytochrome P450 molecules within the "cluster", in the fast phase of the reduction, without transitional motion. The slow phase of reduction represents the reduction of molecules not directly associated with the cluster. The "non rigid" model has been further supported by experiments in which partially purified cytochrome P448 has been incorporated into the membrane leading to an enhanced (up to 5 fold) microsomal benzo(α)pyrene hydroxylase activity, showing that added cytochrome P448 can become a functional part of the monooxygenase system (Yang and Strickhart, 1975). The enhancement was not instantaneous and a temperature dependent incorporation of cytochrome P448 into the microsomal membrane was observed.

1.1.7 The role of microsomal lipids in cytochrome P450 mediated monooxygenation

Lu and Coon (1968) resolved the microsomal cytochrome P450 system into three components, each necessary for maximal fatty acid hydroxylating activity in a reconstituted system: cytochrome P450, NADPH-cytochrome P450 reductase and a heat stable factor extractable into organic solvents which was subsequently shown to be phosphatidylcholine (Strobel, 1970). Since then phosphatidylcholine has been shown to be essential for the achievement of maximal rates of fatty acid (laurate), hydrocarbon (hexane, cyclohexane, octane) drug (benzphetamine, hexobarbital, ethylmorphine) carcinogen (benzpyrene) and aniline metabolism by reconstituted cytochrome P450 systems (Strobel et al., 1970; Lu et al., 1972a,b; Lu and West, 1972; Björkhem et al., 1973; Lu et al., 1973; Guengerich and Coon, 1975; van der Hoeven et al., 1974).

Strobel et al. (1970) proposed that the lipid component in reconstituted systems was essential for electron transfer from NADPH to cytochrome P450 via NADPH-cytochrome P450 reductase but phosphatidylcholine is not an electron carrier so its mode of action remains unknown. More recently Guengerich and Coon (1975) showed that phosphatidylcholine lowered the apparent binding constant and K_m for benzphetamine, increased the magnitude of the spectral change seen upon interaction of this substrate with cytochrome P450 and increased V_{max} for benzphetamine metabolism. Lu et al. (1974) showed that certain non-ionic detergents at appropriate concentrations substitute for lipid in supporting benzphetamine N-demethylation. Lu et al. (1974) concluded that lipids play a physical rather than a chemical role in maintaining monooxygenase activity. A

similar conclusion was reached by Eletr et al. (1973) who found that an abrupt change in microsomal membrane fluidity occurred at 19°C and 32°C. This was detected by following the electron spin resonance of lipophilic nitroxide radicals. These results have been correlated with the apparent breaks at 19°C and 32°C in the Arrhenius plot of the UDP-glucuronyltransferase reaction. Stier and Sackmann (1973) studied the effect of temperature on the NADPH-dependent reduction of a lipophilic nitroxide spin labelled substrate with liver microsomes from phenobarbital treated rabbits. A break in the Arrhenius plot was observed at 32°C and this led to the suggestion that NADPH-cytochrome P450 reductase was surrounded by a halo of phospholipids which, unlike the bulk of microsomal lipids, underwent a phase transition at 32°C. NMR and ESR studies by Stier (1976) suggested that the binding of cytochrome P450 to lipids imposed a high degree of order on the lipids and that the lipoprotein complex changed its conformation at about 36°C.

Cinti et al. (1979) reported that, in the absence of added substrate, rat liver microsomes contain large amounts of high-spin cytochrome P450. In contrast the spin equilibrium of the purified soluble haemoprotein was predominantly in the low-spin configuration. It was suggested that membrane components might modulate the spin equilibrium of cytochrome P450 and thus change the spin state. A subsequent study by the same group (Gibson et al., 1980a) revealed that the whole lipid extract of rat liver microsomes, when added to purified cytochrome P450, resulted in a shift in the high-spin form from 17% to 53% at 20°C. Furthermore, they attributed this change largely to the free fatty acid fraction in the lipid extract which may behave as endogenous substrates.

1.1.8 Induction and multiple forms of cytochrome P450

The increased activities of the microsomal drug-metabolising enzymes following pretreatment with a wide range of drugs, pesticides, food additives, steroids, polycyclic hydrocarbons and other xenobiotic compounds is now well known and extensively documented (Conney,1967). Stimulation of these enzymes occurs when the inducing compounds are administered to the living animal, are perfused through the isolated liver or other organs (Juchau et al,1965) or are added to mammalian cell cultures in vitro (Nebert and Gelboin,1968a,b). Enzyme induction acts to increase the rate of production and/or decrease the rate of degradation of an enzyme and must not be confused with agents such as ethylisocyanide, acetone and metyropone which bring about an in vitro activation of monooxygenase reactions (Cinti,1978).

There is increasing evidence that different inducers elevate the levels of different forms of cytochrome P450. Treatment with phenobarbital causes an increase in cytochrome P450 levels while 3-methylcholanthrene pretreatment causes a change in the absorbance maximum of the reduced carbon monoxide difference spectrum from 450nm to 446-448nm (Alvares et al,1967). This altered form has an altered ethyl isocyanide difference spectrum (Sladek and Mannering,1966). The haemoproteins induced by phenobarbitone and 3-methylcholanthrene exhibit major differences in their catalytic properties. Phenobarbitone increases the metabolism of a wide range of substrates whereas 3-methylcholanthrene is more specific in its action (Conney,1967).

A number of substrates of the monooxygenase system are metabolised, after pretreatment with inducing agents, to give different products in microsomal suspensions. These include Warfarin (Pohl et al.,1977; Kaminsky et al.,1980), steroids (Kremers et al.,1978, Conney et al.,1973), benzo(α)pyrene (Rasmussen and Wang,1974) and biphenyl (Burke and Bridges,1975). Kremers et al. (1978) investigated the effect of inducers on the activity of 16 α -hydroxylase using four steroid substrates and concluded that there are at least two cytochrome P450 forms in addition to cytochrome P448. More recently Kaminsky et al. (1980) reported that, on the basis of three criteria (regio- and stereoselectivity of Warfarin metabolism and immunological inhibition), there is one distinct form of cytochrome P450 induced by 3-methylcholanthrene and two to four forms in phenobarbitone treated rats. Uninduced rat microsomes contain two distinct cytochrome P450 forms, one of which is identical with a phenobarbital induced form.

Sodium dodecylsulphate (SDS) polyacrylamide gel electrophoresis has been used to separate microsomal proteins. A preferential induction of proteins in the 45,000-60,000 molecular weight region has been observed in phenobarbitone and 3-methylcholanthrene pretreated rats (Alvares and Siekevitz,1973; Welton and Aust,1974), mice (Huang et al.,1976) and rabbits (Haugen et al.,1975). Proteins induced by polycyclic aromatic hydrocarbons are in general of a higher molecular weight than those induced by phenobarbitone. Other inducers have been shown to induce proteins with a molecular weight different to those induced by both 3-methylcholanthrene or phenobarbitone, for instance, nitrogen-containing carcinogens (Cameron et al.,1976) and isosafrole (Dickins et al.,1978). A large number of inducing agents are now recognised, these include endogenous and contraceptive steroids (Selye,1970), pregnenolone 16 α -carbonitrile (PCN) (Lu et al.,1972a), polyhalogenated compounds such as DDT (Hart and Fouts,1965),

polychlorinated biphenyls (Ryan et al,1977;Alvares and Kappas,1977), flavones such as β -naphthoflavone (Boobis et al,1977) and ethanol (Joly et al,1977).

The investigation of multiple forms of cytochrome P450 is particularly important since early work with reconstituted systems indicated that substrate specificity resides within the cytochrome P450 fraction and that NADPH-cytochrome P450 reductase and phospholipid fractions could be used interchangeably (Lu et al,1971,1973; Nebert et al,1973). Much of the evidence for multiple forms of cytochrome P450 has been obtained from studies on purified preparations. This has been reviewed recently by Guengerich (1979). A number of techniques are in use to investigate purified cytochrome P450 preparations, these include spectral, catalytic, electrophoretic, peptide mapping, amino acid analysis and immunochemical methods. Guengerich (1979) concluded that there were at least 8-12 forms of cytochrome P450 present in rats under varying conditions. At least 20 different cytochrome P450 types have been found in rabbits, rats and mice.

The molecular basis of induction is thought to involve de novo protein synthesis and administration of protein synthesis inhibitors will prevent the increase in microsomal monooxygenase activity. The effects of a number of inhibitors such as cycloheximide, actinomycin D and α -amanitin have been studied. These inhibit peptidyl transferase, DNA-dependent RNA synthesis and RNA polymerase II respectively and all inhibit induction of cytochrome P450 (Cutroneo and Bresnick,1973; Gelboin and Blackburn,1963; Jacob et al,1974). In addition, induction may be associated with reduced degradation or increased stabilization of components involved in protein synthesis (Seifert and Vacha,1970; Black et al,1971; Pousada and Lechner,1972). It has been suggested that the primary event in induction

is the combination of the inducer with a receptor protein. This mechanism has been proposed for polycyclic aromatic hydrocarbons (Poland and Kende,1976). Lipid peroxides, produced by an uncoupling of electron flow from cytochrome P450 by the binding of poor receptors of active oxygen and subsequent release of singlet oxygen, might control the induction of cytochrome P450 (Paine,1978).

1.1.9 Denaturation and destruction of cytochrome P450

Cytochrome P450 can be converted into its inactive form (cytochrome P420) by a variety of agents which act by blocking or oxidising the thiolate haem ligand or disrupting the membrane environment. These agents include high concentrations of neutral salts such as potassium thiocyanate and sodium iodide (Imai and Sato,1967a), sulphhydryl reagents such as p-chloromercuribenzoate (Mason et al, 1965), cholate (Klingenberg,1958), deoxycholate, phospholipase A and steapsin (Omura and Sato,1964a,b), trypsin and urea (Mason et al,1965). NADPH dependent lipid peroxidation is thought to cause the destruction of cytochrome P450 haem (Levin et al,1973a,b; Jacobson et al,1973). A number of compounds cause a loss of cytochrome P450 on metabolism including allyl substituted barbiturates (Levin et al, 1973a).

A loss of cytochrome P450 occurs after the administration to animals of heavy metals such as cobalt (Tephly and Hibbeln,1971), nickel and platinum (Maines and Kappas,1977) and cadmium (Hadley et al,1974). Heavy metals may cause this conversion by reaction with sulphhydryl groups and/or induction of haem oxygenase (De Matteis,1978).

1.1.10 Sex differences in drug metabolism

Kato (1974) has reviewed the sex related differences in drug metabolism. Hepatic microsomes isolated from male rats can hydroxylate many drugs more rapidly than those from female rats (Conney, 1967; Gillette, 1963; Quinn et al, 1958; Kato and Onoda, 1966) and this is paralleled by greater drug induced spectral changes in cytochrome P450 from male rats, indicating a higher binding capacity of cytochrome P450 (Schenkman et al, 1967; Kato and Onoda, 1970).

Hepatic microsomes from newborn animals have little or no ability to metabolise drugs (Fouts and Adamson, 1959; Jondorf et al, 1959). During the first four weeks after birth there is a similar increase in mixed-function oxidase activity in male and female rats (Conney, 1967). Thereafter sex differences begin to appear, becoming maximal at about 50 days (Quinn et al, 1958; Kato et al, 1962; Murphy and Dubois, 1958).

In male rats, castration decreases the rate of oxidation of some drugs and the binding capacity of cytochrome P450 for these compounds. The administration of androgens restores both deficiencies to the level of intact male rats whereas oestrogen antagonises these effects. The hydroxylation of hexobarbital and the N-demethylation of aminopyrine, as well as the binding capacity of cytochrome P450 with hexobarbital and aminopyrine, are androgen-dependent. The hydroxylation of aniline and zoxazolamine, as well as the binding capacity, are androgen-independent. Thus castration of male rats increased K_m and K_s for androgen-dependent hydroxylations while comparable values for androgen-independent hydroxylations were unaffected (Kato and Onoda, 1970; Schenkman et

al,1967).

The effect of 3-methylcholanthrene treatment on the activity of the microsomal drug metabolising enzymes is sex related. The androgen-dependent activities are decreased in male rats but not significantly affected in female rats whereas androgen-independent activities are increased in both sexes (Kato and Takayanagi,1966). Treatment with thyroxine, morphine and alloxan, adrenalectomy and starvation decreased the metabolism of hexobarbital and aminopyrene and the binding capacity of cytochrome P450 in male but not female rats (Kato and Gillette,1965a,b). These states do not decrease the metabolism of aniline or zoxazolamine or the binding capacity of cytochrome P450 for these drugs in rats of either sex. The reduction in binding capacity of cytochrome P450 might be responsible for the decrease in drug oxidation seen in male rats during these states. These conditions may interfere with the action of androgen to increase the binding capacity of cytochrome P450 (Kato et al,1971; Kato,1974). Androgen has an action on steroid hydroxylation similar to its effect on drug metabolism.

Sex differences in drug metabolism are pronounced in rats, though some strains of mice exhibit a difference in the metabolism of type 'I' substrates (Van den Berg,1978; Noordhoek et al,1978). Guinea pigs, hamsters, rabbits, dogs and monkeys do not show a sex mediated difference in drug metabolism (Quinn et al,1958; Kato et al,1962; Kato et al,1968).

1.2 DRUG METABOLISM IN PREGNANCY

1.2.1 The extent and character of drug consumption during pregnancy

The necessity for closely monitoring the extent and character of drug consumption during pregnancy and relating this to adverse effects on the mother and her offspring has been widely recognised following the thalidomide tragedy of the early 1960's. Drugs administered during pregnancy have as their principal target the mother, while, with very few exceptions (eg. corticosteroids administered to the mother to enhance foetal lung maturation), no therapeutic effect on the foetus is intended.

A number of studies have appeared in which the nature of drug consumption during pregnancy has been monitored (Peckham and King, 1963; Hill, 1973; Forfar and Nelson, 1973; Forrest, 1976; Brocklebank et al, 1978; Doering and Stewart, 1978). Doering and Stewart (1978) in Florida, reported that an average of eleven drug products were consumed in the prenatal period and seven in the perinatal period. This finding was based on a study of 168 patients. The authors pointed out that the average number of drugs consumed does not accurately reflect the number of different chemical entities to which the foetus is exposed since many products contain up to four or five different ingredients. Aspirin appears to be the most common drug consumed during pregnancy and the maternal and foetal effects of this analgesic have been recently reviewed by Corby (1978).

1.2.2 Factors affecting drug metabolism during pregnancy

During pregnancy there are many characteristic alterations in maternal physiology that influence the pharmacokinetic properties of drugs (Krauer and Krauer, 1977). These are reflected in a variation in the plasma concentrations of some drugs in pregnancy compared with the non-pregnant state (Eadie et al., 1977). The influence of pregnancy on drug absorption, distribution and elimination will be briefly discussed.

(1) Drug Absorption

Gastrointestinal absorption: The gastrointestinal absorption of drugs is influenced by several factors such as drug formulation, diet, the chemical composition and pH of the intestinal secretions, gastric emptying time, intestinal motility and intestinal blood flow. In pregnancy, the motility of the stomach and gut and the composition of the intestinal secretions change. A reduction in intestinal motility and an increase of 30-50% in gastric and intestinal emptying time during pregnancy has been reported (Davison et al., 1970; Parry et al., 1970; Prescott, 1974).

Secretions of the stomach have a different biochemical activity (Hunt and Murray, 1958) and there is a reduction in gastric acid secretion and an increase in the secretion of mucus. These alterations result in an increased gastric pH and buffer capacity which influences the ionisation of weakly acid or basic drugs and hence their pharmacokinetics.

Pulmonary absorption: This is known to be influenced by haemodynamic and ventilatory factors. Inhalation agents cross the alveolar membrane at a higher rate during pregnancy since an increased tidal volume produces a

relative hyperventilation (Templeton and Kelman,1976). Furthermore, alveolar drug uptake is also favoured by a higher pulmonary blood flow due to an increased cardiac output.

Intramuscular absorption: Measurements of regional blood flow show a general increase in peripheral tissue perfusion during pregnancy. In late pregnancy blood flow is markedly slowed down in the lower limbs due to an increased hydrostatic pressure in the venous system (Ginsberg and Duncan,1967).

(2) Drug Distribution

Many factors modify drug distribution in pregnancy, including an increased circulating blood (plasma) volume and an increased cardiac output due to a raised heart beat and a greater stroke volume (Walters and Leng Ling,1975). The plasma volume increases by about 50% during pregnancy and cardiac output by about 30%. Renal blood flow is increased by 50% by the end of the first trimester of pregnancy, although hepatic blood flow does not seem to alter (Munnell and Taylor,1947). There is evidence that the plasma or serum protein binding of drugs decreases during pregnancy (Crawford and Hooi,1968; Levy et al,1975). This results in an increase in the free fraction of a drug in the plasma and usually causes a corresponding elevation in total clearance (Levy and Yacobi,1974) combined with changes in the intensity and pharmacological effects of drugs (Levy,1976). A reduction of the serum protein binding of a number of weakly acidic drugs has been reported during pregnancy in the rat (Stock et al,1980). This was associated with an increase in the unbound form of the drug in the plasma and was due, in part, to the accumulation of endogenous displacing agents.

(3) Drug Elimination

Drug elimination is defined as the sum of drug excretion and drug metabolism. Distribution and elimination are interrelated and simultaneously operating processes and any factor affecting one will also affect the other.

Renal drug elimination: Creatine clearance is elevated by 50% during pregnancy and this suggests an increased clearance for drugs which are primarily removed by the kidney (Krauer and Krauer,1977).

Hepatic drug elimination: This will be dealt with in section 1.2.5.

1.2.3 Foetal drug metabolism

A large number of drugs are now known which have undesirable side effects on the developing foetus (Hill and Stern,1979). A number of investigators have found that in laboratory animals the foetal hepatic cytochrome P450 mediated mixed-function oxidase system remains undeveloped (<5% of adult values) even late in gestation (Dallner et al,1966; Jori and Briatico,1973; Hart et al,1962; Rane et al,1973; Gillette,1973; Vainio,1975; Th Cresteil et al,1979). Adult enzyme levels are reached 3-4 weeks after birth (Gram et al,1969; Henderson,1971; McLeod et al,1972; Bresnick and Stevenson,1968). Microsomal levels of cytochrome P450 and NADPH-cytochrome P450 reductase represent less than 14% of adult levels in the full term rat foetus. Drug metabolism in the human foetus, by contrast, develops at a much earlier stage, first becoming apparent in late organogenesis (12-50 days). In the full term human foetus, the hepatic microsomal drug enzyme activity represents 35-40% of adult values (Rane and

Ackeman, 1972).

In most mammalian species studied the hepatic microsomal monooxygenase system will respond to drugs only during the last few days of gestation (Fouts and Devereux, 1972; Hart et al., 1962; Dixon and Willson, 1968; Pantuck et al., 1968), if at all (Schlede et al., 1973; Bresnick and Stevenson, 1968). This is apparently the case for both barbiturate and polycyclic hydrocarbon inducing agents (Conney, 1967). Thus Hart et al. (1962) found that the metabolism of hexobarbital and aminopyrine could be stimulated by phenobarbitone pretreatment only during the last 4 days of foetal life in the rabbit. It has been suggested that this is due to the relatively small amounts of inducing agent actually reaching the foetal liver (Bresnick and Stevenson, 1968; Dixon and Willson, 1968). Recent work in the rat by Th Cresteil et al. (1979) has suggested that 3-methylcholanthrene pretreatment induced a net biosynthesis of cytochrome P450 in the near term foetal liver whereas phenobarbitone pretreatment produces a premature transformation of rough into smooth endoplasmic reticulum thus decreasing ribosomal protein 'contamination' of the 105,000g pellet. As a result, the specific microsomal content of cytochrome P450 appears to be increased although there is no true induction of the haemoprotein. The development of the foetal and neonatal microsomal monooxygenase system has been reviewed by Short et al. (1976).

1.2.4 Placental drug metabolism

The placenta contains very low levels of cytochrome P450, though it does exhibit certain mixed-function oxidase activity, especially that concerning the synthesis of estrogens and the aromatization of androgens. Treatment of pregnant rats with a variety of polycyclic aromatic hydrocarbons known to be present in cigarette smoke produced a large increase in placental benzo(α)pyrene hydroxylase activity (Welch et al., 1969, 1971). Cigarette smoking also markedly increased the activity of benzo(α)pyrene and 3-methyl-4-monomethylaminoazobenzene demethylase in the human placenta. In the placenta of non-smoking mothers these enzyme activities are almost undetectable (Welch et al., 1969). The rat placenta does not show any induction of the drug metabolising enzymes after pretreatment with phenobarbitone, and although pretreatment with 3-methylcholanthrene enhances the activity of benzo(α)pyrene hydroxylase many other monooxygenase activities such as biphenyl 4-hydroxylase, 4-chloromethylaniline N-demethylase, UDP-glucuronyltransferase and p-nitrobenzoic acid reductase remain unaffected (Lake et al., 1973). Similar results were found in the rabbit with the exception that phenobarbitone caused an increase in UDP-glucuronyltransferase activity (Lake et al., 1973). Juchau et al. (1974) reported that there was a marked increase in benzo(α)pyrene hydroxylation and a decrease in cholesterol metabolism in the human term placenta as the number of cigarettes smoked per day increased. It was proposed that smoking might, via its effect on cholesterol metabolism, cause a decrease in the placental synthesis of progestational hormones and oestrogens which are essential for the maintenance of pregnancy. The association between smoking in pregnancy and deleterious effects on foetal wellbeing is now well established (Hill and

1.2.5 Maternal drug metabolism in pregnancy

In recent years a number of publications have appeared on the subject of drug metabolism in pregnancy. These have been concerned primarily with hepatic microsomal Phase I drug metabolism in the rat and rabbit; studies involving the human have dealt with the in vivo pharmacokinetics of a drug.

Pregnancy can influence the activity of certain enzymes not directly concerned with drug metabolism, thus β -glucuronidase activity is increased in rat liver and serum (Bernard and Odell,1950) and in human serum (Fishman,1947; Pulkkinen and Willman,1968). Other serum enzymes have been shown to decrease in activity during pregnancy in women; acetylcholinesterase, lactate dehydrogenase and succinic dehydrogenase are all affected in this way (Visilieu et al,1967). The enzyme system responsible for conjugating bromosulphthalein with glutathione, which is present in the soluble fraction of liver preparations, is decreased in activity during the last trimester of pregnancy in rats (Combes and Stakehem,1962).

Pharmacokinetic studies in the human have revealed an impairment in the disappearance of injected 4-¹⁴C-cortisol from the plasma of pregnant women near term and a decreased rate of appearance of cortisol metabolites in the urine (Migeon et al,1957). Crawford and Rudofsky (1966) found that the metabolism of pethidine and promazine is reduced during pregnancy in the human. This finding was based on a study of the urinary excretion of

these drugs and their metabolites but it has been criticised for the use of too few subjects and for not controlling the pH of the urine (Editorial in the Lancet, 1966). In contrast, Peiker et al. (1979) have found that the half life of orally administered methaqualone was significantly decreased during the last trimester of pregnancy in the human though there was no significant difference in the volume of distribution. Similarly, Ray et al. (1979) have found a decrease in the half life of chlorazepate elimination during pregnancy in the human. The influence of pregnancy on the plasma levels and clearance of primidone has been studied in the mouse (McElhation et al., 1977a,b). On the basis of plasma level determinations following orally dosed primidone it was concluded that there may be an increased rate of metabolism of primidone in the 14 day pregnant mouse compared with the non-pregnant mouse.

The conjugation (Phase 2 metabolism) of drugs and xenobiotics with glucuronic acid was first shown to be inhibited during pregnancy by Cessi (1952) and this has been attributed to inhibition of glucuronyl transferase(s) by high circulating levels of pregnane-3 α ,20 α -diol and other progestational and oestrogenic steroids (Lathe and Walker, 1958; Hsia et al., 1963; Hartiala et al., 1963). Conjugation with sulphate has also been shown to be decreased during pregnancy in the rat and is thought to be due to high levels of oestrogens (Pulkinnen, 1966). Since these early studies various workers have reported decreased hepatic glucuronyl transferase activity during pregnancy of o-aminophenol (Feuer and Liscio, 1969), 4-methylumbelliferone (Neale and Parke, 1973), bilirubin and p-nitrophenol (Halac and Sicignano, 1969) and oestrone and oestradiol (Vore and Soliven, 1979). In contrast, Blake et al. (1978) have failed to find a change in glucuronyl transferase activity during pregnancy using 5-(p-hydroxyphenyl)-5-phenylhydantoin as substrate.

Maternal hepatic Phase '1' metabolism during pregnancy has been the subject of a number of investigations since the early 1960's when it was reported that coumarin and biphenyl hydroxylation were decreased to 50% of normal levels during late pregnancy in the rabbit (Creaven and Parke, 1965). These investigations were concerned primarily with determining the effect of pregnancy on hepatic mixed-function oxidase activity rather than a characterization of the mechanism involved in these changes. The results presented have often been contradictory and confusing and for this reason the various studies have been dealt with in some detail in the following review. This is followed by a summary of the major findings.

Feuer and Liscio (1969) demonstrated that the duration of sleeping induced by phenobarbitone was increased during pregnancy in the rat when dosed on a weight basis. This confirmed work by King and Becker (1963). The authors were also able to show that the hydroxylation of 4-methylcoumarin was reduced during pregnancy and that pretreatment with phenobarbitone or 3-methylcholanthrene induced an increase in enzyme activity in pregnant rats but not to the level of that in induced non-pregnant animals. Induction of the hepatic microsomal mixed-function oxidase system in the pregnant rat has been reported previously (Inscoe and Axelrod, 1969; Pantuck, 1968) although a decrease in enzyme activity in untreated pregnant rats was not found. Kato et al. (1968a) have reported that the effect of phenobarbitone to increase the activities of microsomal drug metabolising enzymes is greater in pregnant rats than in non-pregnant rats

Guarino et al. (1969a) investigated alterations in the kinetic properties of aniline hydroxylase and ethylmorphine N-demethylase associated with pregnancy in the rat. Values for K_m and V_{max} were

determined in control and pregnant rats 6, 20, and 23 (1 day post partum) days after mating. It was found that during pregnancy K_m did not alter significantly while V_{max} was significantly reduced on day 20 of pregnancy. Cytochrome P450 levels paralleled this change in V_{max} , being significantly reduced at day 20 of pregnancy. The authors proposed that high levels of circulating steroids present during pregnancy competitively inhibited microsomal drug metabolism. Neale and Parke (1973) however, point out that it is more likely that the decreased activity of the enzyme system in the full term rat is due to a reduction of the enzymic haemoprotein cytochrome P450 rather than competitive inhibition from steroids. Studies by Peters (1973) using livers from 20 day pregnant rats showed that pregnancy tended to decrease V_{max} for methadone metabolism while K_m remained unchanged. The reported decrease was not, however statistically significant. Gabler and Falace (1970) found that microsomes prepared from the livers of late pregnancy rats hydroxylated aniline at a rate 32% slower than similar preparations from non-pregnant females. Furthermore, the clearance of 5,5'-diphenylhydantoin from several tissues of pregnant rats was markedly slower than from non-pregnant rats.

A survey of the effects of pregnancy on the hepatic microsomal Phase '1' and '2' metabolism of drugs by Neale and Parke (1973) has shown that the full term pregnant rat exhibits decreases in the order of 30% in the specific activities of biphenyl 4-hydroxylase and 4-methylumbelliferone glucuronyltransferase and in the level of cytochrome P450, while biphenyl 2-hydroxylase, p-nitrobenzoic acid reductase and microsomal protein levels are unchanged. Liver weight is elevated by up to 40% in pregnancy and when expressed per whole liver weight there is an increase in both microsomal protein and nitroreductase levels in the pregnant rat and no change in cytochrome P450, glucuronyltransferase or biphenyl hydroxylases when

compared with the non-pregnant rat. The authors suggested that these findings could explain the results obtained for hexobarbital sleeping times since it was found that hexobarbital, when administered to rats at doses related to pregnant weight, significantly increased the sleeping time in pregnant animals. At doses related to non-pregnant weight the sleeping time remained unchanged. Dean and Stock (1975) have pointed out, however, that administering hexobarbital during late pregnancy in a dose based on non-pregnant weight will not give plasma levels equivalent to those in the non-pregnant rat. Neale and Parke determined the effect of inducing agents on the drug enzyme system and found that phenobarbitone and 3-methylcholanthrene pretreatment increased enzyme activities in pregnant and non-pregnant animals to such an extent as to annul the inhibitory effect of pregnancy. The authors suggested that the reduction in microsomal enzyme activity during pregnancy in the rat was due to a parallel decrease in the level of cytochrome P450. A different situation was found in the rabbit where there was no increase in liver weight during pregnancy. Glucuronyltransferase and coumarin 7-hydroxylase activities were significantly decreased during pregnancy while cytochrome P450, microsomal protein, nitroreductase and biphenyl 4-hydroxylase were unchanged from non-pregnant control levels. Devereux and Fouts (1975) have reported that hepatic dimethylaniline demethylation and N-oxidation are unchanged during pregnancy in the rabbit.

Schlede and Borowski (1974) reported that K_m values for ethylmorphine N-demethylase and benzo(α)pyrene hydroxylase did not alter during pregnancy compared with non-pregnant control rats. V_{max} values for these enzymes were found to be significantly decreased during gestation (14 and 21 days) when expressed per gram liver weight and unchanged if expressed per total liver. Cytochrome P450 levels were unaltered during pregnancy if expressed

per gram liver and elevated if the total liver weight was taken into account. Phenobarbitone pretreatment resulted in a significant increase in the V_{max} for benzo(α)pyrene hydroxylase and ethylmorphine N-demethylase in both pregnant and non-pregnant rats though this was larger in the latter group. Phenobarbitone pretreatment caused an elevation in cytochrome P450 levels in non-pregnant rats but not in pregnant rats, a finding also reported by Neale and Parke (1973). Hexobarbital sleeping time was unaffected by pregnancy and decreased after phenobarbital pretreatment.

Dean and Stock (1975) have found a progressive depression in the in vitro hepatic microsomal enzyme metabolism of four drug substrates (aniline, p-nitroanisole, aminopyrine and p-nitrobenzoic acid) during pregnancy. This decrease was most pronounced on day 20 of pregnancy, being greatest with aniline p-hydroxylation and least with p-nitrobenzoic acid reduction (45% and 80% of non-pregnant levels respectively). The depressed metabolism which correlated with prolonged in vivo hexobarbital sleeping times was paralleled by a decrease in hepatic microsomal cytochrome P450 levels and was significant even when calculated per total liver weight. A rapid reversal of this depression was found just prior to parturition. It was suggested that the lower levels of hepatic microsomal enzyme activity might reflect a biological control mechanism to ensure the elevated levels of progesterone required to maintain the pregnant state. At the approach of term, when the elevated levels are no longer required, the inhibition of metabolism would be released.

Feuer and Kardish (1975) studied the effect of pregnancy and the in vivo administration of a number of progesterone metabolites on the hepatic microsomal monooxygenase system in the rat. The authors found that pregnancy (18-20 days) resulted in a significant decrease in the activity

of the microsomal drug enzymes aniline p-hydroxylase, aminopyrine N-demethylase and coumarin 3-hydroxylase and a decrease in microsomal total phospholipid, phosphatidylcholine and phosphatidylethanolamine together with phospholipid synthesis. Microsomal levels of cytochrome P450 and cytochrome c reductase were also significantly decreased. The effect of pregnancy could be mimicked by the pretreatment of non-pregnant females with reduced metabolites of progesterone and it was proposed that increased levels of these metabolites occurring in pregnancy were responsible for the changes observed in drug metabolism. Pretreatment of non-pregnant rats with 5 α -pregnan-3 β -ol-20-one or 5 α -pregnan-3 β , 20 β -diol produced a decrease in microsomal drug hydroxylation and microsomal phospholipids.

Pretreatment with the hydroxylated progesterone metabolite, 16 α -hydroxyprogesterone produced an opposite effect. The authors suggested that these opposite reactions provided evidence that during pregnancy the formation of the endoplasmic reticulum and associated decrease in drug metabolism might be regulated by a balance displayed in the production of the various progesterone metabolites. The role of the reduced metabolites of progesterone in the control of microsomal drug enzyme activity has been discussed previously by Kardish and Feuer (1972) who followed progesterone metabolism in the liver of pregnant (18-20 day) and non-pregnant rats.

During pregnancy they found a decrease in 16 α -hydroxylase activity and an increase in Δ^4 -5 α -hydrogenase activity. The drug enzyme coumarin 3-hydroxylase showed a significant decrease in activity in the pregnant state. More recently, data presented by Feuer and colleagues (Dhami et al, 1979a,b) has shown that pregnancy in the rat is associated with a decrease in microsomal and total hepatic phospholipid fatty acids paralleling the decrease in phospholipids. The ratio of unsaturated to saturated fatty acids did not, however, appear to alter significantly in pregnancy. The work of this group has been reviewed recently (Feuer, 1979)

The effect of pregnancy on hepatic microsomal drug metabolism in rabbits and rats has been investigated by Gut et al. (1976). In pregnant Dutch-Belted rabbits on the 29th day of gestation, the rate of biotransformation of aminopyrine, benzphetamine and hexobarbital by hepatic 9000g supernatant fractions were several fold lower than in non-pregnant females. The liver to body weight ratio was not changed by pregnancy and the optimum pH for in vivo aminopyrine metabolism was between 7.3 and 7.8 for both groups. A different situation was found in the rat where aniline hydroxylase was the only enzyme found to be decreased during pregnancy. Cytochrome P450 levels were unchanged during pregnancy. The duration of hexobarbital sleeping time in pregnant rats (given a standard dose) was not significantly different from that in the non-pregnant rats. The authors suggested that the larger volume of distribution of hexobarbital in the pregnant rat possibly reflected the ability of the foetus to function as a storage compartment since it does not metabolise drugs to any degree.

Tabei and Heinrichs (1976) reported on the effect of pregnancy, in the rat and rabbit, on hepatic dehydroepiandrosterone (DHA) 7 α -, 7 β - and 16 α -hydroxylase and aminopyrine N-demethylase activity. In the rat aminopyrine N-demethylase and DHA 7 α -hydroxylase were significantly elevated early in pregnancy but at the end of gestation enzyme activities had fallen to below the non-pregnant levels. Cytochrome P450 levels remained unchanged during pregnancy. In rabbits cytochrome P450 levels were significantly decreased early in pregnancy (8 days) but not at the later stages. Aminopyrine N-demethylase activity followed these changes. Mukhtar et al. (1978) failed to find a decrease in hepatic cytochrome P450 in late pregnancy (19-20 days) rats compared with non-pregnant controls although a small but significant decrease in arylhydrocarbon hydroxylase activity was found.

The kinetic constants for phenytoin metabolism have been determined in the pregnant and non-pregnant rat (Blake et al, 1978). In late pregnancy (21-22 days) V_{max} for the hydroxylation of phenytoin to 5-(p-hydroxyphenyl)-5-phenylhydantoin was significantly decreased while V_{max} for 5-(3,4,-dihydroxy-1,5-cyclohexadien-1-yl)-5-phenylhydantoin formation was unchanged. Michaelis constants (K_m) for these reactions were unaltered during pregnancy. If the increased liver size in pregnancy was accounted for the total phenytoin hydroxylase activity in pregnant rats was not significantly different from that of non-pregnant controls.

Some general conclusions concerning the effect of pregnancy on the hepatic microsomal mixed-function oxidase system are summarised below:

(1) Hepatic Phase '1' drug metabolism has been determined during pregnancy in the rat and rabbit. Pregnancy in the rat is associated with a marked increase in liver weight; a similar change is not however found in the rabbit.

(2) Hepatic monooxygenase activity, when expressed per unit liver weight, has been shown to be decreased during pregnancy in the rat by most investigators. This decrease appears to be progressive and Dean and Stock (1975) have found it to be rapidly reversible just prior to parturition. When expressed per total liver, monooxygenase activity is decreased or unchanged in the pregnant rat compared with the activity in the non-pregnant controls.

(3) Microsomal levels of cytochrome P450 have been reported variously as increasing, decreasing or remaining unchanged during pregnancy (when expressed per unit liver weight).

(4) The kinetic constants K_m and V_{max} have been measured for a number of drug substrates. V_{max} is reduced during pregnancy in the rat when expressed per unit liver weight while K_m is unchanged.

(5) Microsomal phospholipids have been determined during pregnancy in the rat by Feuer and colleagues. Pregnancy appears to be associated with a decrease in total and component phospholipids. The ratio of saturated to unsaturated phospholipid fatty acids is not altered during pregnancy.

(6) Monooxygenase inducing agents such as phenobarbitone and 3-methylcholanthrene elevate drug enzyme activity in both pregnant and non-pregnant rats. Some investigators have reported that induction abolishes the inhibitory effect of pregnancy while others have found that it does not.

(7) In rabbits there appears to be a decrease in mixed-function oxidase activity during pregnancy when expressed per unit liver weight or per total liver. Cytochrome P450 levels remain unchanged during pregnancy.

(8) A number of authors have discussed the possibility that elevated plasma steroids, particularly progesterone, are responsible for the observed changes in hepatic monooxygenase activity during pregnancy. Steroids could act in a number of ways to mediate this effect, for instance via competitive inhibition of drug metabolism, a decrease in cytochrome P450 levels or by affecting the microsomal membrane (phospholipid) environment of the mixed-function oxidase system. There is a lack of information concerning the effect of progesterone and other steroids on drug metabolism during pregnancy. Feuer and colleagues have studied the effect of the in vivo administration of progesterone metabolites on in

vitro drug metabolism. The effect of progesterone, the predominant steroid of pregnancy, was not, however, studied.

1.3 AIMS OF THIS PROJECT

This project is concerned with a detailed investigation of the effect of steroids and other hormones, that are found to be elevated during pregnancy, on the maternal hepatic mixed-function oxidase system. In addition, the role of cytochrome P450 and microsomal phospholipids in mediating the pregnancy-induced alterations in drug metabolism will be studied. It is hoped that from this a rationalisation of the changes occurring in hepatic drug metabolism during pregnancy in the rat can be made.

CHAPTER 2. MATERIALS AND METHODS

2.1 Materials

Cofactors

β -Nicotinamide adenine dinucleotide phosphate (NADP sodium salt, 98%), β -Nicotinamide adenine dinucleotide (NAD, 98%), β -Nicotinamide adenine dinucleotide phosphate reduced form (NADPH, Tetrasodium salt, 95-97%), β -Nicotinamide adenine dinucleotide reduced form (NADH, disodium salt, 98%), Glucose-6-phosphate (G6P, disodium salt, 98%) and Flavin mononucleotide, 95-97%) were obtained from Sigma London Chemical Co.Ltd., Poole, Dorset, England.

Adenosine-5'-triphosphoric acid disodium dihydrogen salt (ATP, 98%) was obtained from BDH Chemicals, Ltd., Poole, Dorset, England.

Steroids

Pregesterone, oestradiol, cortisol, testosterone and oestriol were obtained from Sigma London Chemical Co.Ltd., Poole, Dorset, England.

Pregnanolone (5 β -pregnane-3 β -ol-20-one), pregnanediol (5 β -pregnane-3,20-dione) and 17 α -hydroxyprogesterone were obtained from Steraloids Ltd., Croydon.

Aniline hydrochloride, *p*-aminophenol, NED (N-1-Napthylethylenediamine dihydrochloride) and *p*-nitrobenzoic acid were obtained from BDH Ltd.,

Poole, Dorset, England,.

p-Aminobenzoic acid was obtained from Sigma London Chemical Co.Ltd.,
Poole, Dorset, England.

PPO (2,5-diphenyloxazole) and DMPOPOP (1,4-di-2-(4-methyl-5-phenyloxazolyl) benzene) were obtained from the Packard Instrument Co., Switzerland.

Specific reagents are dealt with in the Methods section (2.2). All other reagents were of Analar grade.

2.2 Methods

2.2.1 Animals

Female, nulliparous (unless stated) Wistar albino rats (170-250g) were obtained from the University of Surrey Animal Breeding Unit. Animals were kept in polypropylene cages covered with stainless steel tops (North Kent Plastic Cages Ltd.) on sterolite bedding (U.P.Usher Ltd.). Animals were allowed food (Spratts Animal Diet No 1) and water ad libitum. A twelve hour light/dark cycle was in operation (0700-1900 light) at a temperature of 22° and 50% humidity.

2.2.2 Mating of Animals

Mating was carried out on sawdust bedding (Lee and Co., Chertsey, Surrey). A proven male breeder was placed into a large cage containing 6 females (170-190g) during the morning and left for 24 hours. The day of removal of the male was considered to be day 1 of gestation. Pregnancy was indicated by the presence of a vaginal plug and confirmed by abdominal palpation at day 7. Animals were transferred to a sterolite bedding at day 1 of pregnancy or at least seven days before death.

The rats were killed at various times during pregnancy and post-partum. When using post-partum rats the litter was removed from the mother within 16 hours of delivery. Non-pregnant rats in the same weight range at day 0 of pregnancy (170-190g) were used as controls.

2.2.3 Preparation of liver fractions

Rats were killed by cervical dislocation at 9.00am +/- 1 hour and the livers were rapidly excised and placed in ice cold 1.15% (w/v) KCl. The livers were washed, blotted dry and weighed. Subsequent operations were carried out at 0-4°. The livers were scissor minced and homogenised in three volumes (w/v) of ice cold 1.15% (w/v) KCl (ie. 1ml = 250 mg liver; 25%) using a motor driven Potter-Elvehjem glass-Teflon homogeniser (3 return strokes). This was designated 'liver homogenate'.

Liver homogenate was centrifuged in 50ml polycarbonate centrifuge tubes at $10,000g_{av}$ (11,000rpm) for 20 minutes at 4° using an 8x50 aluminium angle-head rotor in an M.S.E. "High Speed 18" refrigerated centrifuge. This removed cellular debris, nuclei, mitochondria and lysosomes. The supernatant was decanted and designated the "microsomal supernatant" and comprised the microsomal and soluble fraction.

The microsomal supernatant was further centrifuged at $105,000g$ (40,000 rev/min) for 1 hour at 4° using an 8x25ml aluminium angle head rotor in an M.S.E. "Superspeed 50" refrigerated centrifuge. The supernatant obtained was discarded and the pellet washed carefully in homogenising medium. The washed pellet was finally resuspended in 20mM Tris-HCL (pH 7.4) buffer containing 250mM sucrose and 5.4mM EDTA to give a concentration equivalent to 500mg wet weight of liver per ml. This was designated the "microsomal suspension" and was stored in 2.0ml aliquots at -20° .

In the determination of glucose-6-phosphatase, glucose 6-phosphate dehydrogenase, malic enzyme, glucokinase, hexokinase, aspartate aminotransferase and glutamate dehydrogenase liver fractions were prepared as follows.

Liver homogenate was prepared as described above in 1.15% (w/v) KCl containing 0.2mM $KHCO_3$ adjusted so that 1g liver = 3ml homogenate. A sample of this homogenate was retained for enzyme assays. The remaining homogenate was centrifuged at $105,000g_{av}$ (40,000 rev/min) for 1 hour at 4° using an 8x25 aluminium angle-head rotor in an M.S.E. "Superspeed 50" refrigerated centrifuge. The supernatant was carefully decanted to avoid contamination from the upper layer of fat and designated the "soluble

fraction".

2.2.4 Serum preparation

Blood was obtained from rats under a light ether anaesthetic by cardiac puncture. Rats were killed by cervical dislocation immediately after blood collection. After clotting in Soveril tubes the blood was centrifuged in an M.S.E. bench centrifuge at 2000rpm for 15 minutes. Serum was carefully withdrawn from the tube using a pasteur pipette and stored in glass vials (Payne 1x5/8" stoppered glass tubes) at -20°.

2.2.5 Animal pretreatment

2.2.5.1 Phenobarbitone

Sodium phenobarbitone (BDH Ltd.) was administered as a solution in 0.9% (w/v) saline. Rats received 80mg/kg phenobarbitone by intra-peritoneal (i.p) injection, once daily (at 9am +/- 1hour) for three consecutive days and were killed 24 hours after the last injection. Pregnant rats received phenobarbitone on days 17, 18 and 19 of gestation and were killed on day 20. Control rats received saline alone.

2.2.5.2 3-Methylcholanthrene

3-Methylcholanthrene (Sigma Chemical Co.Ltd.) was administered as a solution in corn oil. Rats received 20mg/kg by i.p injection daily (at 9am +/- 1 hour) for three days and were killed 24 hours after the last injection. Pregnant rats received 3-methylcholanthrene on days 17, 18 and 19 of pregnancy and were killed on day 20. Control rats received corn oil alone.

2.2.5.3 Progesterone

Chronic progesterone treatment

Rats were treated chronically with progesterone in two ways:

(a) Chronic progesterone treatment by i.p injection

Progesterone was administered (10mg/kg) in corn oil. Rats were injected i.p (at 9am +/- 1 hour) for 12 days and killed 24 hours after the last injection. Control rats received corn oil alone.

(b) Progesterone implants

Autoclaved silicone rubber implants (1cmx0.4cm) containing 32mg progesterone were obtained from Dr.N.F.Cunningham (M.A.F.F. Central Veterinary Laboratory, Surrey). The implants were placed intradermally on the flank of the right leg of rats under a light ether anaesthetic. The wound was closed with Michel clips (Holborn Surgical, London). Control rats were sham operated. Rats were killed 7, 14 and 21 days after insertion of the implant.

Acute progesterone treatment

Rats received progesterone (40mg/kg) i.p in corn oil (at 9am +/- 1 hour) and were killed 60 minutes later. Control rats received corn oil alone.

2.2.5.4 Oestradiol

Oestradiol -17 β was administered by i.p injection in corn oil. Rats received 10mg/kg oestradiol for 10 days and were killed 24 hours after the last injection. Control rats received corn oil alone.

2.2.5.5 Increased dietary choline

Diet Preparation

Spratts No.1 powdered animal diet was mixed thoroughly with choline chloride (Sigma Chemical Co.Ltd.). The final diet contained 1g choline chloride per 100g diet (1% choline diet).

Diet administration

Pregnant and non-pregnant animals (170-190g) received a 1% choline diet ad libitum for 20 days. Body weight gain was monitored throughout the period of feeding. Control animals received a normal diet (Spratts No.1 powdered diet) containing 45mg choline chloride / 100g. Pregnant animals were killed on day 20 of gestation.

2.2.5.6 Human placental lactogen

Source

Human placental lactogen (Ref.No. 70/144) was obtained from the National Institute for Biological Standards and Control (NIBSAC), London.

Administration

Non-pregnant rats received daily (9am +/- 1 hour) i.p injections of 0.22mg human placental lactogen in 0.9% saline (containing 0.22% (w/v) lactose) for six days and were killed on the morning of the seventh day. Control rats received 0.9% saline (containing 0.22% (w/v) lactose) alone.

2.2.5.7 Rat placental homogenate

Preparation of placental homogenate

Placental homogenate was prepared according to the method of Linkie and Niswender (1973). Pregnant rats (13 days) were killed by cervical dislocation and the placentae (5-14 per donor rat) removed. Placentae were dissected free of uterine attachments and embryos and were placed in ice cold saline (0.9% (w/v) NaCl). Combined placentae were homogenised in saline (3.5 placentae per ml) and centrifuged (Beckman J6) at 1000g for 20 minutes at 4°. The supernatant was stored in 3.0 ml aliquots in glass vials at -20° until the morning of administration.

Administration

Non-pregnant rats received daily (9am +/- 1 hour) i.p injections of placental homogenate (2.0 placental equivalents per rat) for 6 days and were killed on the seventh day. Control rats received saline alone.

2.2.6 Drug enzyme assays

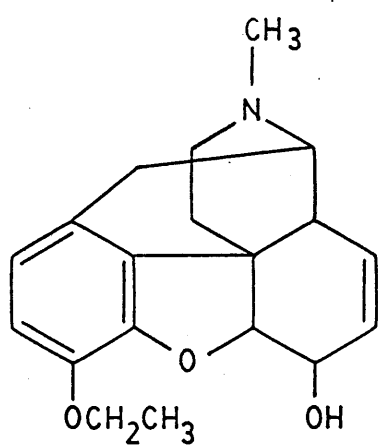
Three drug enzyme activities were measured in the hepatic microsomal supernatant (10,000g supernatant). These were ethylmorphine N-demethylase, aniline p-hydroxylase and p-nitrobenzoic acid reductase.

2.2.6.1 N-demethylation of ethylmorphine

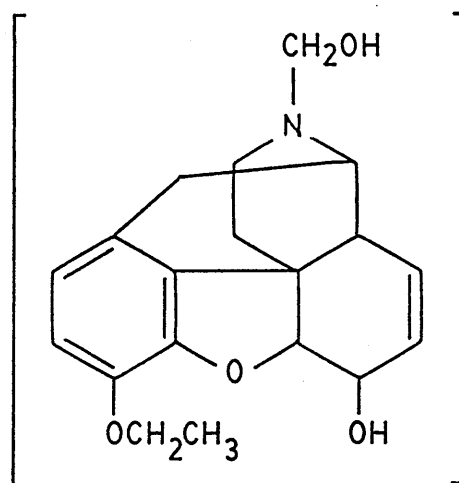
The N-demethylation of ethylmorphine is followed by measuring the formaldehyde formed from the methyl group. Formaldehyde is trapped by semicarbazide. Trapped formaldehyde is then heated with the Nash reagent to form a yellow complex (Nash,1953) The incubation mixture was essentially that of Holtzman et al. (1968), developed in our laboratory by Ioannides (1973).

Reagents

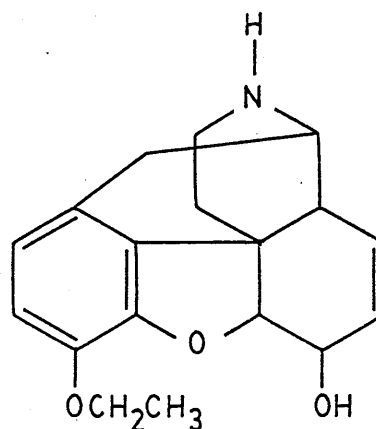
- (1) 0.3M Tris-HCl pH 7.6
- (2) 60mM Ethylmorphine in 0.3M Tris-HCl pH 7.6
- (3) 1.2mM Formaldehyde
- (4) 100mM MgCl₂
- (5) 2% (w/v) Semicarbazide HCl pH 7.0 in 0.3M Tris-HCl
- (6) 30mM Glucose 6-phosphate in 0.3M Tris-HCl (prepared fresh)



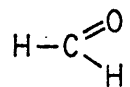
Ethylmorphine



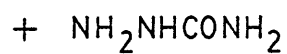
Unstable intermediate



+ HCHO



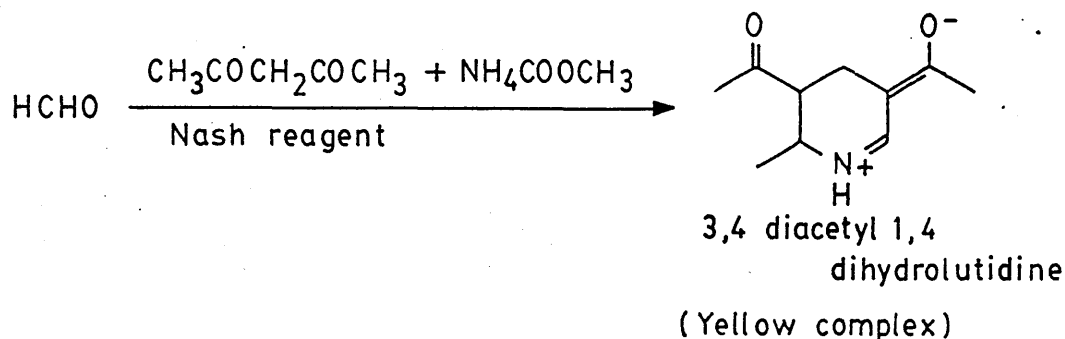
Formaldehyde



Semicarbazide



'Trapped'
Formaldehyde



- (7) 2.5mM NADP in 0.3M Tris-HCl (prepared fresh)
- (8) 15% (w/v) ZnSO_4
- (9) Saturated Ba(OH)_2 solution
- (10) Saturated $\text{Na}_2\text{B}_4\text{O}_7$ solution
- (11) Nash reagent: 4M Ammonium acetate containing 4ml/l acetylacetone
 (Prepared fresh)

Method

The incubation was carried out in soveril tubes (8.0ml). The incubation contained:

0.3M Tris-HCl pH 7.6	0.50ml
100mM MgCl_2	0.10ml
60mM Ethylmorphine	0.25ml
2% (w/v) semicarbazide HCl	0.20ml
30mM Glucose-6-phosphate	0.50ml
2.5mM NADP	0.50ml
Microsomal supernatant (25%)	0.50ml
Total Volume	2.55ml

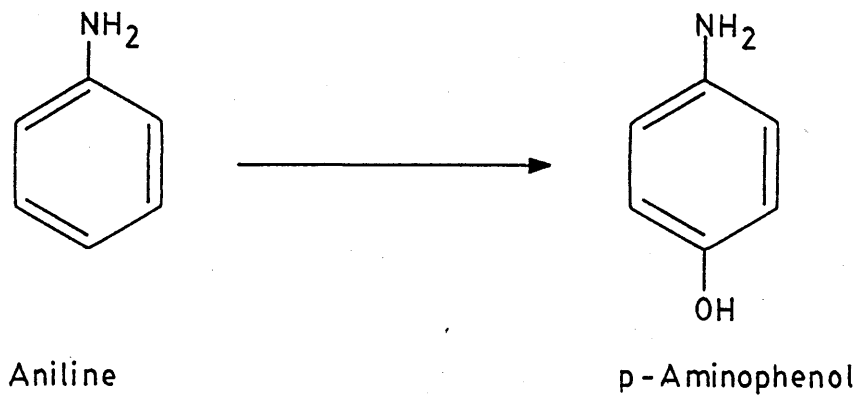
The reaction mixture, excluding microsomal supernatant, was preheated to 37° and the reaction initiated by the addition of microsomal

supernatant. Incubation was carried out for 10 minutes at 37° in a Mickle shaking water bath. The rate of shaking was kept constant at 60 cycles per minute. The reaction was terminated by addition of 15% ZnSO₄ (1.0ml) followed by a mixture (2:1) of saturated solutions of Ba(OH)₂ and Na₂B₄O₄ (1.0ml). The tubes were centrifuged to remove protein at 2,500rpm for 10 minutes in a Beckman J6 centrifuge. Aliquots of supernatant (2.0ml) were added to Nash reagent (2.0ml) in clean test tubes. The mixture was incubated for 40 minutes at 37° in a Mickle shaking water bath (60 cycles per minute).

The yellow colour was measured at 412nm in a Gilford 250 spectrophotometer. Suitable blanks and standards (0.3μmoles) were carried through the same procedure.

2.2.6.2 Aniline p-hydroxylation

Aniline, a type 2 substrate, is metabolised by rat liver microsomes to p-aminophenol. The method is essentially that of Guarino et al. (1969b), developed in our laboratory by Ioannides (1973).



Reagents

- (1) 0.3M Tris-HCl buffer pH 7.6
- (2) 40mM Aniline hydrochloride pH 7.6 in 0.3M Tris-HCl
- (3) 100mM MgCl_2
- (4) 10mM NADP + 100mM Glucose-6-phosphate in 0.3M Tris-HCl (prepared fresh)
- (5) Solid sodium chloride
- (6) Peroxide free ether containing 1.5% (w/v) isoamylalcohol
- (7) 1mM p-Aminophenol in 0.1M HCl
- (8) Alkali phenol: 0.5M Tripotassium orthophosphate + 1% (w/v) phenol (prepared daily)

Method

The incubation was carried out in soveril tubes (15ml). The incubation contained:

0.3M Tris-HCl ph 7.6	0.5ml
40mM Aniline hydrochloride	0.5ml
100mM MgCl_2	0.1ml
10mM NADP + 100mM Glucose-6-phosphate	0.2ml

Microsomal supernatant (25%)	0.5ml
Total Volume=	1.8ml

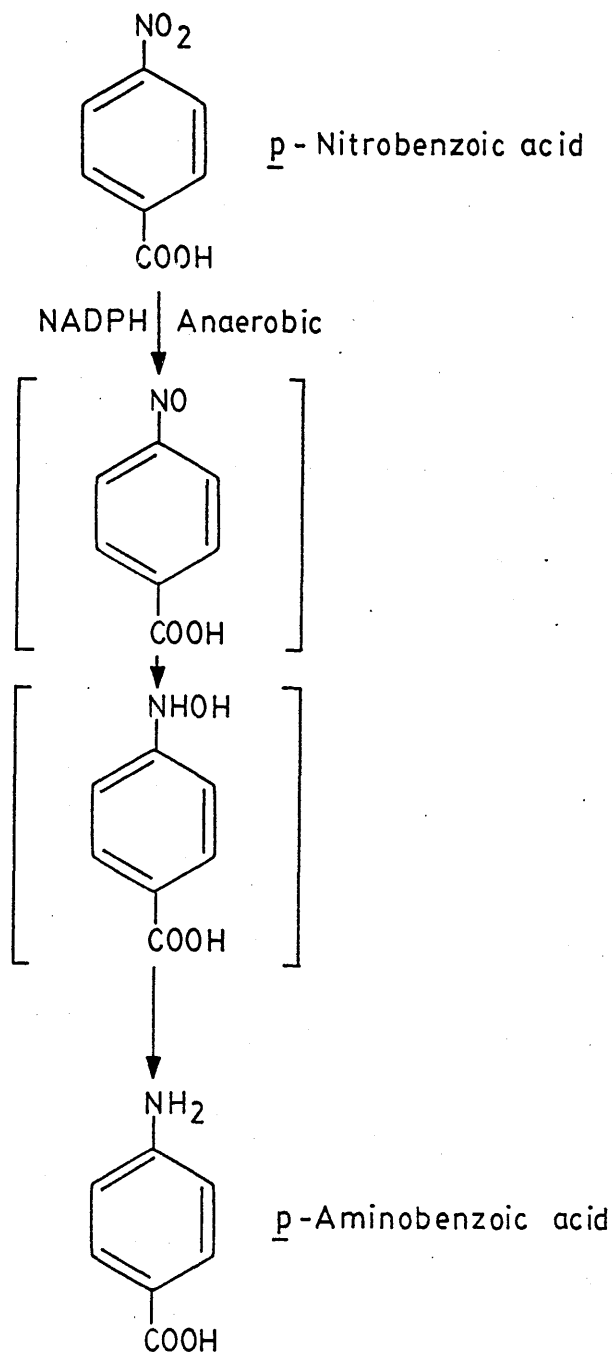
The reaction mixture, excluding microsomal supernatant, was preheated to 37°. The reaction was initiated by the addition of microsomal supernatant and incubation was carried out for 15 minutes at 37° in a Mickle shaking bath. The rate of shaking was kept constant at 60 cycles per minute. Longer incubation times have been shown to result in p-aminophenol disappearance (Mazel, 1972).

The reaction was stopped by the addition of solid NaCl (approx. 1g) and peroxide free ether containing 1.5% (w/v) isoamylalcohol (12ml). p-Aminophenol was extracted into ether on a rotary shaker for 20 minutes. Ether aliquots (10.0ml) were subsequently added to alkali phenol (4.0ml) in clean soveril tubes and extracted for a further 5 minutes.

The ether layer was removed using a glass pipette attached to a vacuum pump. The blue phenol-indophenol complex was measured at 620nm, 30 minutes after addition of phenol in a Gilford 250 spectrophotometer. Suitable blanks and standards (0.1 μ moles p-aminophenol) were carried through the same procedure.

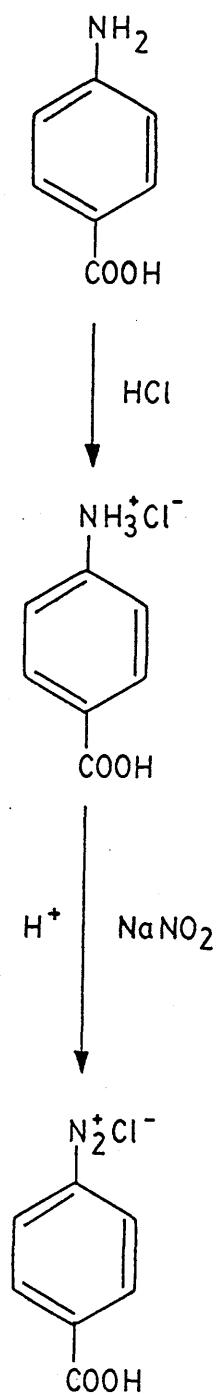
2.2.6.3 Reduction of p-nitrobenzoic acid

Microsomal nitroreductase activity can be determined by measuring the quantity of p-aminobenzoic acid formed from the reduction of p-nitrobenzoic acid, probably through the formation of nitroso- and hydroxylamine intermediates.

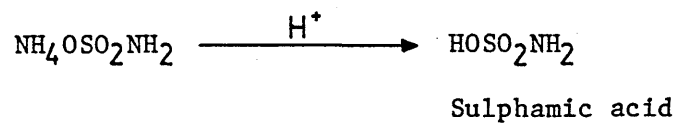


The p-aminobenzoic acid formed is diazotised and then coupled to N-1-naphthyl^henediamine (NED) to yield a coloured complex (Bratton and Marshall, 1939).

The p-aminobenzoic acid is diazotised by addition of sodium nitrite.



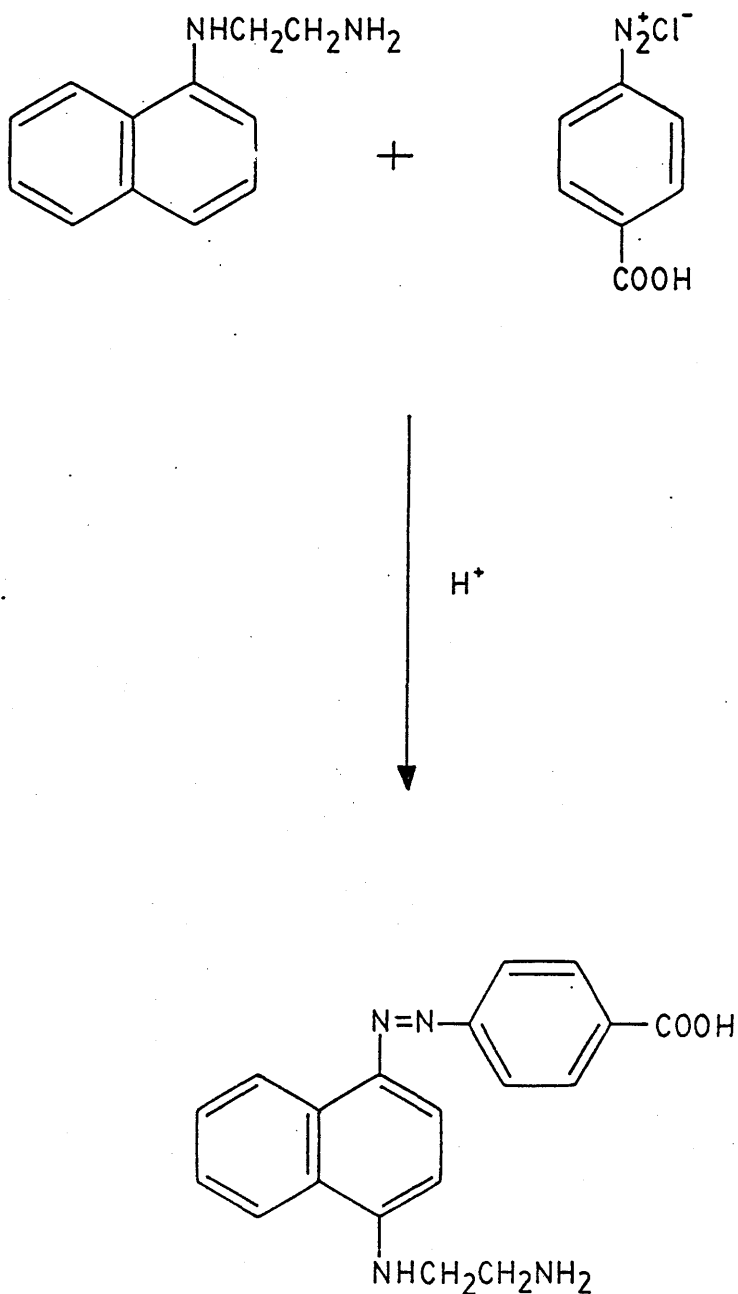
Excess sodium nitrite is removed with ammonium sulphamate



Then



The diazonium salt is coupled with NED:



Incubation was carried out using the method of Fouts and Brodie (1957), developed in our laboratory by Ioannides (1973).

Reagents

- (1) 0.05M Tris-HCl pH7.6
- (2) 10mM p-Nitrobenzoic acid in 0.05M Tris-HCl pH7.6
- (3) 125 μ M p-Aminobenzoic acid in 0.05M Tris-HCl pH 7.6
- (4) 50mM MgCl₂
- (5) 6.6mM NADP in 0.05M Tris-HCl (prepared fresh)
- (6) 250 μ M FMN in 0.05M Tris-HCl (prepared fresh)
- (7) 50mM Glucose-6-phosphate in 0.05M Tris-HCl (prepared fresh)
- (8) 12.5% (w/v) Trichloroacetic acid
- (9) 0.1% (w/v) NaNO₂
- (10) 0.5% (w/v) Ammonium sulphamate
- (11) 0.1% (w/v) NED (N-1-naphthylenediamine)

Method

The incubation was carried out in soveril tubes (8.0ml). The incubation contained:

10mM p-Nitrobenzoic acid	2.0ml
50mM MgCl ₂	0.5ml
6.6mM NADP	0.5ml
250 μ M FMN	0.5ml
50mM Glucose-6-phosphate	0.5ml
Microsomal supernatant (25%)	0.5ml

Total Volume= 4.5ml

Tubes containing the reaction mixture were kept on ice while N₂ was flushed through (1-2 lb/in²) for 10 minutes to maintain anaerobic conditions.

Tubes were transferred to a Mickle shaking water bath at 37° and the incubation was carried out under N₂ for 30 minutes. The rate of shaking was kept constant at 60 cycles per minute. The reaction was terminated by the addition of 12.5% (w/v) trichloroacetic acid (0.5ml). After centrifugation at 2,500rpm for 10 minutes in a Beckman J6 centrifuge an aliquot of the supernatant (2.0ml) was used for diazotization in a clean test tube. Tubes were allowed to stand for 3 minutes after addition of 0.1% NaNO₂ (0.5ml). This was followed by 0.5% ammonium sulphamate (0.5ml) and finally 0.1% NED (0.5ml). The volume was made up to 10ml with distilled water and the mauve colour was determined 30 minutes later at 545nm in a Gilford 250 spectrophotometer. Suitable blanks and standards (0.25 μ moles) were carried through the same procedure.

2.2.7 Studies on the effect of in vitro additions of steroids on drug enzyme activity in the liver

Steroids were dissolved in a suitable volume of dimethylformamide (DMF) (BDH Ltd.) and added to the drug enzyme incubation mixture such that the effects of 10⁻⁹ to 10⁻³M (final concentrations) could be observed. Steroids were added prior to the initiation of the enzyme reaction. The volume of DMF added to the incubation mixture represented 0.56% of the total volume. Control incubates received DMF alone.

Drug enzyme assays were performed as described elsewhere (see 2.2.6).

2.2.8 Measurement of cytochrome P450

Cytochrome P450, the terminal mixed-function oxidase, was measured by making use of its ligand binding properties. Carbon monoxide combines with the reduced form of cytochrome P450 to form a single peak at 450nm when compared with untreated microsomes. The method used was described by Sladek and Mannering (1966).

Microsomal suspension (25%) was diluted threefold with 0.1M phosphate buffer pH 7.4. Diluted suspension (5ml) was divided equally between two 1cm glass cuvettes. A baseline of equal light absorbance between 400 and 500nm was established using a Pye Unicam SP1800 recording spectrophotometer in the dual beam mode. The contents of both cuvettes were reduced by the addition of solid sodium dithionite (1-2mg) and carbon monoxide was bubbled through the contents of the sample cuvette for 20 seconds. A difference spectrum was obtained between 400 and 500nm. The absorbance change at the absorbance maximum (approx. 450nm) was measured relative to that at 490nm, and the concentration of cytochrome P450 was calculated using an extinction coefficient of $91\text{mM}^{-1}\text{cm}^{-1}$ (Omura and Sato, 1964a).

2.2.9 Measurement of cytochrome b5

Cytochrome b5 was measured as the difference spectrum of NADH reduced microsomes against oxidised microsomes. The method of Schenkman et al. (1967) was used. Microsomal suspension (25%) was diluted threefold with 0.1M phosphate buffer pH 7.4. Diluted microsomal suspension (5ml) was

divided equally between two 1cm glass cuvettes.

A base line of equal light absorbance was established between 390nm and 450nm in a Pye Unicam SP 1800 recording spectrophotometer in the dual beam mode. Into the sample cuvette 0.1ml NADH (1mg/ml) was added and a difference spectrum was obtained between 390nm and 450nm. The concentration of cytochrome b5 was determined by measuring the difference in absorbance between 410 and 426nm (ie. trough and peak) using an extinction coefficient of $185\text{mM}^{-1}\text{cm}^{-1}$.

2.2.10 NADPH-cytochrome c reductase

This enzyme is thought to be the same as NADPH-cytochrome P450 reductase. Direct measurement of cytochrome P450 reduction is difficult to carry out routinely so cytochrome c is used as an alternative electron acceptor. The reduction of cytochrome c was monitored spectrophotometrically by using a modified version of the method of Williams and Kamin (1962). Use is made of the difference in absorption between oxidised and reduced cytochrome c at 550nm.

Reagents

- (1) 0.05M Potassium phosphate buffer (pH 7.6) containing 1mM KCN
- (2) 0.1mM Cytochrome c (Boehringer Mannheim GmbH)
- (3) 30mM NADPH in 0.05 phosphate buffer

Method

Into two cuvettes the following were added:

	Test	Reference
Phosphate buffer	1.7ml	1.8ml

Cytochrome c (0.1mM)	1.0ml	1.0ml
Microsomal suspension (25%)	0.2ml	0.2ml
NADPH (30mM)	0.1ml	-
Total Volume=	3.0ml	3.0ml

The cuvettes were preincubated at 25° for 5 minutes. After drawing a baseline the reaction was commenced by the addition of NADPH and followed at 550nm in a Pye Unicam SP 1800 recording spectrophotometer. The initial velocity was taken as the measure of cytochrome c reduction. An extinction coefficient of $18.5\text{mM}^{-1}\text{cm}^{-1}$ was used to calculate the rate of reduction.

2.2.11 Ligand induced binding spectra

Binding spectra were elicited as described by Schenkman et al. (1967). Microsomes (5ml of a suspension containing 2mg protein/ml in 66mM Tris-HCl, (pH 7.4) were divided equally between two cuvettes and placed in a thermostated cuvette holder at 37° in a Varian Cary 219 split beam spectrophotometer. A baseline of equal light absorbance between 350 and 500nm was established. The ligand was added to the sample cuvette in μl quantities of a solution in buffer (Aniline) or N,N-dimethyl formamide (DMF) (Ethylmorphine, Progesterone and 17 α -hydroxyprogesterone) using a Hamilton 10 μl syringe (Hamilton Bonaduz AG, Switzerland). An equal volume of solvent was added to the reference cuvette and the contents of both cuvettes were mixed by gentle inversion. A difference spectrum between 350 and 500 nm was recorded.

2.2.12 Haemoprotein spin state analysis

The spin state of microsomal cytochrome P450 was determined by a modified method of Sligar (1976).

A sample of microsomal suspension was diluted to 1nmol cytochrome P450 per ml in 50mM phosphate buffer, pH 7.25 containing 20% (w/v) glycerol and 10mM EDTA. This was placed in a cuvette in a Perkin-Elmer 356 dual wavelength spectrophotometer with the monochromators fixed at 390 and 420nm. A thermistor temperature probe (type FM, Edale Instruments, Cambridge, England) was placed carefully in the cuvette such that the tip was immersed but the light path was not obscured and connected to a temperature read-out device with a full scale expansion of 0° to 50° (Model C, Edale Instruments, Cambridge, England). The temperature of the cuvette was raised from approximately 14° to 40° by a heated water bath connected to the cuvette housing and the increase in $\Delta A_{390-420}$ was recorded simultaneously with the corresponding cuvette temperature.

The absorbance values of cytochrome P450 corresponding to 100% low-spin material and 100% high-spin material were used as free floating parameters in conjunction with the observed $\Delta A_{390-420}$ values in a one parameter fit procedure described by the equation:

$$K = \frac{A_{LS} - A}{A - A_{HS}}, \text{ assuming } A_{HS} - A_{LS} = 126 \text{mM}^{-1} \text{cm}^{-1}$$

(Cinti et al., 1979).

K is the spin equilibrium constant (defined as the ratio of high-spin to low-spin cytochrome P450). A_{LS} is the delta absorbance of 100% low-spin

cytochrome P450, A_{HS} is the delta absorbance of 100% high-spin material and A is the observed ΔA 390-420 at any given temperature. The spin equilibrium constant was fitted to a van't Hoff plot by reiterative analysis (Cinti et al., 1979), on line to the University of Surrey Computing Centre.

2.2.13 Determination of total lipid and phospholipids

2.2.13.1 Extraction of lipids

Lipids were extracted from liver homogenate (25%) or microsomal suspension (50%) according to the method of Folch et al. (1957).

Reagents

- (1) Chloroform/Methanol (2/1)
- (2) Pure solvents upper phase: Chloroform/Methanol/Water (3/48/47)
- (3) 0.88% (w/v) KCl

Method

An aliquot (0.25ml) of liver sample was pipetted (in duplicate) into a clean 8ml soveril tube. To this was added 4.25ml chloroform/methanol. The tube was stoppered and the contents shaken on a rotary shaker for 10 minutes. The mixture was filtered through a filter paper (Whatman No.1; 7.0cm), moistened with Chloroform/Methanol into a second soveril tube. The remaining contents of the first tube were re-extracted, with a further addition of Chloroform/Methanol (3.0ml), for 10 minutes and then filtered

through the same filter paper to give a total volume of 7.5ml in the second soveril tube. An aliquot (1.5ml) of 0.88% (w/v) KCl was added and the tube shaken for 5 minutes, followed by centrifugation at 2,000rpm on an MSE Bench centrifuge for 5 minutes. The upper phase was removed using a pasteur pipette attached to a vacuum source. Care was taken to ensure that the lower phase was not disturbed. The lower phase was washed three times by the careful addition of small quantities of 'pure solvents upper phase'. These were removed and the lower phase and remaining upper phase combined by the addition of methanol.

The resulting solution was dried down under N₂ in a water bath at 30°. The lipids were redissolved in ice cold chloroform (1.0ml) and the tube was kept tightly stoppered on ice. The lipid extract was used for the determination of total lipid and phospholipid and was stored for up to two weeks at -20°. The efficiency of lipid extraction by this method was found to be >95%.

2.2.13.2 Determination of hepatic total lipids

Total lipids were determined using a Boehringer-Mannheim GmbH Diagnostica Test Kit (Cat.No.124303) for Total Serum Lipids which was modified to enable liver lipids to be measured.

This assay is based on the reaction of lipids with sulphuric and phosphoric acids and vanillin to form a pink coloured complex. This reaction will detect triglycerides, phospholipids and cholesterol.

Reagents

- (1) Colour reagents: phosphoric acid (14mol/l) and vanillin (13mmol/l)
- (2) Lipid standard: total lipids 1,000mg/100ml
- (3) Sulphuric acid (concentrated)

Method

Aliquots (0.1ml) of liver homogenate lipid extract (from 2.3.13.1) were pipetted into clean tubes. Chloroform was removed under N_2 at 37° and distilled water (50 μ l) was added. The following tubes were prepared:

	blank	standard	sample
			(in 50 μ l distilled water)
Lipid standard	-	0.05ml	-
Sulphuric acid	-	2.0ml	2.0ml

The tubes were mixed well, plugged with cotton wool and placed in a boiling water bath for 10 minutes. The tubes were cooled in cold water and the following was pipetted into clean test tubes:

	blank	standard	sample
from solutions above	-	0.01ml	0.01ml
Sulphuric acid	0.10ml	-	-
Colour reagent	2.50ml	2.50ml	2.50ml

The tubes were mixed thoroughly and stood at $20-25^\circ$ for 30 minutes. The pink colour was determined at 546nm in a Gilford 250 spectrophotometer.

2.2.13.3 Determination of total microsomal phospholipids

Phospholipids were determined using the method described in the Boehringer-Mannheim GmbH Diagnostica Test Kit (Cat.No. 124974).

Phospholipids are oxidised to phosphate with perchloric acid and hydrogen peroxide. Phosphate forms a yellow coloured complex with molybdate and vanadate in the presence of nitric acid.

Reagents

- (1) Vanadate solution: Ammonium vanadate (21mM) in 0.28M Nitric acid
- (2) Molybdate solution: Ammonium molybdate (40mM) in 1.25M sulphuric acid
- (3) Standard phosphorus: Disodium hydrogen phosphate (6.44mM)
- (4) Perchloric acid (70%)
- (5) Hydrogen peroxide (30%)

Method

Aliquots of microsomal lipid extract (from 2.2.13.1) (250 μ l) were pipetted into soveril tubes. Chloroform was removed under N₂ at 37° and distilled water (100 μ l) added to the dried lipid extract. The following tubes were prepared:

	blank	standard	sample (in 100 μ l distilled water)
Perchloric acid	0.5ml	0.5ml	0.5ml
Standard phosphorous	-	0.1ml	-
Hydrogen peroxide	0.2ml	0.2ml	0.2ml

The tubes were mixed and placed into an oil bath at 20-25°. The tubes were heated to 180-200° for approximately 20 minutes and then cooled to 20-25°. After cooling distilled water (2.0ml), Vanadate solution (1.0ml) and molybdate solution (1.0ml) were added to all the tubes. After 10 minutes the absorbance was read at 405nm in a Gilford 250 spectrophotometer.

2.2.13.4 Separation and determination of component microsomal phospholipids

Individual phospholipids were separated by thin layer chromatography using a method modified from that of Skipsky et al. (1962).

Reagents

- (1) Solvent system: chloroform (50) : methanol (25) : glacial acetic acid (7) : water (3)
- (2) Iodine crystals (resublimed: obtained from BDH Chemical Ltd., Poole, Dorset)
- (3) Phospholipid standards (2-10mg/ml in chloroform)

<u>Standard</u>	<u>Source</u>
Phosphatidylcholine	Bovine liver III-L
Phosphatidylethanolamine	Bovine brain I
Phosphatidylinositol	Soya bean
Phosphatidylserine	Bovine brain I
L- α -lysophosphatidylcholine	Egg yolk I
Sphingomyelin	Bovine brain I
L- α -phosphoric acid	Egg lecithin I

All standards were obtained from Sigma Chemical Co. (London) and were >98% pure. Standards were stored at -20° in the dark.

Method

Aliquots (500 μ l) of microsomal lipid extract (from 2.2.13.1) were dispensed into flat-bottom glass vials (2x5/8") and the volume reduced to approximately 100 μ l under N₂ at room temperature. The extract was applied to a TLC plate (silica gel G 1500, acid fast, 0.25mm x 20cm x 20cm glass plates obtained from Schleicher and Schull, West Germany) with a 100 μ l microcap dispenser (Drummond Sci.Co., USA) and the chloroform removed under a cold stream of air from a hairdryer. The glass vial was washed twice

with chloroform (50 μ l). The samples were spotted as horizontal streaks approximately 1.0-1.5cm in width. This was found to give better resolution of the component phospholipids. The TLC plates were developed in a TLC tank saturated with the running solvent (chloroform (50) : methanol (25) : glacial acetic acid (7) : water (3)). The run was terminated when the solvent had travelled 15cm from the origin.

The spots were visualised by placing the TLC plate in a tank containing iodine crystals for 5-10 minutes. The iodine vapour in the tank detected most organic compounds, dissipated within 24 hours and did not interfere with subsequent phospholipid analysis. The fractions were identified by comparison with pure phospholipid markers.

<u>Phospholipid standard</u>	<u>Rf value</u>
L- α -lysophosphatidylcholine	0.10
Sphingomyelin	0.30
Phosphatidylcholine	0.42
Phosphatidylinositol	0.44
Phosphatidylserine	0.56
Phosphatidylethanolamine	0.79
Phosphatidic acid	0.95

The phospholipid spots were scraped into soveril tubes to which distilled water (100 μ l) was added. The amount of each phospholipid was determined as described in 2.2.13.3. The presence of silica gel did not interfere with the phosphate determination. After colour development the tubes were centrifuged at 2,500rpm for 5 minutes in a Beckman J6 centrifuge and the supernatant removed for colour determination. The efficiency of recovery of known amounts of phospholipid separated using this method was >95%.

2.2.14 Gel electrophoresis of microsomal protein

The method of Laemmli (1970), developed in our laboratory by Fennell (1980), for sodium dodecyl sulphate (SDS)-polyacrylamide disc gel electrophoresis was used in the analysis of hepatic microsomal proteins.

Reagents

(1) Gel stock: 30% (w/v) acrylamide containing 0.8% (w/v)

N,N-methylenebis-acrylamide (Biorad Biochemicals,USA)

(2) Buffer 1: 1.5M Tris-HCl (pH8.8) containing 0.4% (w/v) SDS (Biorad Biochemicals,USA)

(3) Buffer 2: 500mM Tris-HCl (pH 6.8) containing 0.4% (w/v) SDS

(4) Buffer 3: 25mM Tris-HCl (pH 8.3) containing 192mM glycine and 0.1% (w/v) SDS

(5) Buffer 4: 62.5mM Tris-HCl (pH 6.8) containing 2.3% (w/v) SDS, 15% (w/v) glycerol, 5% (w/v) 2-mercaptoethanol and 0.001% (w/v) bromophenol blue (Biorad Chemicals,USA)

(6) Staining solution: isopropanol (25) : acetic acid (10) : water (65) containing 0.05% (w/v) Coumassie Blue G250 (Biorad Biochemical,USA)

(7) Destaining solution: isopropanol (10) : acetic acid (10) : water (80)

(8) 3% (w/v) glycerol

(9) N,N'-tetramethylenediamine (TEMED) (Biorad Biochemicals,USA)

(10) 10% (w/v) ammonium persulphate (prepared fresh)

(11) 1.5% (w/v) agar (Difco Laboratories,USA)

(12) Protein standards:

<u>Protein</u>	<u>Mol.wt.</u>	<u>Source</u>
Phosphorylase a	92,500	Rabbit muscle (Boehringer Mannheim GmbH)

Bovine serum albumin	68,000	Grade IV (Sigma Chemical Co.)
Catalase	60,000	Beef liver (Sigma Chemical Co.)
Glutamate dehydrogenase	53,000	(Boehringer Mannheim GmbH)
Chicken egg albumin	43,000	Grade V (Sigma Chemical Co.)
Creatinine phosphokinase	40,000	Rabbit muscle (Sigma Chemical Co.)
Lactate dehydrogenase	36,000	Rabbit muscle, type IX (Sigma Chemical Co.)
Cytochrome c	11,700	Horse heart, type VI (Sigma Chemical Co.)

Protein standards were prepared as 0.1mg/ml in Buffer 4.

Method

Electrophoresis was carried out at room temperature in a vertical slab gel apparatus made in the Biochemistry Department workshop. The gel was contained in a glass and perspex cuvette of internal dimensions 120mmx100mmx1.5mm. The perspex component of the cuvette was washed in detergent, rinsed in tap water and then distilled water and dried with tissue. The glass plates of the gel cuvette were treated similarly and in addition rinsed in methanol and then acetone and left to dry. The cuvette was assembled and sealed with 1.5% (w/v) molten agar and then clamped in a vertical position.

The lower (running) gel was prepared by mixing Buffer 1 (10ml), gel stock (13.3ml) and water (16.6ml). Polymerization was initiated by the addition of TEMED (20 μ l) and ammonium persulphate solution (240 μ l). The

solution was poured into the glass cuvette to a height of 80mm. In order to ensure a flat interface between the running gel and the stacking gel a layer of distilled water was introduced above the running gel mixture. When polymerization was complete the water layer was removed.

The upper (stacking gel) was prepared by mixing Buffer 2 (2.5ml), gel stock (1.0ml) and water (6.5ml). Polymerization was initiated by the addition of TEMED (20 μ l) and ammonium persulphate solution (60 μ l) and the mixed gel was added to the cuvette above the running gel. A perspex comb was introduced into the stacking gel before polymerization to form the sample wells. When the polymerization was complete the comb was removed and a small amount of Buffer 3 was introduced into the sample wells to keep them separate. The lower spacer was removed from the cuvette and the cuvette was then placed in the electrophoresis tank. Buffer 3 was added to the upper and lower reservoirs, and air bubbles trapped underneath the gel were removed using a syringe.

Microsomal suspensions were diluted to a final concentration of 1mg protein/ml in sample buffer (4) and placed in a boiling water bath for 3 minutes. After cooling the samples were introduced into the sample wells with a 25 μ l Hamilton glass syringe. The amount of protein applied was 10-20 μ g. A constant current of 20mA was applied until the bromophenol blue entered the running gel whereupon it was increased to a constant current of 40mA. When the bromophenol blue was within 5mm of the end of the gel the electrophoresis was stopped and the cuvette removed from the tank. The glass plates were separated and the gel removed for staining.

The gels were immersed overnight in the Coomassie blue stain. The gel background was destained by immersing in progressive changes of destaining

solution over a period of 48 hours. The gels were stored in 3% glycerol at 4° once the background was cleared.

2.2.15 Hepatic DNA and RNA

Nucleic acid present in liver homogenate is solubilised by treatment with perchloric acid at 70°. DNA can be determined by its reaction with diphenylamine in acid solution (Dische reaction) giving a blue colour (Burton, 1956). RNA is determined colorimetrically after reaction of its ribose component with orcinol (Munro and Fleck, 1966).

2.2.15.1 Solubilization of hepatic nucleic acids

To 2.55ml of liver homogenate (25%) 0.45ml of 3.3M perchloric acid was added. The samples were incubated at 70° for 30 minutes to solubilize nucleic acids. Samples were centrifuged and the supernatant ('hot PCA extract') was retained for DNA and RNA estimation.

2.2.15.2 DNA

Reagents

- (1) 0.5M perchloric acid
- (2) Burton reagent: Glacial acetic acid (500ml) + concentrated sulphuric acid (7.5ml) + diphenylamine (7.5g). Reagents were mixed carefully and stored in a brown glass bottle at room temperature.
- (3) 1.6% (w/v) acetaldehyde: stored at 4°
- (4) Mixed Burton reagent: (prepared fresh) 20ml Burton reagent plus 0.1ml 1.6% (w/v) acetaldehyde
- (5) DNA standard: 100µg DNA/ml in 0.5M perchloric acid. This was heated at 70° for 30 minutes and 0 to 1.0ml used for a standard curve. DNA was obtained from BDH Ltd.

Method

Duplicate 0.2ml aliquots of the 'hot PCA extract' were placed into test tubes and made up to 1.0ml with 0.5M PCA. Mixed Burton reagent (2.0ml) was added, the tubes covered with parafilm and left standing at room temperature in the dark for 18-48 hours. Blanks and standards were treated similarly. The blue colour was measured at 600nm in a Gilford 250 spectrophotometer. A standard curve was constructed and the DNA content of the 'hot PCA extracts' determined.

2.2.15.3 RNA

Reagents

- (1) 20% (w/v) orcinol in 95% ethanol (prepared daily)
- (2) 0.03% (w/v) FeCl_3 in concentrated HCl
- (3) Standard RNA: 100 μg RNA/ml in 0.05M NaOH (0-1.5ml used for the standard curve). RNA was obtained from BDH Ltd.
- (4) 0.05M NaOH

Method

Duplicate 0.1ml aliquots of 'hot PCA extract' were placed into test tubes and made up to 1.5ml with distilled water. This was followed by 0.03% FeCl_3 (1.5ml) and 20% orcinol (0.1ml). The tubes were mixed thoroughly and placed in a vigorously boiling water bath for 30 minutes. A blank and set of standards were treated similarly. Glass marbles were placed on the top of the test tubes while boiling to prevent volume loss. The tubes were cooled in an ice water slurry.

The red colour was read at 655nm in a Gilford 250 spectrophotometer. A calibration curve was constructed and the RNA content of the 'hot' PCA extracts determined.

2.2.16 Determination of hepatocyte size

Hepatocyte size was determined following their isolation by the method of Fry et al. (1976).

Reagents

(1) Phosphate buffered saline 'A' (PBS'A'):

NaCl (8.00g)

KCl (0.20g)

KH_2PO_4 (0.20g)

$\text{Na}_2\text{HPO}_4 \cdot 12\text{H}_2\text{O}$ (2.98g)

Phenol red (0.01g)

The above were dissolved in 1 litre of distilled water.

(2) Hanks B.S.S. ($-\text{Ca}^{++}$ and $-\text{HCO}_3^-$):

NaCl (8.00g)

KCl (0.40g)

KH_2PO_4 (0.06g)

$\text{Na}_2\text{HPO}_4 \cdot 2\text{H}_2\text{O}$ (0.06g)

Glucose (1.00g)

Phenol red (0.01g)

The above were dissolved in 1 litre of distilled water.

(3) Culture medium: Leibowitz L-15 medium supplemented with 10% foetal calf serum and penicillin/streptomycin (100i.u. and 100 g/ml respectively).

(4) 250mM CaCl_2

(5) 0.5mM EGTA

(6) 2.8% (w/v) NaHCO_3

(7) Collagenase (Sigma Type I)

(8) Hyaluronidase (Sigma Type II)

(9) 0.4% Trypan blue solution

Method

- (1) The rat was killed, the abdomen swabbed with 70% alcohol and opened. The liver pieces were excised and placed in a beaker containing PBS'A'.
- (2) Each liver was then briefly blotted on filter paper and placed on a fresh sheet of filter paper supported on a glass plate. A disposable microtome blade (British American Optical Co., Slough) was used to slice the liver. Each new blade was first degreased by wiping it over with carbon tetrachloride followed by 70% alcohol and finally PBS'A'. The liver pieces were sliced to obtain slices approximately 0.5mm thick. The slices were placed in a petri dish containing 10ml PBS'A'.
- (3) When all the pieces had been sliced, the slices plus PBS'A' were transferred to a 250ml conical flask which was then shaken for 10 minutes at 37° (approx. 100 oscillations per minute).
- (4) The flask was removed from the water bath, left for a few seconds (to allow the slices to settle) and the medium carefully removed with a pasteur pipette. Fresh PBS'A' (10ml) was then added to the flask which was reincubated as above.
- (5) Step 4 was repeated once.
- (6) After these washes the slices were incubated as described above (10 minutes shaking at 37°) in 10ml PBS'A' containing 0.5mM EGTA.
- (7) Step 6 was repeated once.
- (8) While the slices were being subjected to EGTA treatment collagenase (5mg) and hyaluronidase (10mg) were weighed into the same container. To these enzymes 10ml Hanks B.S.S ($-Ca^{++}$ and $-HCO_3^-$) was added. When the enzyme was dissolved, 0.2ml of 250mM $CaCl_2$ was added and sufficient 2.8% $NaHCO_3$ to bring the pH to approximately 7.5 (as shown by the phenol red indicator). The enzyme solution was then ready for use.
- (9) When step 7 was completed the EGTA solution was carefully removed and replaced with enzyme solution. The flask was then shaken for 1 hour at

37°.

(10) At the end of this time the cloudy suspension was passed through a single layer of Bolting cloth ("Nybolt", 125 μ m pore size. British agents: John Staniar and Co., Sherbourne Street, Manchester, M3 1FD) into a beaker. The slices remaining in the flask received 5ml fresh PBS'A' and the whole was gently swirled to loosen any extra cells. The solution and slices were then placed and the slices were gently agitated on the cloth with a Pasteur pipette to complete the cell dissociation.

(11) The cell filtrate was transferred to a 50ml glass centrifuge tube which was then spun at 50g_{qv} for 1 minute.

(12) The supernatant was carefully removed and discarded and 10ml fresh PBS'A' was pipetted onto the pellet and once sucked up and down the pipette to disperse the cells.

(13) Steps 11 and 12 were repeated twice

(14) The cell pellet was finally resuspended in 10ml culture medium and after thorough mixing a 0.25 ml sample was mixed with 0.1ml of 0.4% trypan blue solution and the yield and viability determined with an improved Neubauer counting chamber. The cell suspension was then ready for use.

Cell yield = cell count $\times$ $7/5 \times 10^4$ cells per ml

%viability = No. of viable cells $\times$ 100 / Total No. of cells

(15) Hepatic size was determined under the microscope using a graticule eyepiece, calibrated so that 100 divisions was equivalent to 0.25mm (1 division = 2.5 μ)

2.2.17 Hepatic glycogen

The determination of hepatic glycogen was based on the method of Keppler and Decker (1974). Liver homogenate was treated with perchloric acid and the protein removed by centrifugation. The resulting supernatant was incubated with amyloglucosidase releasing free glucose from the glycogen. Free glucose was converted to glucose-6-phosphate with hexokinase and ATP and thence to 6-phosphogluconate with glucose-6-phosphate dehydrogenase. This latter conversion produced reduced NADP which was followed spectrophotometrically at 340 nm.

Reagents

- (1) 0.6M Perchloric acid (PCA)
- (2) 1.0M Potassium bicarbonate
- (3) 0.3M Triethanolamine buffer pH 7.5 containing 4mM MgSO_4
- (4) 0.2M Acetate buffer pH 4.8
- (5) Amyloglucosidase: 50mg/ml Agidex in acetate buffer (prepared daily) (BDH Ltd.)
- (6) 12mM NADP
- (7) 15mM ATP
- (8) Hexokinase / Glucose-6-phosphate dehydrogenase (Sigma Chemical Co.Ltd.)

Method

An aliquot (0.5ml) of liver homogenate (10%) was thoroughly homogenised with 5 volumes of ice cold 0.6M perchloric acid. Immediately after homogenisation 0.2ml of homogenate was pipetted into a test tube. This was neutralised by the addition of 1M KHCO_3 (0.1ml). Amyloglucosidase suspension in buffer (2ml) was added and the tube incubated in a Mickle shaking water bath at 37° for 2 hours. The rate of shaking was kept

constant at 60 cycles per minute.

The reaction was stopped with PCA (1.0ml) and the precipitate removed by centrifugation at 2,500rpm for 15 minutes in an M.S.E. bench centrifuge. An aliquot (0.2ml) was used for glucose determination.

A sample blank was prepared by omitting homogenate and replacing it with 0.6M PCA. An enzyme blank was prepared by centrifuging 0.5ml of homogenate (10%) with 5 volumes of ice cold 0.6M PCA. This was neutralised with solid KHCO_3 , centrifuged and an aliquot of supernatant (0.2ml) taken for glucose determination.

The incubation mixture for the glucose determination consisted of:

Triethanolamine buffer	(2.5ml)
Sample	(0.2ml)
12mM NADP	(0.1ml)
15mM ATP	(0.1ml)

The reaction was started by the addition of $10\mu\text{l}$ of hexokinase / glucose-6-phosphate dehydrogenase. The absorbance was read at 340nm at time 0 and after 15 minutes incubation at 30° . A blank was prepared by replacing sample with buffer.

2.2.18 Progesterone release from silicone implants

Progesterone release, from silicone rubber implants (see 2.2.5.3), was determined according to the method of Symons et al. (1974).

Method

Silicone implants were removed from experimental animals on death, washed briefly with 0.9% (w/v) NaCl (isotonic saline) and then placed into conical flasks with 15ml isotonic saline. The flasks were shaken (100 oscillations/minute) in a Mickle shaking water bath at 37°. Every 24 hours for 3 days the saline was replaced and the progesterone concentration in the old saline determined. Extinction values at 250nm were determined using quartz cuvettes in a Gilford 250 spectrophotometer and compared to those obtained with a range of progesterone standards in saline (0-100µg/L). Saline samples were diluted as necessary to bring the solution within the range of the progesterone curve.

Progesterone release from unused autoclaved silicone implants was also determined. These were shaken at 37° for 21 days in saline which was changed at 24 hour periods.

2.2.19 Liver histology

2.2.19.1 Tissue fixation, processing and section cutting

Fixation

Pieces of tissue were fixed in 10% neutral buffered formalin for several days.

Formula for 10% neutral buffered formalin:

Formalin (40% formaldehyde) (100ml)

Sodium dihydrogen orthophosphate ($\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$) (4.5g)

Anhydrous disodium hydrogen orthophosphate (Na_2HPO_4) (6.5g)

Distilled water (to 1 litre)

Processing

The tissue was embedded in paraffin wax by the following method:

A piece of liver 2-3mm in thickness was cut from each sample and placed in a metal processing container together with the appropriate reference number. The containers were placed in a tissue basket which was then fitted to a Histokinette automatic tissue processor. Tissues were transferred automatically from one beaker of fluid to the next and the processes of dehydration, clearing and impregnation with wax were carried out using the following processing schedule:

Dehydration

70% alcohol

1 hour

85% alcohol	1 hour
95% alcohol	1 hour
100% alcohol I	1 hour
100% alcohol II	1 hour
100% alcohol III	1 hour

Clearing

Toluene I	1 hour
Toluene II	1 hour

Impregnation

Paraffin wax I at 58°	1 hour
Paraffin wax II at 58°	1 hour

The melting point of the wax was 56°. After this process the tissue containers were transferred to a vacuum embedding oven containing paraffin wax at a temperature of 58° for 1/2 hour. This acted as a third wax bath and the reduced pressure aided impregnation by ensuring that any remaining air bubbles and clearing agent were removed. The tissue was blocked out by removing it from the container using a pair of electrically heated forceps and placing it in a plastic mould filled with molten wax. The tissue was orientated so that the surface to be cut rested on the base of the mould. A plastic block was placed in the mould with the reference number label.

Section cutting

Sections were cut from the blocks at a thickness of 7 μ using an American Optical Spencer 820 Rotary microtome. The sections were floated on distilled water at a temperature of 50° until the creases disappeared. The sections were mounted on slides by half submerging a clean slide into the water near the section and withdrawing it, so bringing the flattened

section with it. The slides were placed initially on a drying hot plate and then left in an incubator at a room temperature of 37° overnight to dry.

Sections prepared in this way were stained with Haematoxylin and Eosin or by the Periodic acid-Schiff (P.A.S) technique.

2.2.19.2 Tissue staining with Haematoxylin and Eosin

Sections prepared in 2.2.19.1 were stained with Ehrlich's acid haematoxylin (obtained from R.A.Lamb Ltd) and eosin (colour index No.45380 obtained from R.A.Lamb Ltd.).

Slides were placed in:

- (1) Xylene to remove wax (2 minutes)
- (2) Absolute alcohol (1 minute)
- (3) 70% alcohol (1 minute)
- (4) 50% alcohol (1 minute)
- (5) Distilled water (rinse)
- (6) Ehrlich's acid haematoxylin (15 minutes)
- (7) Tap water (5 minutes)
- (8) Differentiated in acid alcohol (1% HCl in 70% alcohol) by agitating (5 seconds)
- (9) Returned to tap water
- (10) Examined under low power microscope to ensure the sections were sufficiently differentiated
- (11) Tap water (15 minutes)
- (12) Counterstained in 1% aqueous Eosin (2 minutes)

- (13) Rinsed in tap water (1/2 minute)
- (14) Dehydrated in 85% alcohol (1/2 minute)
- (15) Dehydrated in 100% alcohol (1/2 minute)
- (16) Dehydrated in 100% alcohol (1/2 minute)
- (17) Cleared in xylene (1/2 minute)
- (18) Cleared in xylene (1/2 minute)

The sections were mounted in D.P.X. (B.D.H Ltd.) by placing a drop of the mountant on a clean coverslip and pressing gently so that the mountant spread under the coverslip. Slides were examined at magnifications of x40, x100 and x400 using a Vickers M15c microscope. Using this stain the nuclei appeared blue and the cytoplasm pink.

2.2.19.3 Periodic acid-Schiff (P.A.S) staining technique

This technique was used to stain for liver glycogen. The reaction was based on the fact that certain tissue elements were oxidised by periodic acid producing aldehydes which were then demonstrated with Schiff reagent.

Periodic acid cleaves C-C bonds where these carbon atoms have adjacent hydroxyl (-OH) groups or adjacent hydroxyl and amino groups.

Glycogen can be distinguished because it is selectively digested by enzymes (diastase, amylase or 'saliva') into smaller water soluble molecules which can be removed by washing.

Method

Liver sections prepared in 2.2.19.1. were immersed in water and taken through the following procedure:

- (1) Oxidised in 1% periodic acid (5 minutes)
- (2) Washed in gently running water (5 minutes)
- (3) Rinsed in distilled water
- (4) Treated with Schiff reagent (Feulgen reagent obtained from R.A.Lamb Ltd.) (15 minutes)
- (5) Washed in gently running water (10 minutes)
- (6) Counterstained lightly with 1% light green (alcoholic) (colour index 42095, obtained from R.A.Lamb Ltd.) (5-10 seconds)
- (7) Dehydrated in 100% alcohol (1/2 minute)
- (8) Dehydrated in 100% alcohol (1/2 minute)
- (9) Cleared in xylene (1/2 minute)
- (10) Cleared in xylene and mounted in D.P.X. (1/2 minute)

Result

P.A.S. positive substances : bright pink

Other tissue constituents : pale pink

The P.A.S. technique, when used to detect glycogen, included a diastase control.

Control slide

- (1) Immersed in water
- (2) Incubated at 37° in 0.1% diastase (obtained from R.A.Lamb Ltd.) (30 minutes)

(3) Washed in running water (5 minutes)

Control and experimental slides were taken through the P.A.S. procedure simultaneously.

2.2.19.4 Oil Red O staining technique for lipids

This technique was based upon the fact that Oil Red O was more soluble in tissue lipids than in the solvent system used.

Reagents

(1) Staining solution: A saturated solution of oil red O (0.25-0.5%) in propan-2-ol was prepared as a stock solution. For use stock solution (6ml) was diluted with distilled water (4ml) and allowed to stand for 5-10 minutes. The stain was filtered and used within 1-2 hours. Oil red O (colour index 26125) was obtained from R.A.Lamb Ltd.

(2) 60% (v/v) alcohol

(3) Harris's Haematoxlin (obtained from R.A.Lamb Ltd.)

(4) Acid alcohol: 1% HCl in 70% alcohol

(5) Borax: saturated aqueous sodium tetraborate

Method

Liver tissue was fixed (see 2.2.19.1), frozen and cut to 10μ on a Reichert sledge microtome. Sections were stained using the following procedure:

(1) Washed well in water

(2) Placed in stain in a sealed container for 10-15 minutes

- (3) Rinsed in 60% alcohol to clear background
- (4) Washed in tap water
- (5) Nuclei stained lightly in Harris's haematoxylin (2 minutes)
- (6) Washed in water
- (7) Differentiated in acid alcohol (2 or 3 quick dips)
- (8) Washed in water
- (9) Blue-ed in borax for 10 seconds (colour intensity checked microscopically)
- (10) Washed in tap water
- (11) Mounted in glycerin jelly

Results

Lipids stained bright red and nuclei stained blue.

2.2.19.5 Calculation of hepatic mitotic index

Liver sections stained with Haematoxylin and Eosin were observed at a magnification of x200 using a Vickers M15c microscope with a graticule eyepiece. The number of mitoses were counted in 40 grids (approximately 12,000 cells). The grid was moved across the whole section avoiding large vessels. The mitotic index was expressed as the number of mitoses in 10,000 cells.

2.2.20 Progesterone radioimmunoassay (RIA)

Progesterone was determined by a radioimmunoassay developed by the Epsom Hospital Laboratories (West Park Hospital, Epsom, Surrey).

Reagents

(1) Assay buffer: pH 7.0:

0.1mol/l phosphate buffer

1g/l gelatine powder (BDH Ltd.)

1g/l sodium azide (BDH Ltd.)

The ingredients were dissolved in distilled water (gentle warming in 45° water bath) and made up to 1l when cool. The assay buffer was stored at 4°.

(2) Charcoal suspension: 1.75g Norit A charcoal (Sigma Chemical Co. Ltd.) was dissolved in 250ml assay buffer in a bottle containing a 'Teflon' coated stirring magnet. The contents were stirred for 2 hours and stored at 4°. A 24-hour equilibrium period was allowed after preparation before using the suspension for the first time. The suspension was mixed well before use and stirred continuously while dispensing.

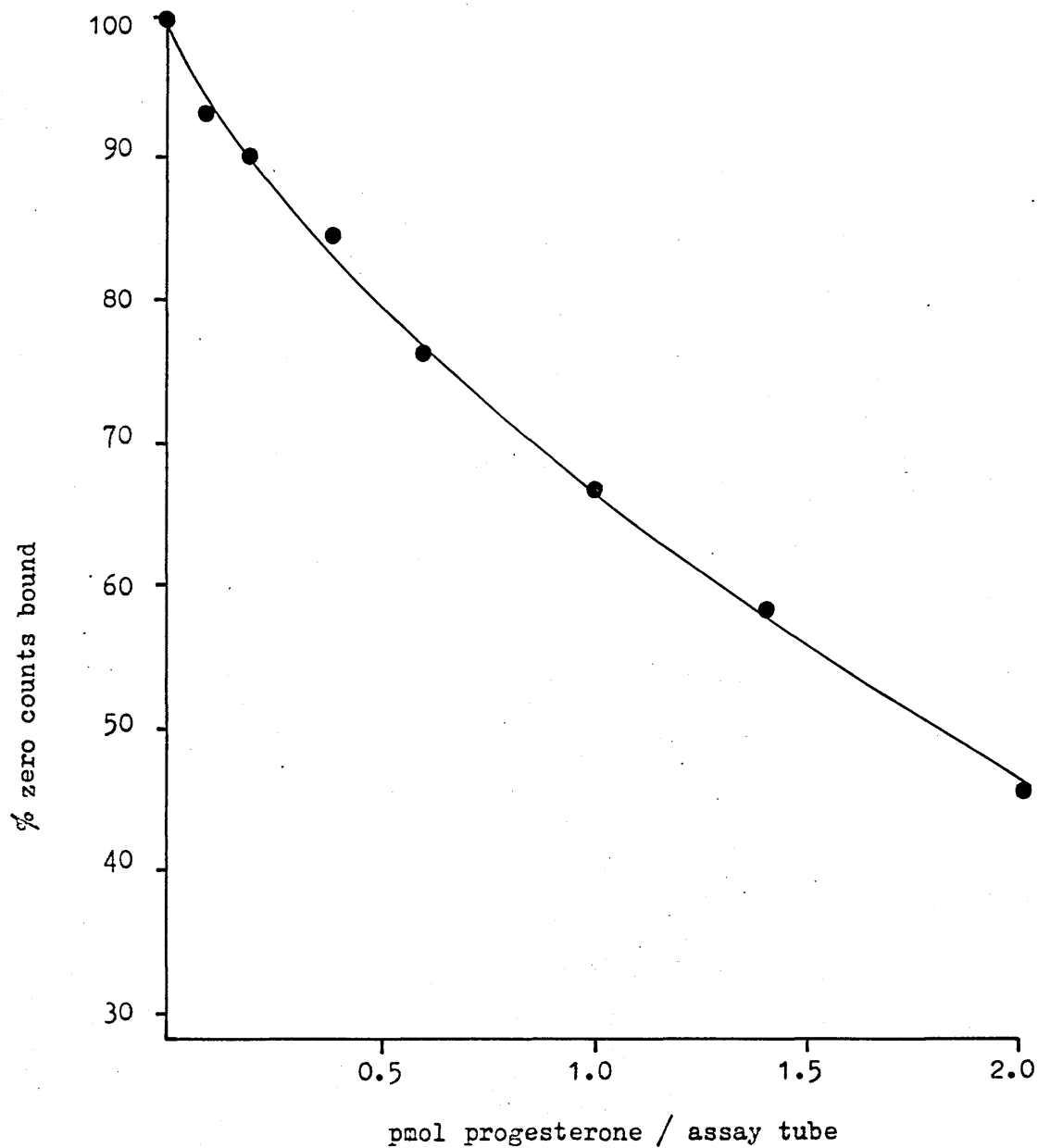
(3) Liquid scintillation solution: 5g PPO (2,5-diphenyloxazole), 0.5g DMPOPOP (1,4-di-2- (4-methyl-5-phenyl oxazolyl) benzene) and 330ml of metapol (Durham Chemical Distributors) were made up to 1 litre with toluene.

(4) Petroleum spirit, 40-60° : (BDH Ltd., AR grade)

(5) Standards (in absolute ethanol): A stock solution of progesterone (2mmol/l) was diluted in stages to 20nmol/L. A range of working standards were prepared from this solution with concentrations 0, 1, 2, 4, 6, 10, 14, 20, 30 and 40nmol/l. All standards were stored at -20°.

(6) Tracer: (1, 2, 6, 7 -³H) progesterone (specific activity 85

Figure 2.1 Progesterone radioimmunoassay standard curve



The standard curve was produced using a final antiserum dilution of 1/4000. The radioactive tracer final dilution was 1/400. The percentage of total counts bound (in the 0 pmol progesterone standard tube) was 48%. None specific binding was <5%.

curies/mmol, obtained from The Radiochemical Centre, Amersham) was diluted to a radioactive concentration of 50mCi/L with the same solvent as already present. This was stored as a stock solution at -20° in its original container. A working solution (1/200 dilution of the stock solution) was prepared by drying down an aliquot of stock solution under N_2 for a minimum time and redissolving immediately in assay buffer.

(7) Antiserum: (Guildhay Antisera HP/S/53-IIA, from the Division of Clinical Biochemistry, University of Surrey, Guildford, England). Neat antiserum was stored at 4° . An aliquot of neat antiserum (100 μ l) was diluted to 5ml in assay buffer to give a dilution factor of 50. This stock solution was stored at 4° . The working antiserum solution (1/2000 dilution of the stock solution) was prepared freshly for each assay batch by diluting an aliquot of stock.

(8) Controls: Within bath and between batch duplication was checked with a series of serum controls.

Method

(1) Water blanks were prepared by pipetting 20 μ l distilled water (in duplicate) into clean 12x75mm glass tubes.

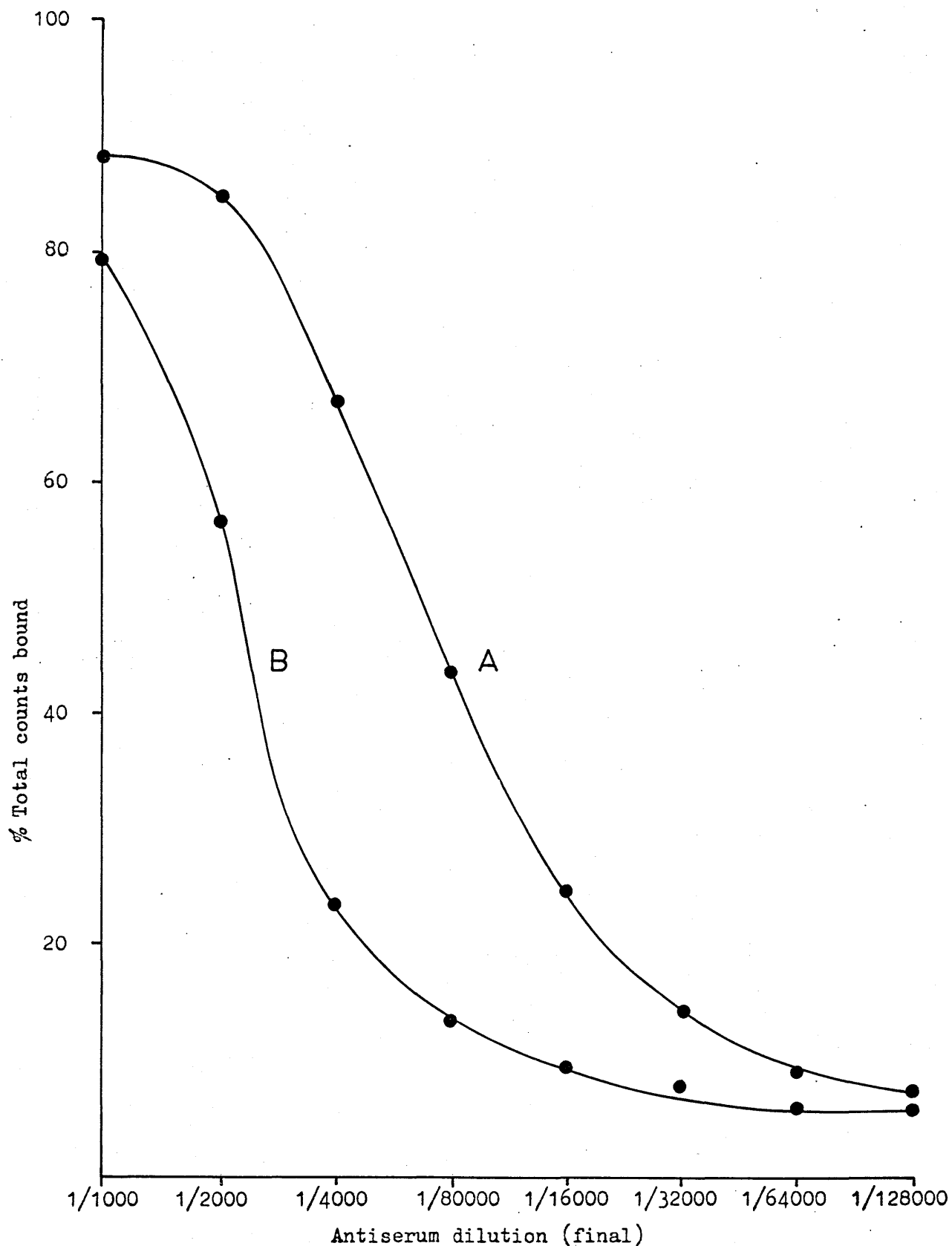
(2) An aliquot (20 μ l) of the serum sample was pipetted (in duplicate) into clean 12x75mm glass assay tubes. Dilutions of the original serum sample were sometimes required depending on the source of the sample.

(3) Petroleum spirit (2ml) was added to the tubes which were sealed with polythene caps.

(4) Progesterone was extracted for 15 minutes on an orbital shaker (Luckman Ltd.) at maximum speed. Care was taken to ensure that the contents of the tube did not come into contact with the polythene caps. After shaking the tubes were allowed to stand for 5 minutes.

(5) The organic layer was carefully decanted into another clean 12x75mm

Figure 2.2 Progesterone antiserum dilution curve



Line A: Antiserum dilution curve with a 1/400 final dilution of tritiated progesterone in the absence of cold progesterone.

Line B: Antiserum dilution curve in the presence of cold progesterone (2 pmoles / assay tube)

glass tube (the aqueous layer remained adhering to the original tube without the need for freezing).

(6) The petroleum spirit was dried under N_2 at 45° in a fume cupboard for a minimum of time.

(7) Standards ($100\mu l$) were pipetted (in duplicate) into clean 12×75 mm glass tubes, giving a range of 0, 0.1, 0.2, 0.4, 0.6, 1.0, 1.4, 2.0, and $3.0 \mu mol/tube$. These were dried under N_2 at 45° for a minimum time.

(8) Duplicate 'total' 12×75 mm glass assay tubes were prepared containing $400\mu l$ assay buffer.

(9) Duplicate 'Norit Blank' 12×75 mm glass assay tubes were prepared containing $200\mu l$ assay buffer.

(10) Working tracer solution ($200\mu l$) was added to all tubes.

(11) Working antiserum solution ($200\mu l$) was added to all tubes except the 'total' and 'Norit blank' tubes.

(12) All tubes were vortexed thoroughly.

(13) The tubes were then incubated for 30 minutes at 37° and then overnight at 4° .

(14) To all assay tubes except the 'totals' $200\mu l$ of stirred charcoal suspension was quickly added. The tubes were vortexed quickly.

(15) The tubes were left in ice-water for 10 minutes followed by centrifugation at 2500rpm for 5 minutes at 4° .

(16) The tubes were returned to the ice-water bath and $400\mu l$ of the supernatant transferred to polythene counting "mini vials" (G.D.Searle and Co.Ltd.).

(17) Liquid scintillation solution ($4.2 ml$) was added, the tubes capped and shaken and placed inside glass counting vials. The tubes were allowed to equilibrate for 30 minutes before counting for 10 minutes. Counting was performed in an LKB Ultra beta liquid scintillation counter.

(18) Serum progesterone levels were determined from a calibrated curve (Figure 2.1). The calibration curve shows the percentage of zero counts bound (ie. the 0 pmol progesterone standard = 100% and other standards were expressed as a percentage of this) against pmol progesterone/assay tube. The progesterone concentration in the original specimen (nmol/l) = 50x amount found (pmol) for undiluted specimen. The avidity of the progesterone antiserum is shown in an antiserum dilution curve (Figure 2.2).

Formal cross reaction of Antiserum HP/S/53-IIA with steroids

Formal cross reaction was measured at 'half displacement' of the progesterone tracer from its zero standard level (data supplied by P.Butler, Equine Research Station, Suffolk).

<u>Steroid</u>	<u>Formal Cross Reaction</u>
Progesterone	100
11-Deoxycorticosterone	0.43%
Corticosterone	0.5%
Cortisol	0.003%
Testosterone	0.06%
17 β -oestradiol	<<0.0006%
Prednisolone	<<0.009%

2.2.21 Microsomal protein

Microsomal protein was determined according to the method of Lowry et al. (1951), modified by Miller (1959).

Reagents

- (1) Copper reagent: prepared fresh by mixing, in order, 10% (w/v) sodium carbonate in 0.5M NaOH (20ml), 2% (w/v) aqueous Sodium Potassium Tartrate (1ml) and 1% (w/v) aqueous Copper Sulphate (1ml).
- (2) Folin-Ciocalteu Phenol Reagent (BDH Ltd.): diluted 1:6 for use
- (3) Bovine serum albumin (Sigma Chemical Co. Ltd.): 250 μ g/ml in water (prepared fresh)

Method

Microsomal suspension was diluted in water to give a protein concentration of less than 250 μ g/ml (normally a final dilution of 160). Diluted microsomal sample (1ml) was added to Copper Reagent (1ml) and left for 10 minutes at room temperature. This was followed by diluted Folin-Ciocalteu Phenol reagent (3mls). The mixture was mixed immediately and incubated for 10 minutes at 50°. The tubes were cooled in iced water and the blue colour read at 540nm in a Gilford 250 spectrophotometer. Soluble blanks and standards (0-250 μ g bovine serum albumin) were taken through the same procedure.

2.2.22 Hepatic non-drug metabolising enzyme assays

2.2.22.1 Glucose-6-phosphatase (EC. 3.1.3.9)

This determination was based on the method of Noordlie and Arion (1966), developed in our laboratory by Curtin (1981).

Reagents

- (1) Tris-maleate buffer 0.05M pH 6.7
- (2) 175mM Glucose-6-phosphate
- (3) 10% (w/v) Trichloroacetic acid (TCA)
- (4) Acid ammonium molybdate: 2.5% (w/v) in 2.5M H_2SO_4
- (5) ANS Reducer: 1-Amino, 2-Naphthol, 4-Sulphonic acid (0.2g) plus sodium metabisulphite (12.0g), dissolved in 70ml distilled water at 40°. This was made up to 100ml after adding $\text{Na}_2\text{SO}_4 \cdot 7\text{H}_2\text{O}$ (2.4g)
- (6) Standard phosphate: 0.324mM KH_2PO_4

Method

Tris-maleate buffer (0.6ml), glucose-6-phosphate (0.2ml) and distilled water (0.6ml) were preheated to 37° in clean test tubes. Aliquots (0.1ml) of liver homogenate (diluted 1 in 10) were added and the tubes incubated for 10 minutes. The reaction was terminated by the addition of 10% (w/v) TCA (1.0ml) and the tubes were cooled on ice. Following centrifugation for 10 minutes at 2,000rpm (M.S.E Bench Centrifuge) aliquots of supernatant (0.5ml) were transferred to clean test tubes together with distilled water (3.9ml), ammonium molybdate (0.4ml) and ANS Reducer (0.2ml). The absorbance was read after 10 minutes at 700nm in a Gilford 250 recording

spectrophotometer. Absorbances were compared with the absorbance of phosphate standards (2.0ml phosphate standard + 2.4ml distilled water + 0.4ml ammonium molybdate + 0.2ml ANS reducer). A zero time blank was included for each sample.

2.2.22.2 Glucose-6-phosphate dehydrogenase (EC. 1.1.1.49)

The determination of Glucose-6-phosphate dehydrogenase was based on the method of Langden (1966), developed in our laboratory by Curtin (1981). The reduction of NADPH was followed at 340nm.



Reagents

- (1) 0.2M Tris-HCl pH 7.5
- (2) 175mM Glucose-6-phosphate
- (3) 75mM MgCl₂
- (4) 10mM NADP

Method

The following was pipetted into clean 1cm micro cuvettes:

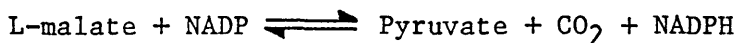
	<u>Sample</u>	<u>Blank</u>
Tris buffer 0.2M pH 7.5	200 μ l	220 μ l
175mM G6P	10 μ l	10 μ l
75mM MgCl ₂	100 μ l	100 μ l
10mM NADP	20 μ l	-
Sample: 10-20 μ l		
soluble fraction		
in water	<u>0.67ml</u>	<u>0.67ml</u>

Total Volume 1.0ml 1.0ml

The cuvette was placed in a Gilford 250 recording spectrophotometer pre-heated to 30°. The cuvette was allowed to warm to 30° for 5 minutes and the reaction was started by the addition of soluble fraction (10-20μl) and followed at 340nm for 5 minutes. Enzyme activity was calculated from the gradient of the slope using an extinction coefficient of 6.22×10^3 l/mol/cm for NADP.

2.2.22.3 NADP-specific malate dehydrogenase, "Malic enzyme" (EC 1.1.1.40)

The determination of malic enzyme activity was based on the method of Hsu and Lardy (1969) developed in our laboratory by Curtin (1981).



Reagents

- (1) Triethanolamine buffer 0.2M pH 7.5
- (2) 10mM Malate: neutralised to pH 7.4 with KOH
- (3) 120mM MnCl₂
- (4) 10mM NADP

Method

The following was pipetted into clean 1cm micro cuvettes:

	<u>Sample</u>	<u>Blank</u>
TEA buffer 0.2M pH 7.5	250μl	300μl
10mM Malate	50μl	--
120mM MnCl ₂	50μl	50μl
10mM NADP	50μl	50μl

Sample:

10 μ l soluble fraction

in water	0.6ml	0.6ml
Total Volume	1.0ml	1.0ml

The cuvettes were placed in a Gilford 250 recording spectrophotometer and allowed to warm to 30° for 5 minutes. The reaction was initiated by the addition of soluble fraction (10 μ l) and followed at 340nm for 5 minutes. Enzyme activity was calculated from the gradient of the slope using an extinction coefficient of 6.22×10^3 l/mol/cm for NADP.

2.2.22.4 Glucokinase (EC 2.7.1.2) and Hexokinase (EC 2.7.1.1)

The determination of glucokinase/hexokinase was based on the method of Sharma et al. (1963) developed in our laboratory by Curtin (1981). Glucose is phosphorylated with ATP to glucose-6-phosphate. This is oxidised with NADP to 6-phosphogluconate and this is further oxidised with NADP to 3-keto 6-phosphogluconate. Thus two moles of NADP are reduced for every mole of glucose phosphorylated. Both glucokinase and hexokinase can be determined since hexokinase is active at very low glucose concentrations.

Reagents

- (1) Glycylglycine buffer 0.25M pH 7.5
- (2) 75mM MgCl₂
- (3) 1.0M KCl
- (4) 750mM glucose
- (5) 3.75 mM glucose
- (6) 10mM NADP

(7) 100mM ATP

(8) Glucose-6-phosphate dehydrogenase (40i.u./ml)

Method

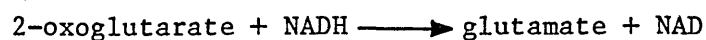
The following was pipetted into four clean 1cm micro cuvettes:

	<u>Total</u>	<u>Hexokinase</u>	<u>Glucose</u>	<u>Control</u>
			<u>dehydrogenase</u>	
	(ml)	(ml)	(ml)	(ml)
Glycylglycine	0.15	0.15	0.15	0.15
MgCl ₂	0.10	0.10	0.10	0.10
KCl	0.10	0.10	0.10	0.10
750mM glucose	0.10	--	0.10	--
3.75mM glucose	--	0.10	--	--
NADP	0.05	0.05	--	--
ATP	0.05	0.05	--	--
G6PDH	0.01	0.01	0.01	0.01
Sample: soluble				
fraction (10 μ l)				
in water	0.19	0.19	0.24	0.34
Total Volume	0.75ml	0.75ml	0.75ml	0.75ml

The cuvettes were preincubated for 5 minutes at 30° in a Gilford 250 recording spectrophotometer. The reaction was initiated by the addition of soluble fraction (10 μ l) and the reaction followed at 340nm. An extinction coefficient of 6.22×10^3 l/mol/cm for NADP was used.

2.2.22.5 Glutamate dehydrogenase (EC 1.4.1.2)

Glutamate dehydrogenase activity was assayed by the method of Herzfeld (1972), developed by Curtin (1981) in our laboratory.



Reagents

- (1) 0.25M sucrose
- (2) 1.75M ammonium chloride
- (4) 1.0M phosphate buffer pH 7.5
- (5) 3mM NADH

Method

Liver homogenate (10%) was diluted in 0.25M sucrose

to a final dilution of 1%. This was sonicated for 3x10 seconds using a sonicating probe (Type 1130/1A, Dawe Instruments Ltd., London). The following was pipetted into clean 1cm microcuvettes:

	Sample	Blank
Phosphate buffer	100 μ l	100 μ l
2-oxoglutarate	50 μ l	50 μ l
NH ₄ Cl	200 μ l	200 μ l
NADH	50 μ l	--
Distilled water	0.59ml	0.64ml
Sample:		
(1% sonicated homogenate)	10 μ l	10 μ l
Total	1.00ml	1.00ml

The cuvettes were preincubated at 30° for 5 minutes in a Gilford 250 spectrophotometer. The reaction was initiated by the addition of liver sample (10 μ l) and followed at 340nm. An extinction coefficient of 6.22x10³ l/mol/cm for NAD was used.

2.2.22.6 Aspartate aminotransferase (EC 2.6.1.1)

Aspartate aminotransferase activity was assayed by the method of Wergedal and Harper (1964) as developed in our laboratory by Curtin (1981).

Aspartate
aminotransferase

Aspartate + 2oxoglutarate $\longrightarrow$ Oxaloacetate + glutamate

Malate dehydrogenase

Oxaloacetate + NADH $\longrightarrow$ Malate + NAD

Reagents

- (1) 0.25M sucrose
- (2) 1M Tris-HCl, pH 7.6
- (3) 60mM 2-oxoglutarate
- (4) 0.8M L-aspartate
- (5) 4mM NADH
- (6) Malate dehydrogenase 5,000i.u./ml (Boehringer Mannheim GmbH)

Method

Liver homogenate (10%) was diluted 1 in 10 in 0.25M sucrose.

This was sonicated for 3x10 seconds using a sonicating probe

(Type 1130/1A, Dawe Instruments Ltd., London). The following was pipetted into clean 1cm microcuvettes:

	Sample	Blank
Tris-HCl pH 7.6	100 μ l	100 μ l
2-oxoglutarate	110 μ l	110 μ l
L-aspartate	100 μ l	100 μ l
NADH	50 μ l	--
Distilled water	0.62ml	0.67ml
Sample:		
1% sonicated homogenate	10 μ l	10 μ l
	-----	-----
Total	1.00ml	1.00ml

The cuvettes were preincubated at 30° for 5 minutes in a Gilford 250 recording spectrophotometer. The reaction was initiated by the addition of sample (10 μ l) and followed at 340nm. An extinction coefficient of 6.22×10^3 l/mol/cm for NAD was used.

CHAPTER 3 HEPATIC DRUG METABOLISM DURING PREGNANCY AND POST-PARTUM

IN THE RAT

3.1 Introduction

Pregnancy induced changes in hepatic drug metabolism in the rat are well documented (see Chapter 1, 1.2.5). What is lacking is a clear explanation of the nature of the changes. These can only be evaluated by a characterisation of the mixed-function oxidase system during gestation. This chapter will be concerned with the analysis of microsomal drug enzyme activity throughout pregnancy and the relationship between factors known to affect drug metabolism such as cytochrome P450 and microsomal phospholipids. In addition the relationship between liver weight changes and hepatic drug metabolism will be discussed.

The effect of pretreatment with inducing agents such as phenobarbitone and 3-methylcholanthrene has been examined as it may be helpful in understanding the processes involved in determining mixed-function oxidase activity in pregnancy. Similarly, a dietary supplement of choline may serve as a useful tool by which the relationship between microsomal drug metabolism and phospholipids could be evaluated. This has also been examined.

3.2 Experimental

(a) Body weight, liver weight, hepatic drug enzyme activities (Chapter 2, 2.2.6), cytochrome P450 (Chapter 2, 2.2.8), cytochrome b5 (Chapter 2, 2.2.9) and microsomal protein (Chapter 2, 2.2.21) were determined in non-pregnant, pregnant (7, 15, 17 and 20 days) and post-partum (1 and 5 days). NADPH-cytochrome c reductase activity (Chapter 2, 2.2.10) was determined in non-pregnant and pregnant (20 days) rats.

(b) Hepatic microsomal total phospholipids, phosphatidylcholine and phosphatidylethanolamine have been determined (Chapter 2, 2.2.13) in groups of non-pregnant, pregnant (15 and 20 days) and post-partum (1 and 7 days) rats.

(c) Hepatic DNA, RNA, lipid, glycogen, hepatic size and hepatic non-drug metabolising enzyme activities have been determined (Chapter 2) in the livers from non-pregnant and pregnant (20 days) rats. Liver sections from these groups were analysed histologically (Chapter 2, 2.2.19).

(d) The effect of a choline supplemented diet on hepatic microsomal drug enzyme activity, cytochrome P450, cytochrome b5, protein and phospholipids was determined in pregnant (20 days) and non-pregnant rats. Rats were fed on a 1% choline chloride diet for 19 days (Chapter 2, 2.2.5.5) and the results were compared with those obtained from pregnant (20 days) and non-pregnant rats maintained on a normal diet.

(e) The effect of inducing agents on hepatic drug metabolism in pregnant (20 days) and non-pregnant rats was investigated. Rats were injected i.p. with phenobarbitone (80mg/kg) or 3-methylcholanthrene (20mg/kg) for three days and killed 24 hours later (Chapter 2, 2.2.5). Control non-pregnant rats received saline or corn oil alone.

3.3 Results

3.3.1 Maternal body weight and liver weight changes during pregnancy and

post-partum

During pregnancy, maternal liver weight and body weight increased significantly above non-pregnant control levels (Figure 3.1). This increase was greatest on day 20 of gestation when liver weight and body weight were elevated by 37% and 44% respectively. Following parturition there was a significant decrease in liver and body weight. In the post-partum rat (1 and 5 days) liver and body weights though declining were still significantly elevated above non-pregnant control levels.

3.3.2 Maternal hepatic drug metabolism during pregnancy

During pregnancy there was a progressive decrease in the activities of ethylmorphine N-demethylase, aniline p-hydroxylase and p-nitrobenzoic acid reductase per gram liver (Figure 3.2). The largest decrease was found on day 20 of pregnancy (74%, 73% and 53% of non-pregnant levels respectively). Enzyme activities were still significantly depressed 1 day post-partum but had returned to non-pregnant control levels by 5 days post-partum. Microsomal levels of cytochrome P450 were not significantly different from non-pregnant control levels when expressed per milligram microsomal protein (Figure 3.4). Cytochrome b5 was significantly decreased

in the 7-day pregnant rat but unchanged at other time points (Figure 3.4).

When expressed per total liver (Figure 3.3), drug enzyme activities in the pregnant and post-partum rat were unchanged or increased above non-pregnant control levels with the exception of p-nitrobenzoic acid reductase activity which was significantly reduced on day 20 of pregnancy and 1 day post-partum (Figure 3.3) Total hepatic cytochrome P450 and b5 levels were significantly increased above non-pregnant control levels at all time points in pregnancy and post-partum (Figure 3.4).

Microsomal protein was significantly elevated above non-pregnant control levels on days 7 and 17 of pregnancy but unchanged at other time points (Table 3.1). NADPH-cytochrome c reductase activity was unchanged in the 20 day pregnant rat (46.3 ± 2.5 S.E.M (n=4) μ moles cytochrome c reduced / gliver/ h) compared to the non-pregnant rat (38.8 ± 2.5 S.E.M. (n=4) μ moles cytochrome c reduced / gliver / h).

3.3.3 Maternal hepatic microsomal phospholipids during pregnancy and

post-partum

Total phospholipids and phosphatidylcholine were significantly decreased and phosphatidylethanolamine significantly increased in the 15 and 20 day pregnant and 1 day post-partum rat (Figure 3.5) compared with the non-pregnant control levels. These changes were greatest on day 20 of pregnancy when they represented 82%, 77% and 130% on non-pregnant control levels respectively. Microsomal phospholipids had returned to non-pregnant control levels by 7 days post-partum. The ratio of phosphatidylcholine to

phosphatidylethanolamine was significantly decreased during pregnancy and 1 day post-partum but was unchanged at 7 days post-partum compared with non-pregnant control levels.

3.3.4 Liver biochemistry and histology in non-pregnant and pregnant

(20 days) rats.

Hepatic DNA, RNA, lipid, glycogen, enzyme function and hepatocyte size were determined in pregnant (20 days) and non-pregnant rats (Table 3.2 and Figures 3.6, 3.7, 3.8). Hepatic DNA, RNA, total lipid, glycogen and hepatocyte size were unchanged on day 20 of pregnancy compared with non-pregnant control levels. These results were confirmed histologically by the similar appearance of the livers from the two groups using the three staining techniques: haematoxylin and eosin (Figure 3.6), periodic acid-schiff (Figure 3.7) and Oil red O (Figure 3.8). Mitotic activity was assessed histologically in non-pregnant and pregnant rats (Figure 3.9). A significant increase in the mitotic index was seen in early pregnancy. This fell progressively to control levels by day 20 of gestation.

A number of non-drug metabolising enzymes were assayed in the livers from pregnant and non-pregnant animals (Table 3.2). Microsomal glucose-6-phosphatase activity was significantly decreased (to 61% of control values) in late pregnancy and aspartate aminotransferase (determined in the sonicated homogenate) significantly elevated to 131% of non-pregnant control levels.

3.3.5 The effect of a 1% choline diet on hepatic microsomal

monooxygenase activity and phospholipids in pregnant and non-pregnant rats.

A 1% choline diet had no effect on body weight, liver weight or general appearance of either the pregnant or non-pregnant rats. The activities of aniline *p*-hydroxylase, ethylmorphine N-demethylase and *p*-nitrobenzoic acid reductase are shown in Figure 3.10. Microsomal phospholipid levels are shown in Figure 3.11.

Pregnancy resulted in a significant decrease in total phospholipids and phosphatidylcholine and an elevation in phosphatidylethanolamine as discussed earlier (section 3.2.3). A dietary supplementation of choline increased the total phospholipid and phosphatidylcholine levels in pregnant rats to the level found in non-pregnant control animals while significantly decreasing the level of phosphatidylethanolamine. Similarly, non-pregnant rats receiving a choline supplemented diet showed significantly increased levels of phosphatidylcholine and decreased phosphatidylethanolamine but total phospholipid levels remained unchanged. This treatment resulted in a significant increase in the ratio between phosphatidylcholine and phosphatidylethanolamine from 3.0 and 1.8 in non-pregnant and pregnant rats to 4.9 and 6.2 in their choline fed counterparts.

Microsomal cytochrome P450, cytochrome b5 and microsomal protein levels are shown in Figure 3.12. All were unaffected by a dietary choline supplement.

3.3.6 The effect of inducing agents on hepatic microsomal drug metabolism

in pregnant (20 day) and non-pregnant rats

The effect of in vivo pretreatment with phenobarbitone and 3-methylcholanthrene on in vitro hepatic microsomal drug enzyme activity in the pregnant (20 days) and non-pregnant rat has been expressed per unit liver weight (Figure 3.13). Microsomal levels of cytochrome P450, cytochrome b5 and protein are shown in Figure 3.14. Phenobarbitone and 3-methylcholanthrene did not affect the liver or body weight of the animals.

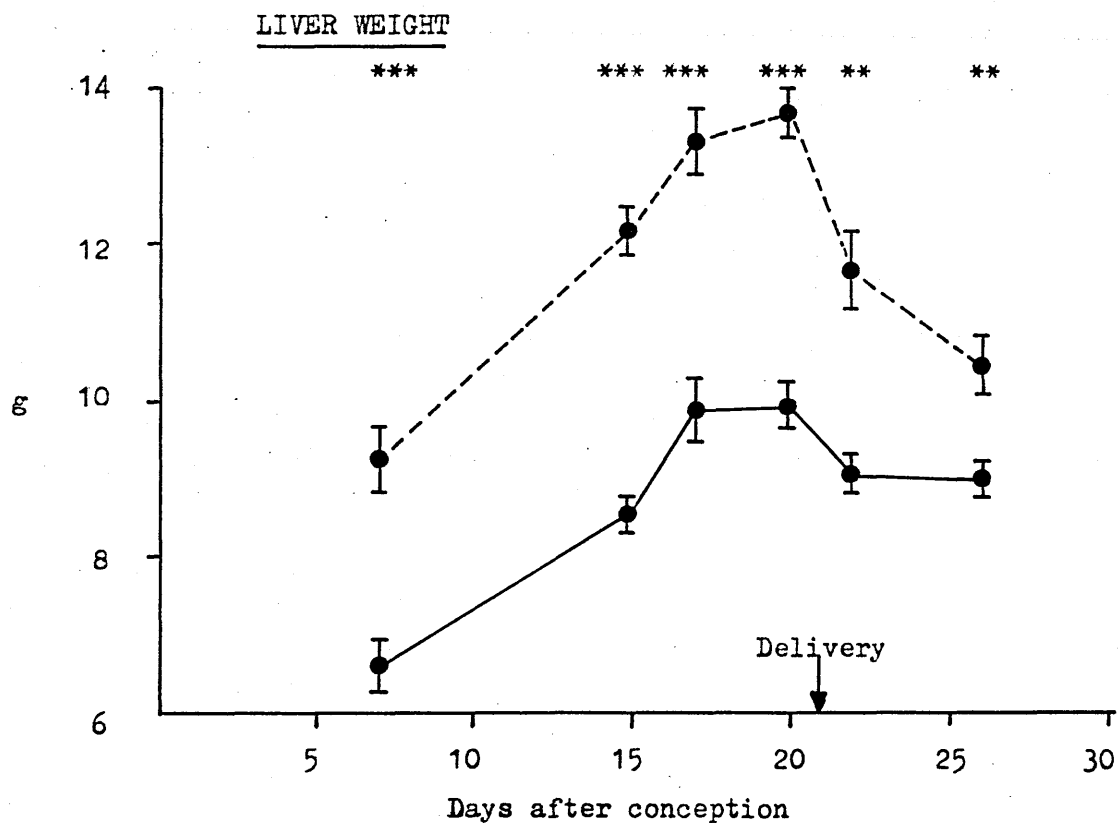
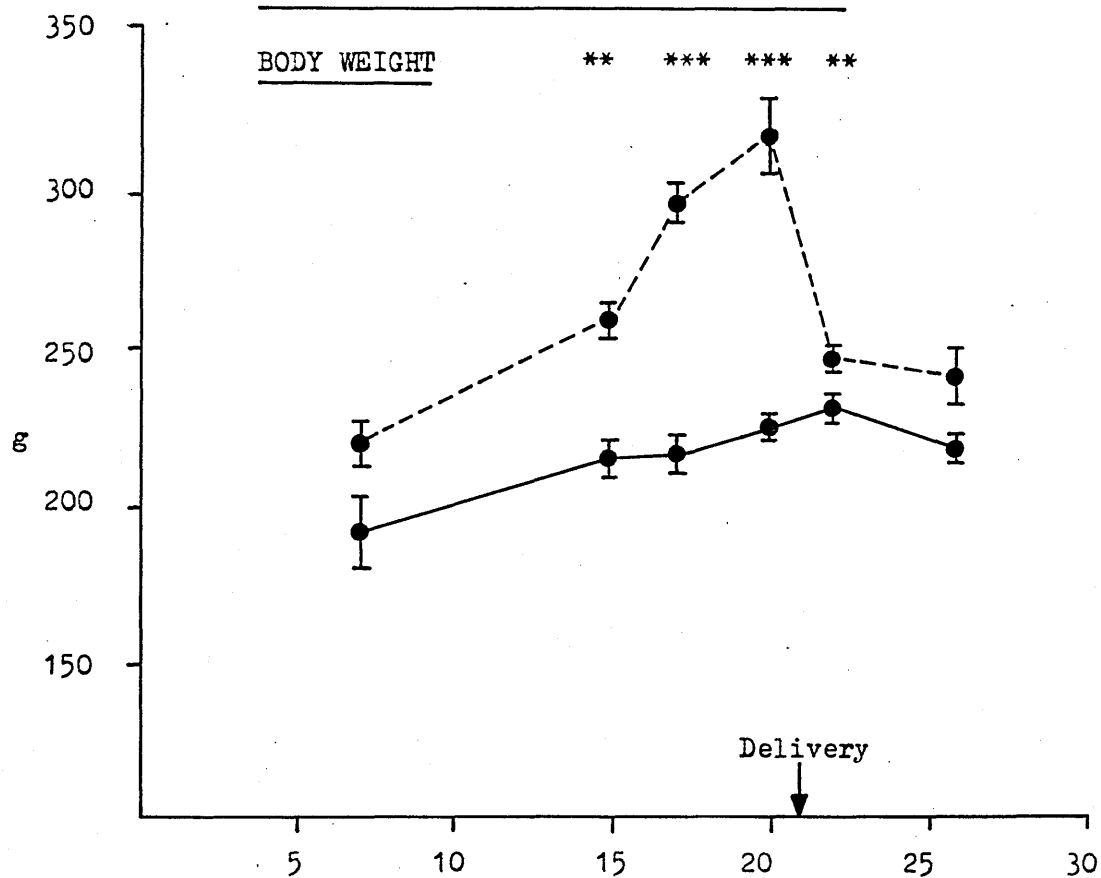
Phenobarbitone pretreatment of pregnant and non-pregnant rats resulted in a statistically significant increase in aniline p-hydroxylase and ethylmorphine N-demethylase. The activity of p-nitrobenzoic acid reductase was significantly increased in non-pregnant but not pregnant rats. Phenobarbitone pretreatment resulted in a significant increase in microsomal protein concentrations in both pregnant and non-pregnant animals. The level of cytochrome P450 was increased by phenobarbitone pretreatment in non-pregnant and pregnant rats. In addition cytochrome P450 levels in the phenobarbitone treated non-pregnant rats were significantly elevated above the levels found in phenobarbitone treated pregnant animals. Cytochrome b5 levels were unaffected by phenobarbitone treatment.

Pretreatment with 3-methylcholanthrene resulted in a significant increase in aniline p-hydroxylase and ethylmorphine N-demethylase activity in pregnant and non-pregnant animals. The activity of p-nitrobenzoic acid reductase was unchanged. Microsomal cytochrome P450 was significantly

increased by 3-methylcholanthrene pretreatment in pregnant and non-pregnant rats. Microsomal protein and cytochrome b5 remained unchanged.

The pregnancy associated decrease in hepatic drug enzyme activity was abolished by pretreatment with phenobarbitone and 3-methylcholanthrene. There were no significant differences in enzyme activities between non-pregnant and pregnant animals following inducer pretreatment.

Changes in body and liver weight during pregnancy and post-partum in the Wistar-albino rat.



Values represent mean $\pm$ SEM (n=4 or 8) for pregnant/post-partum (●---●) and non-pregnant (●—●) female rats. Significant differences (students 't' test) are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3.2 Hepatic microsomal mixed-function oxidase activity during pregnancy and post-partum in the rat expressed per gram liver.

Values represent mean $\pm$ SEM (n=4 or 8) for pregnant/post-partum (●---●) and non-pregnant (●—●) animals. Significant differences (students 't' test) between non-pregnant and pregnant/post-partum animals are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

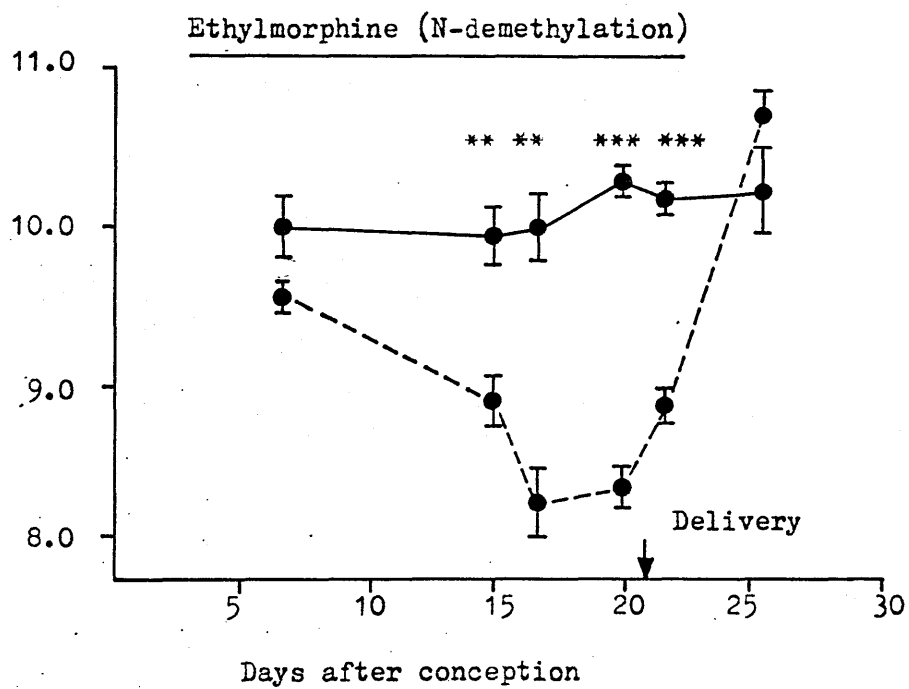
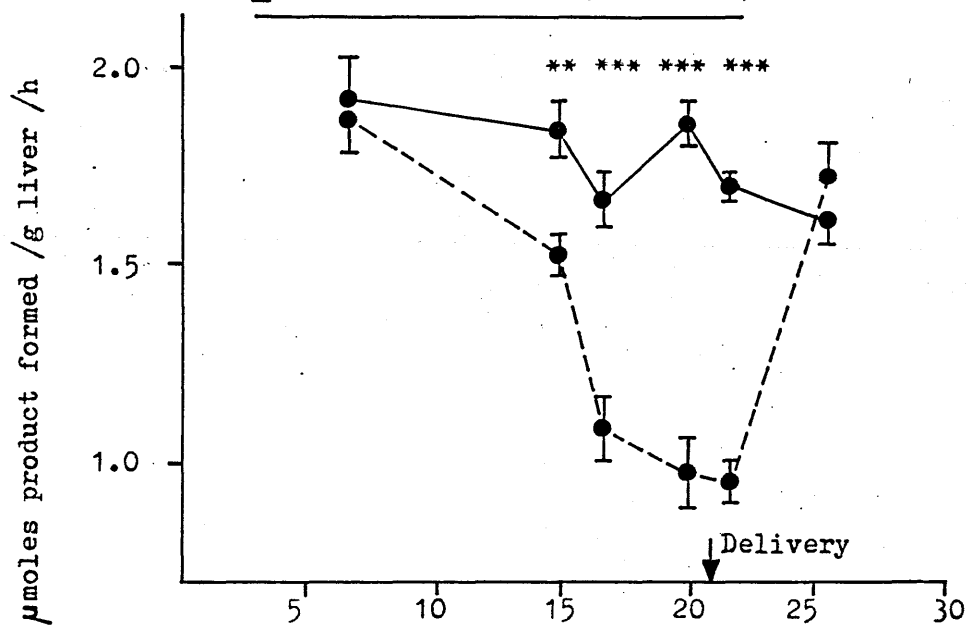
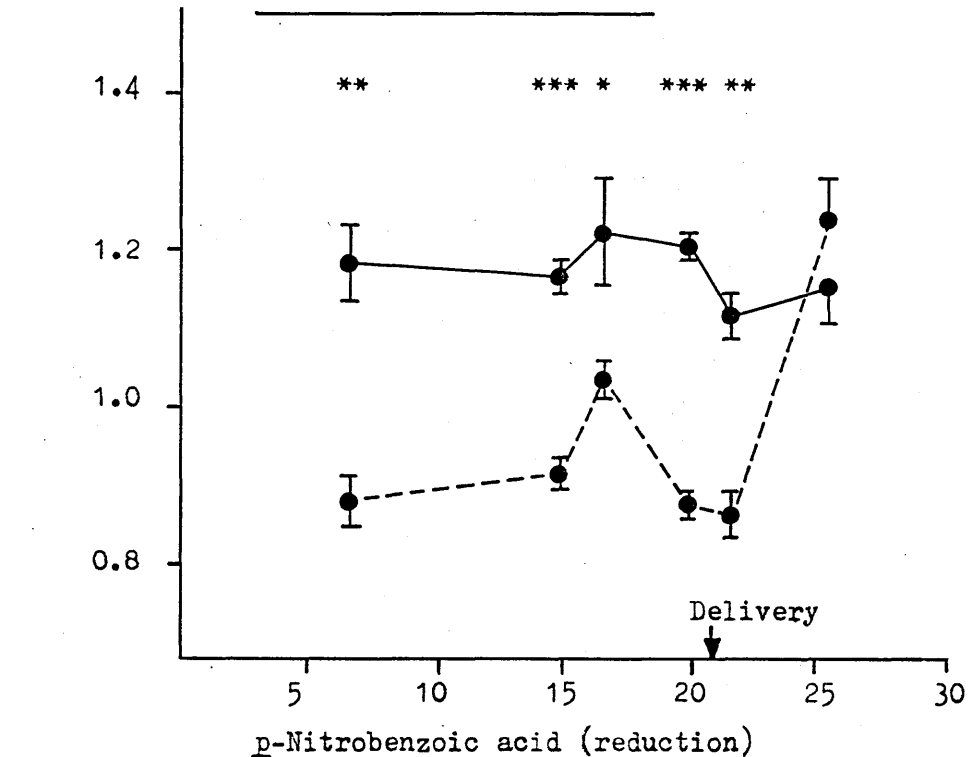


Figure 3.3 Hepatic microsomal mixed-function oxidase activity during pregnancy and post-partum in the rat expressed per total liver

Data from Figure 3.2 has been expressed as enzyme activity per total liver. Values represent mean $\pm$ SEM (n=4 or 8) for pregnant/post-partum (●---●) and non-pregnant (●—●) animals. Significant differences (students 't' test) between non-pregnant and pregnant/post-partum animals are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

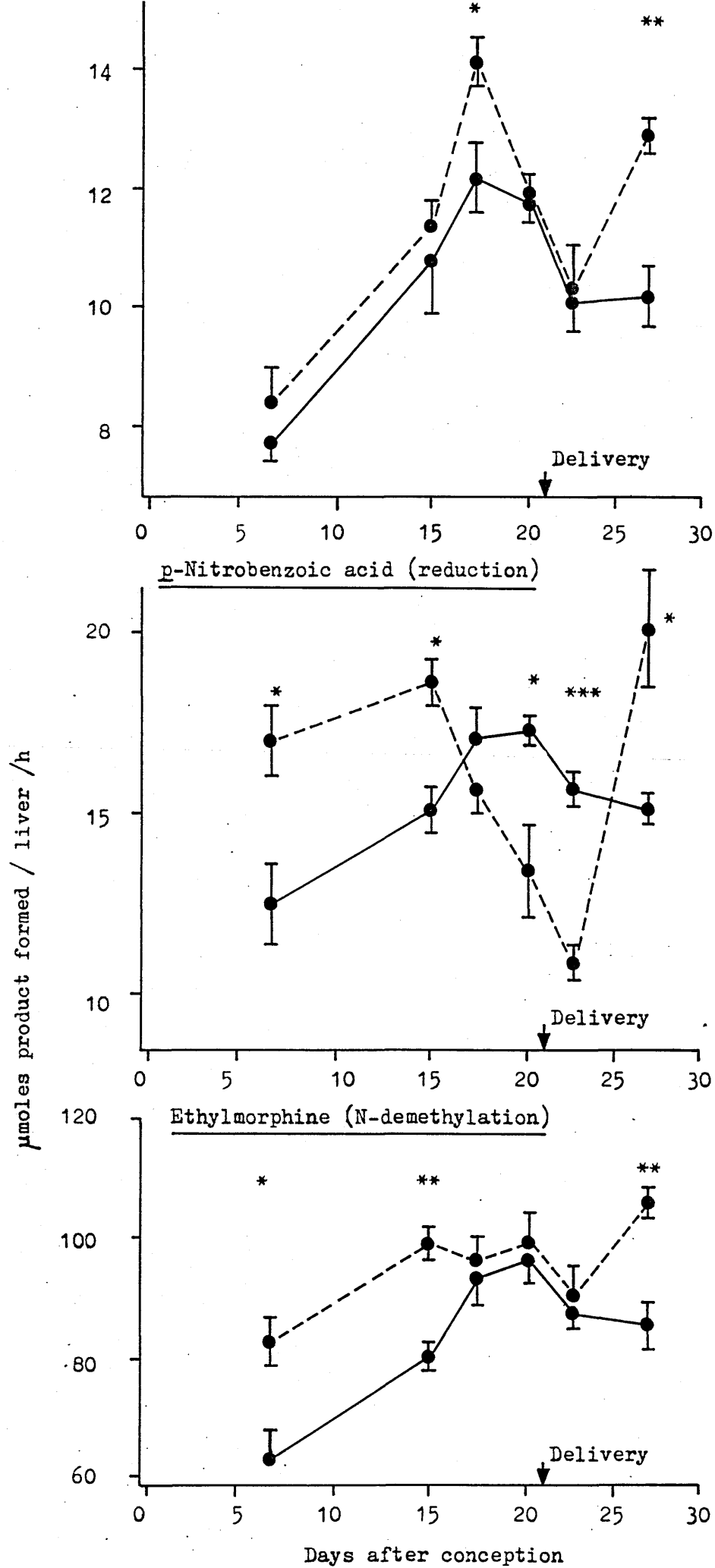
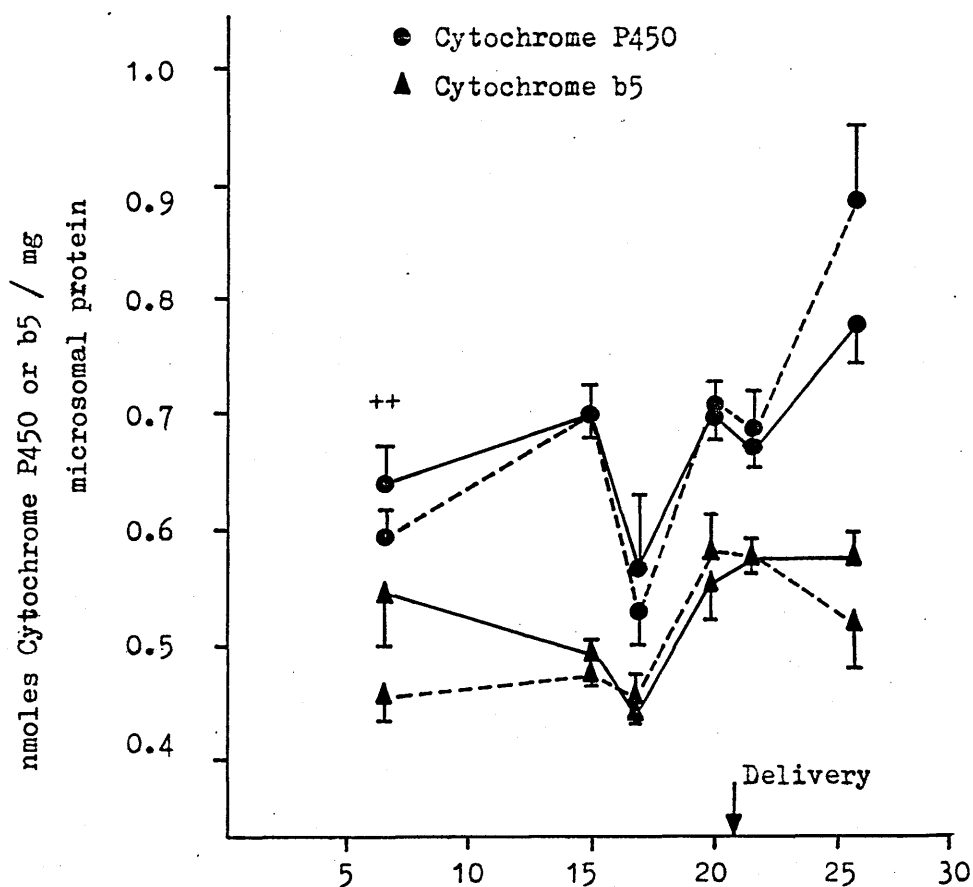


Figure 3.4 Hepatic microsomal cytochrome P450 and b5 during pregnancy and post-partum in the rat expressed per milligram protein (a) or per total liver (b).

Hepatic cytochrome P450 (●) and b5 (▲) in pregnant/post-partum (---) and non-pregnant (—) rats has been expressed per milligram microsomal protein (a) or per total liver (b).

Significant differences (students 't' test) between non-pregnant and pregnant/post-partum animals are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ (cytochrome P450) and + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$ (cytochrome b5).

(a) Hepatic haemoprotein concentration



(b) Total hepatic haemoprotein

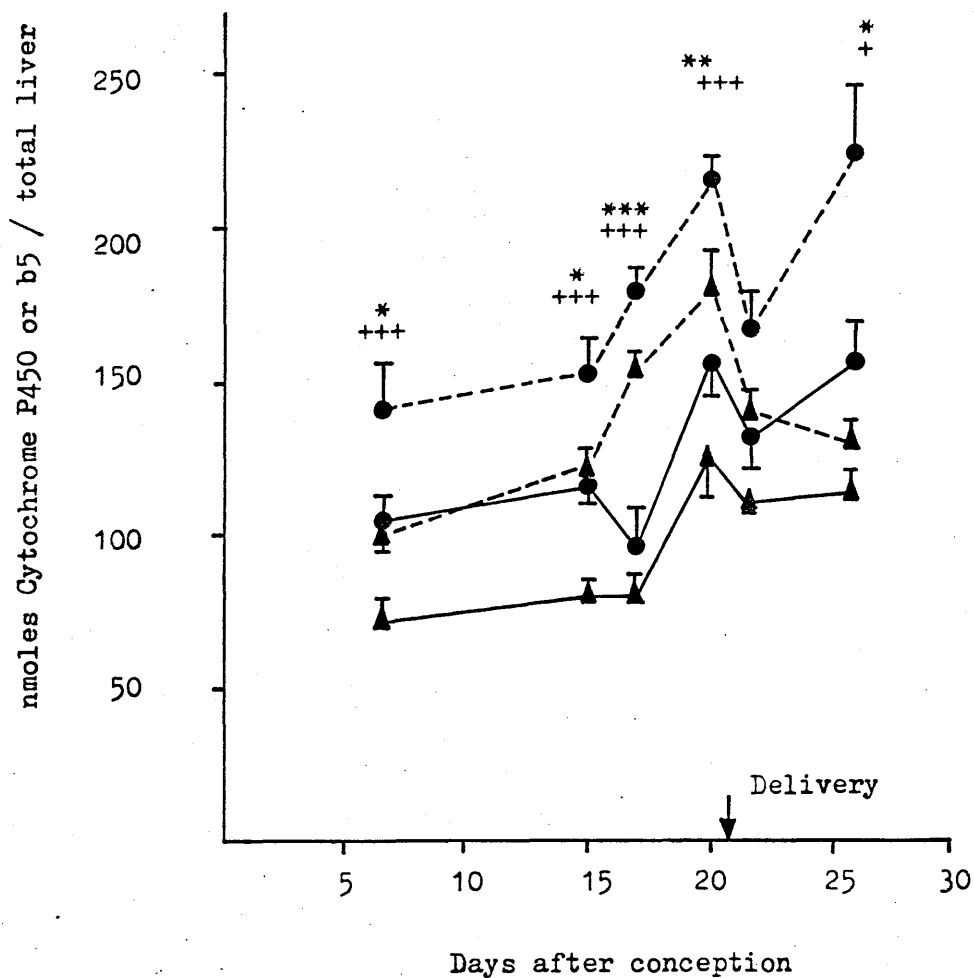
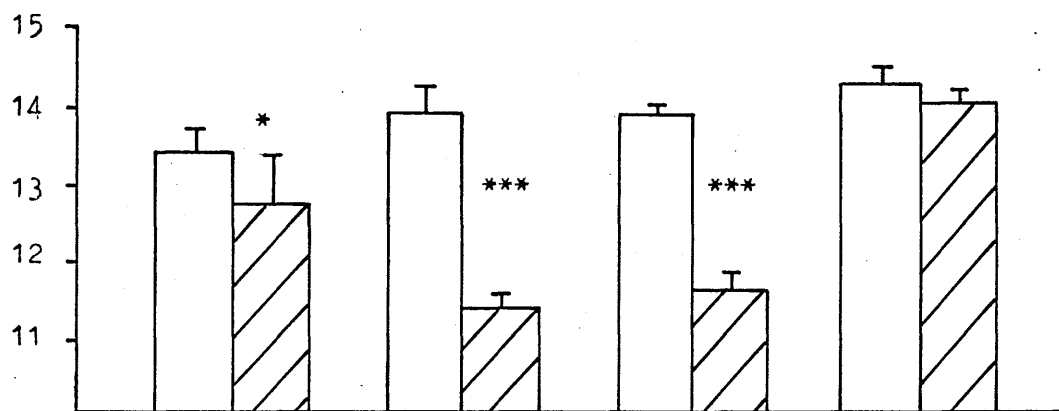


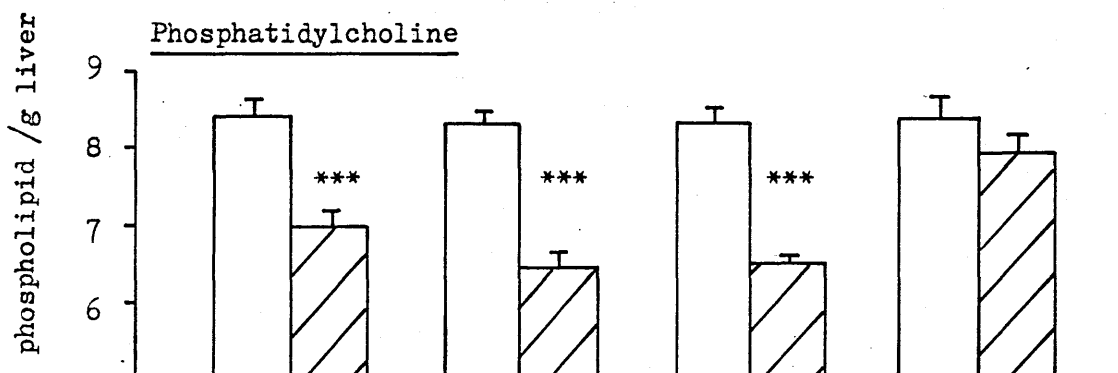
Figure 3.5 Microsomal phospholipid levels during pregnancy and post-partum in the rat.

Values represent mean $\pm$ SEM (n=4) for pregnant/post-partum  and non-pregnant  animals. Significant differences (students 't' test) between non-pregnant and pregnant/post-partum animals are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

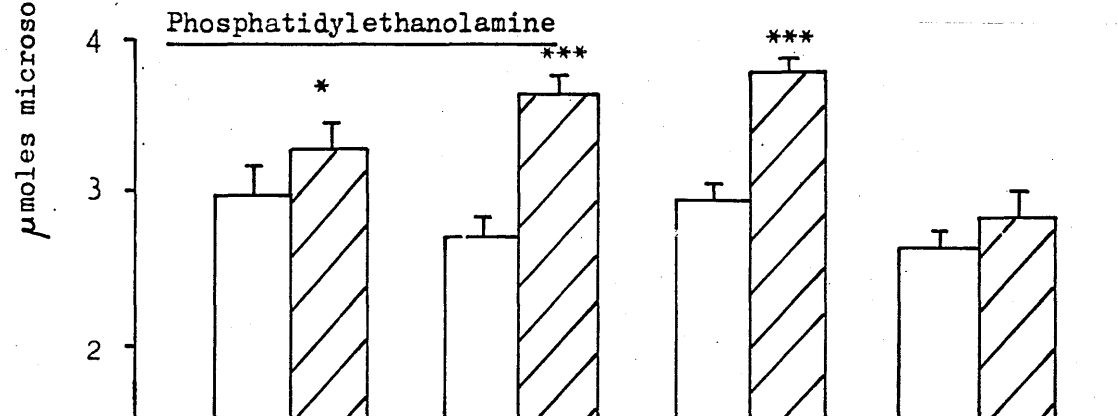
Total phospholipid



Phosphatidylcholine



Phosphatidylethanolamine



Phosphatidylcholine to phosphatidylethanolamine ratio

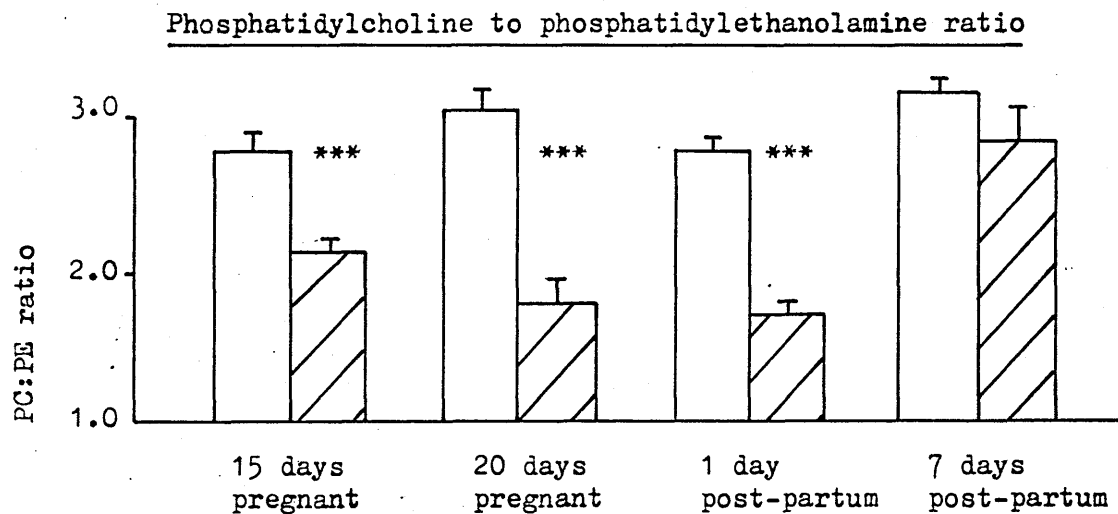
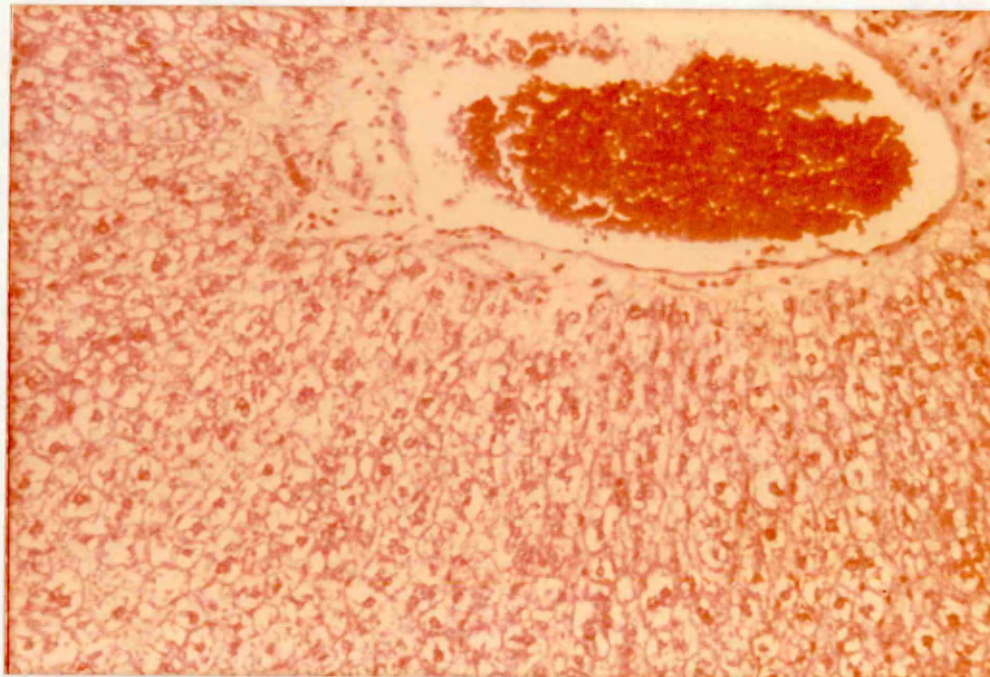


Figure 3.6 Liver from non-pregnant (A) and pregnant (20 days) (B) rats
stained with Haematoxylin and Eosin. (Magnification x100)

(A)



(B)

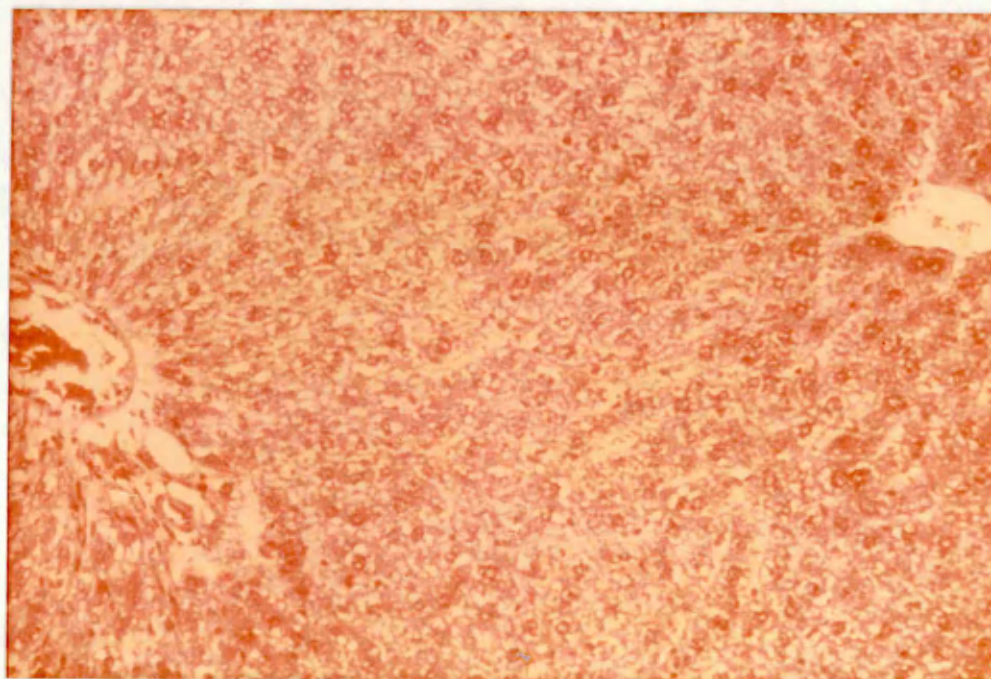


Figure 3.7 Liver from non-pregnant (A) and pregnant (20days) (B) rats
stained with Periodic acid-Schiff. (Magnification x100)

(A)

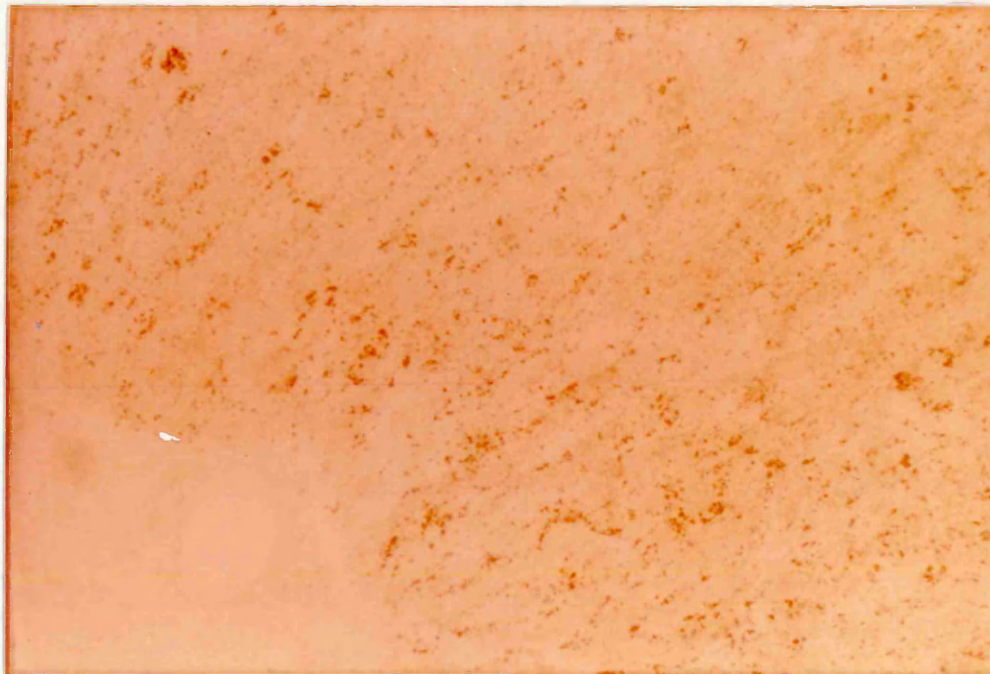


(B)



Figure 3.6 Liver from non-pregnant (A) and pregnant (20 days) (B) rats
stained with Oil Red O. (Magnification x100)

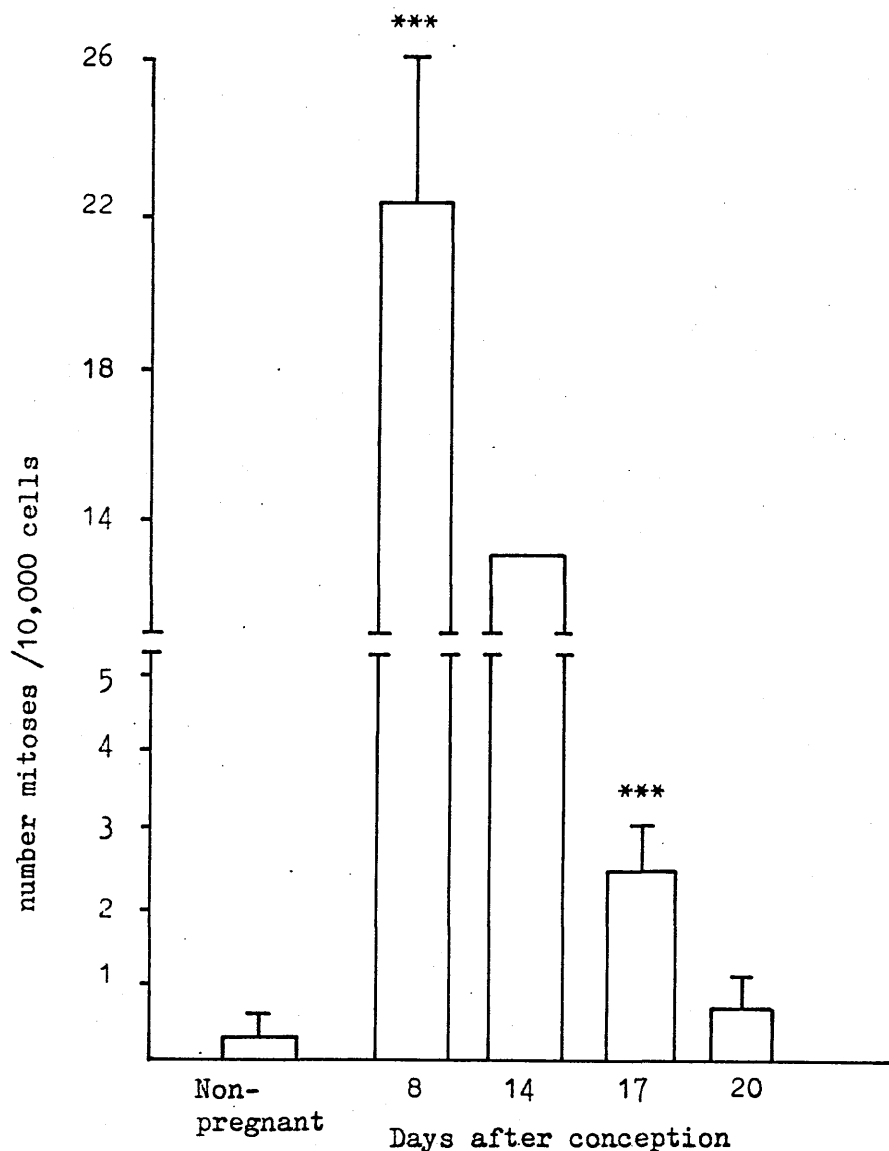
(A)



(B)



Figure 3.9 Hepatic parenchymal mitotic indices in pregnant and non-pregnant rats.



Mitotic indices are expressed as the number of cells in division per counts of 10,000 cells. Values represent mean $\pm$ SEM; n=3 (8 days pregnant), n=4 (non-pregnant, 17 and 20 days pregnant) and n=1 (14 days pregnant). Significant differences (students 't' test) between non-pregnant and pregnant rats are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 3.10 The effect of a choline supplemented diet (1%) on
cytochrome P-450 dependent
aromatic microsomal mixed-function oxidase activity in pregnant
(14-18 days) and non-pregnant rats.

Normal diet

 Non-pregnant (control)

 Pregnant

1% Choline diet

 Non-pregnant

 Pregnant

Values represent mean $\pm$ SEM (n=4 except for pregnant animals fed on a
choline diet where n=3). Significant differences (students 't' test)
between control and test groups are shown as * $p < 0.05$, ** $p < 0.01$,
*** $p < 0.001$.

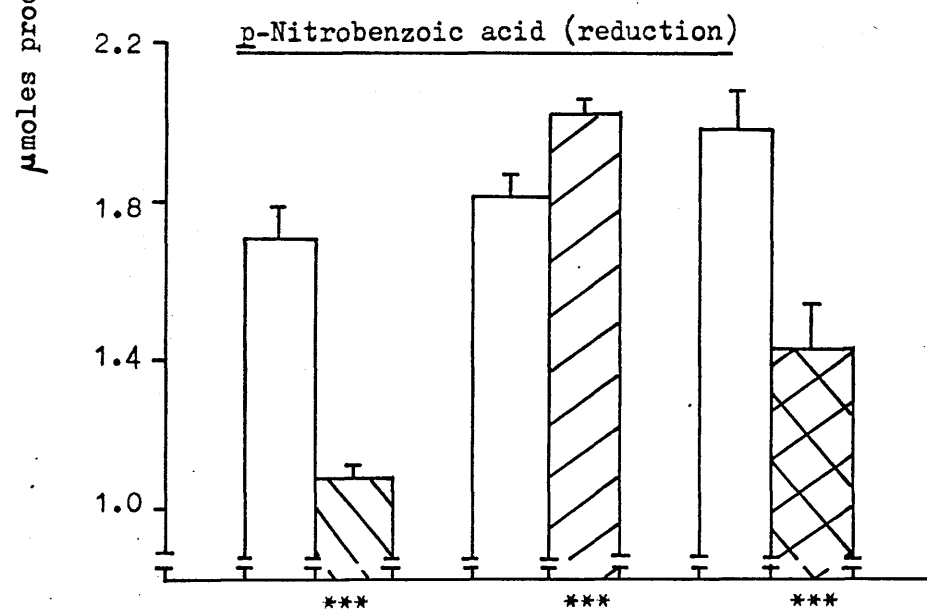
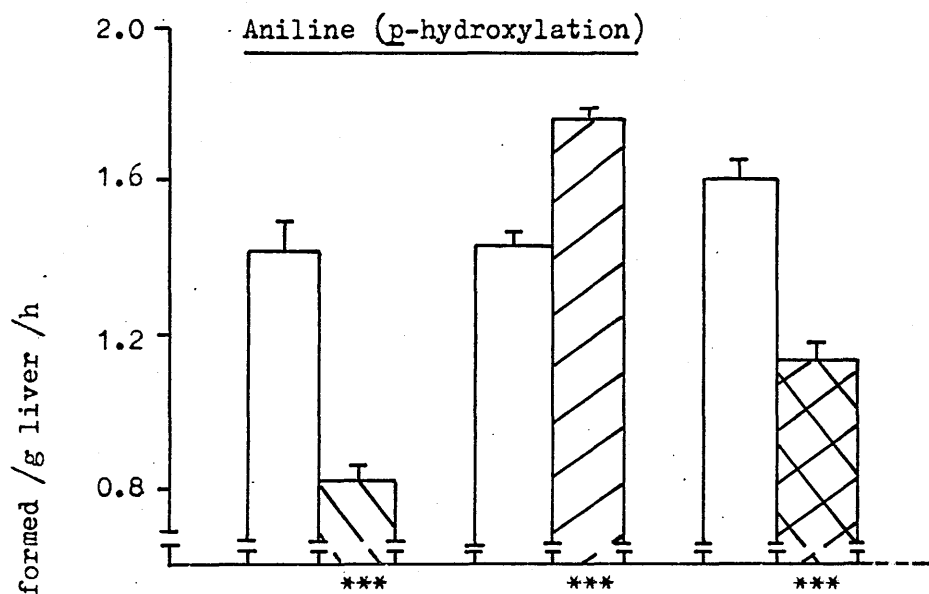
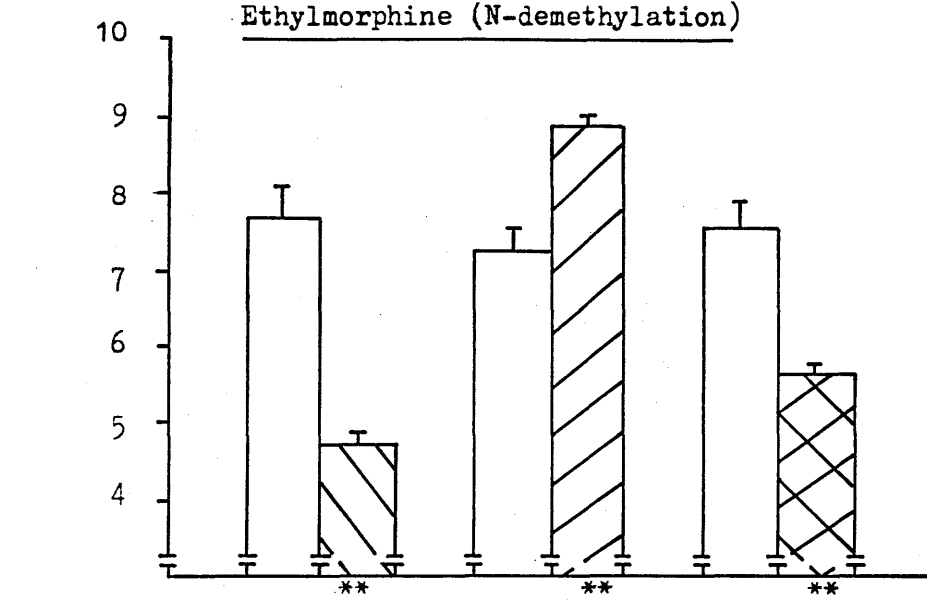
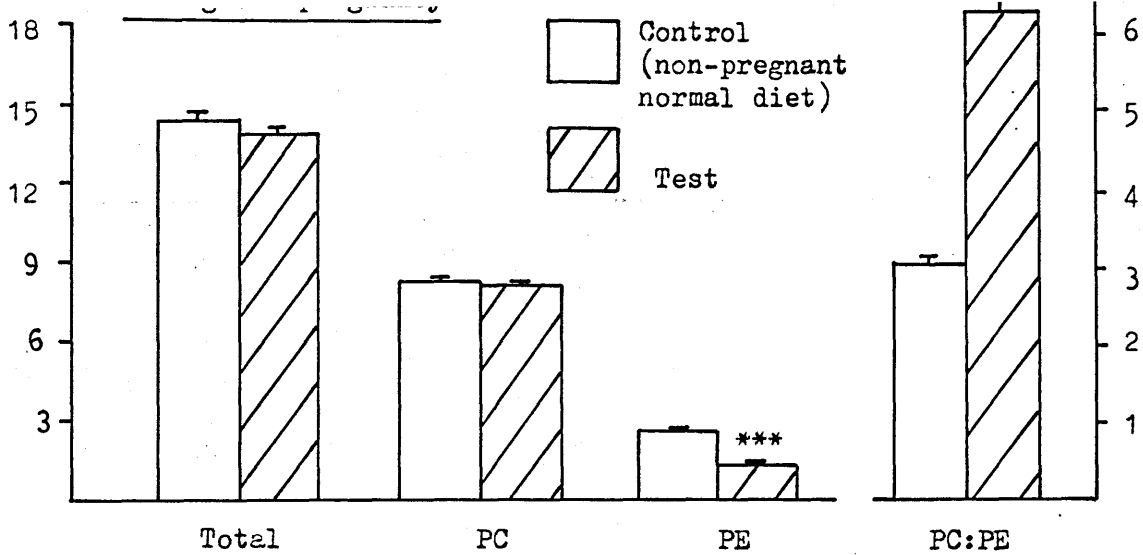


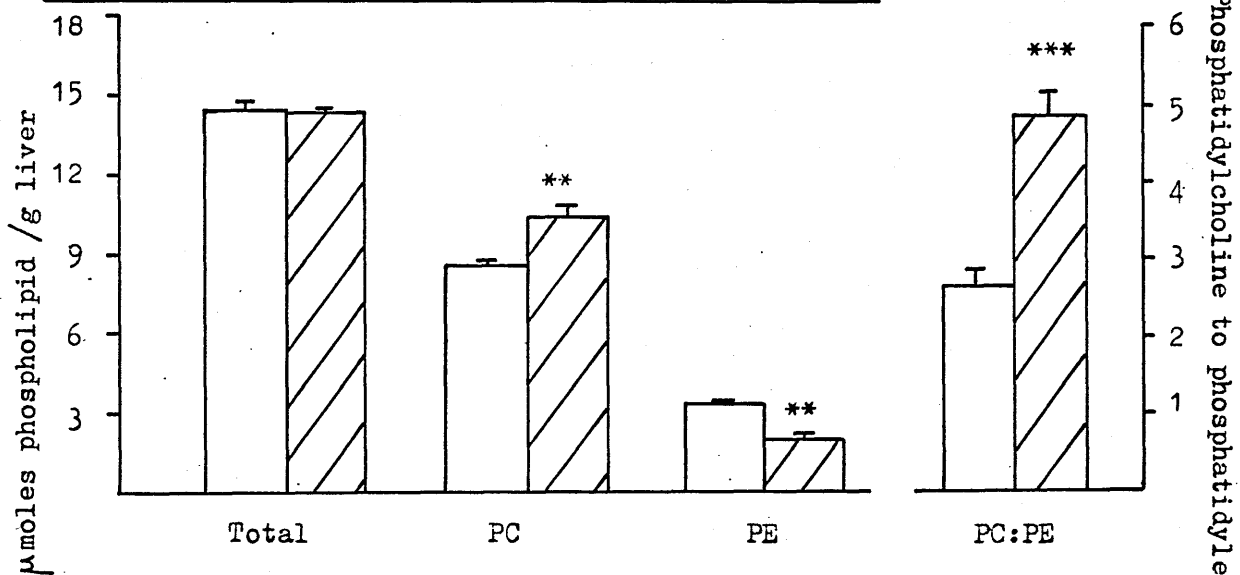
Figure 3.11 The effect of a choline supplemented diet (1%) on hepatic
microsomal phospholipids in pregnant (20 days) and non-pregnant rats.

Each point represents mean $\pm$ SEM (n=4 except for pregnant rats on a choline diet where n=3) for total phospholipids, phosphatidylcholine (PC), phosphatidylethanolamine (PE) and the PC:PE ratio. Significant differences (students 't' test) between control and test animals are shown as

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



(B) Non-pregnant rats on 1% choline diet for 19 days



(C) Pregnant rats (20 days) on normal diet

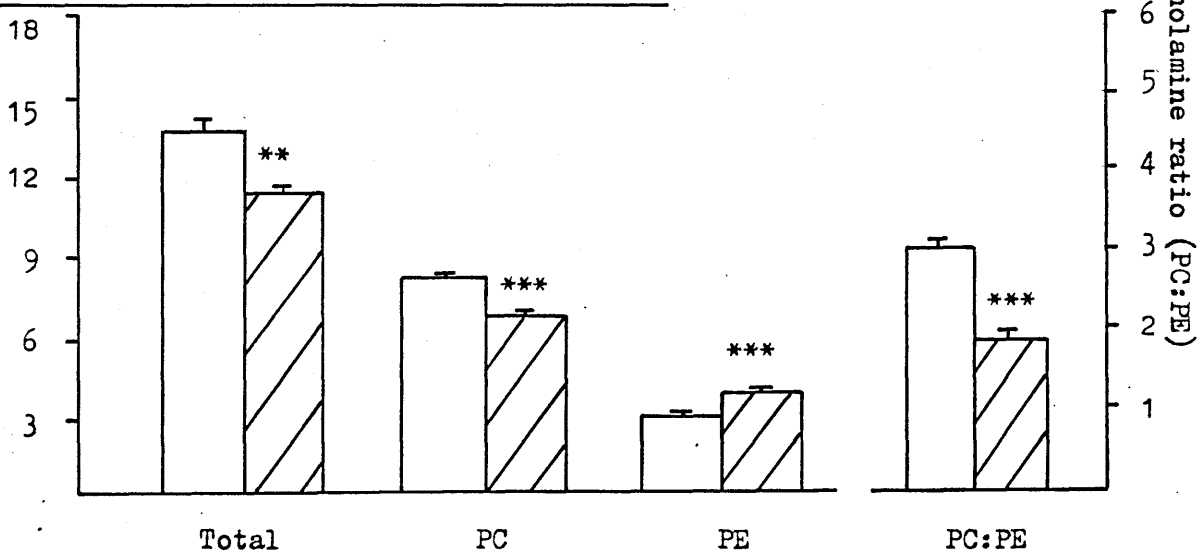
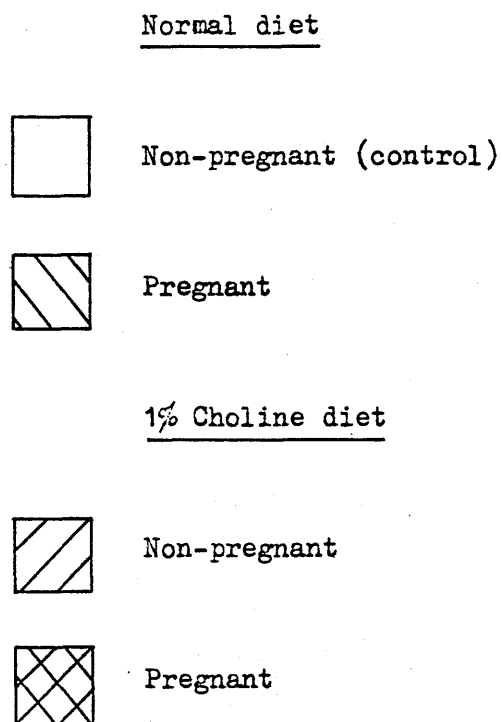
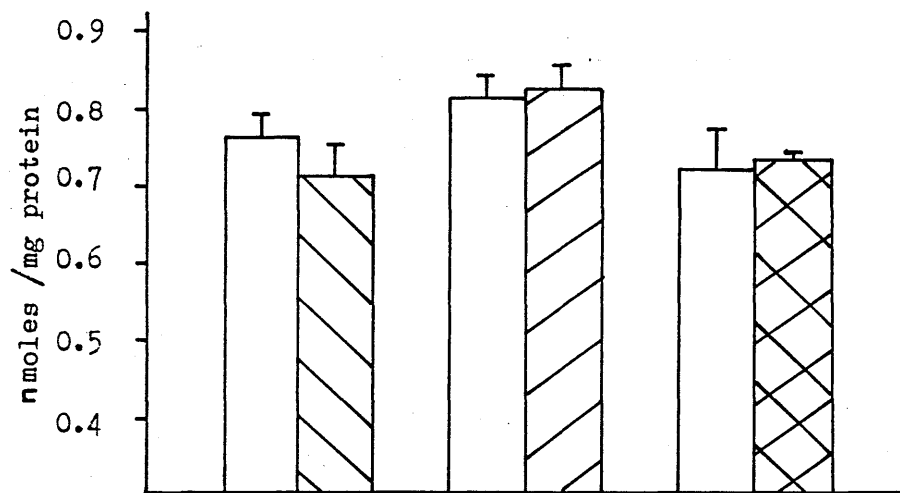


Figure 3.12 The effect of a choline supplemented diet (1%) on
hepatic microsomal cytochrome P450, b5 and protein in pregnant
(20 days) and non-pregnant rats.

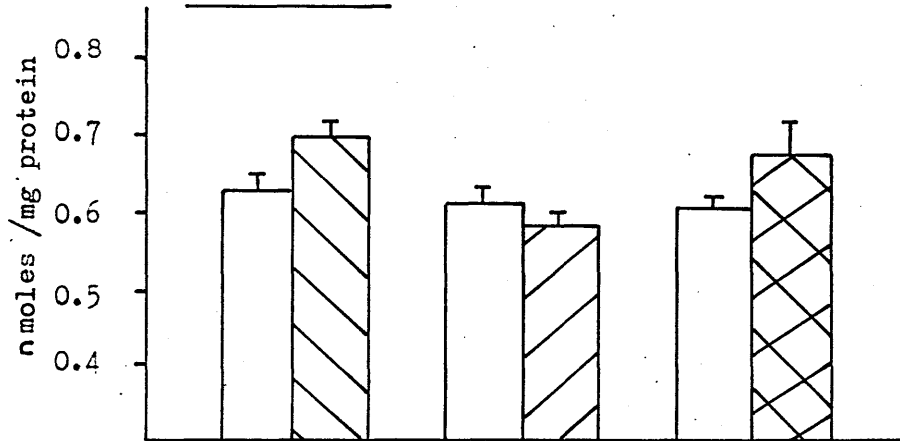


Values represent mean $\pm$ SEM (n=4 except for pregnant animals fed on a choline diet where n=3). There were no significant differences between control and test groups.

Cytochrome P450



Cytochrome b5



Microsomal protein

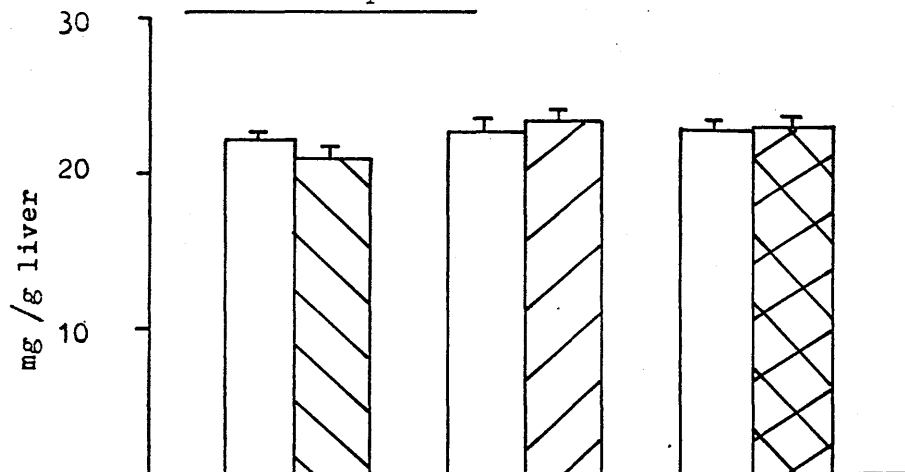


Figure 3.13 The effect of in vivo pretreatment with phenobarbitone and 3-methylcholanthrene on in vitro hepatic microsomal mixed-function oxidase activity in pregnant (20 days) and non-pregnant rats.

Animals were pretreated for three days prior to death with phenobarbitone (80mg/kg) or 3-methylcholanthrene (20 mg/kg).



Untreated non-pregnant control



Inducer pretreated non-pregnant



Inducer pretreated pregnant (20 days)

Values represent mean $\pm$ SEM (n=4). Significant differences (students 't' test) between control and pretreated animals are shown as

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

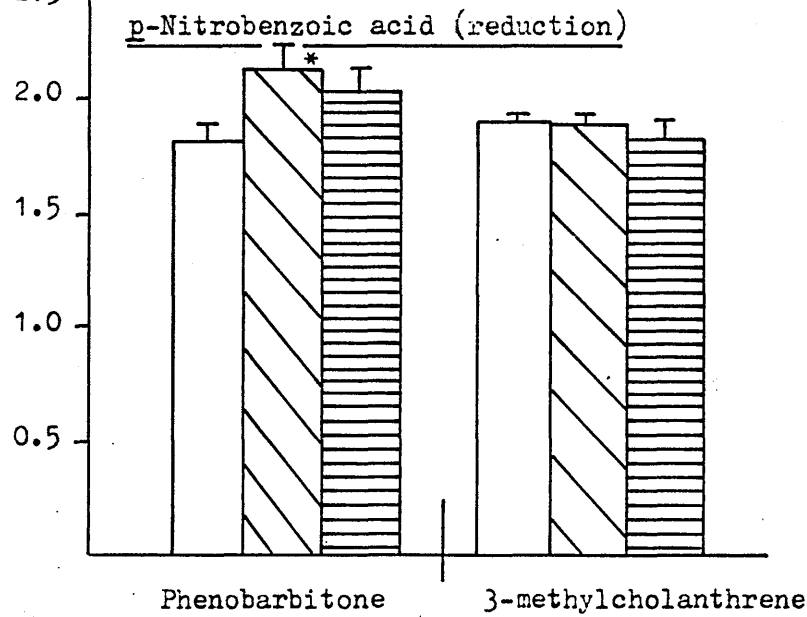
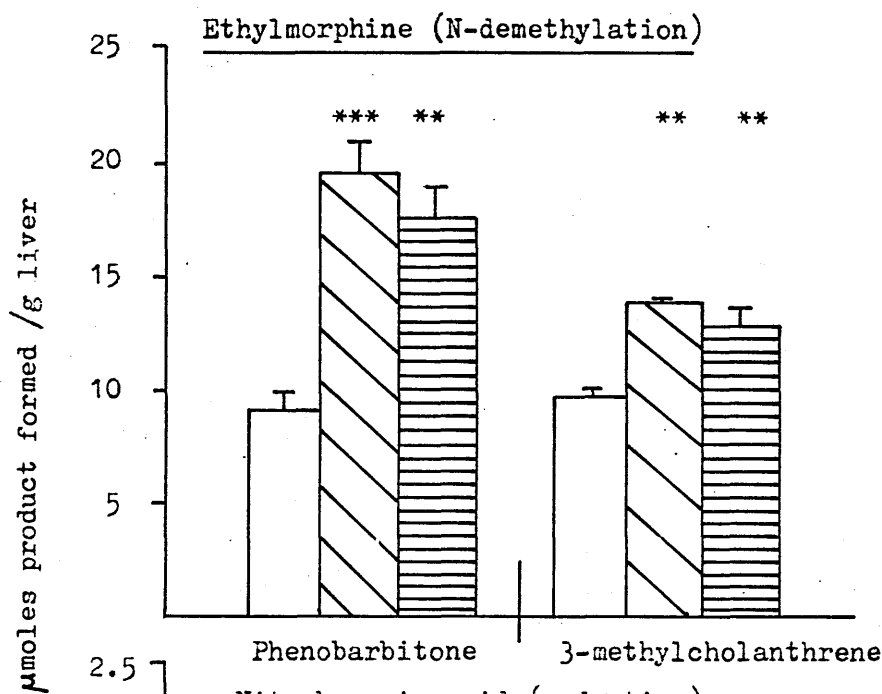
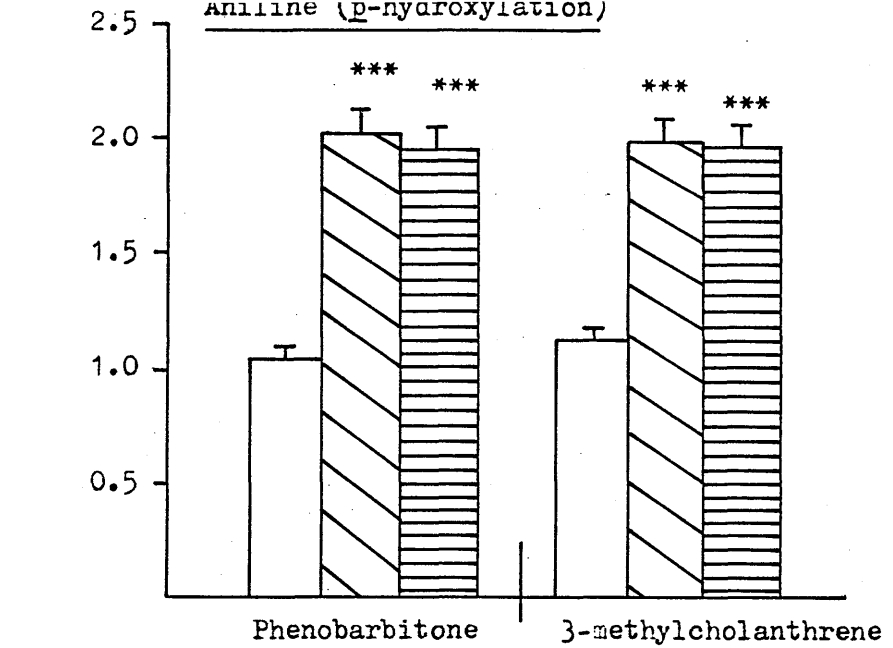


Figure 3.14 The effect of in vivo pretreatment with phenobarbitone and 3-methylcholanthrene on in vitro hepatic microsomal cytochrome P450, b5 and protein in pregnant (20 days) and non-pregnant rats.

Animals were pretreated for three days prior to death with phenobarbitone (80mg/kg) or 3-methylcholanthrene (20mg/kg).



Untreated non-pregnant control



Inducer pretreated non-pregnant



Inducer pretreated pregnant (20 days)

Values represent mean $\pm$ SEM (n=4). Significant differences (students 't' test) between control and pretreated animals are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$. Significant differences between pretreated non-pregnant and pregnant animals are shown as + $p < 0.05$, ++ $p < 0.01$, +++ $p < 0.001$.

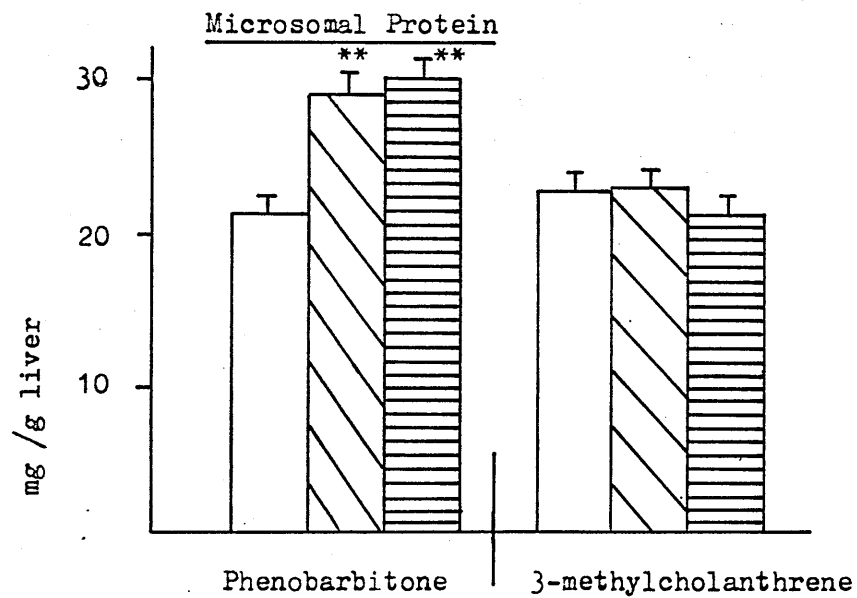
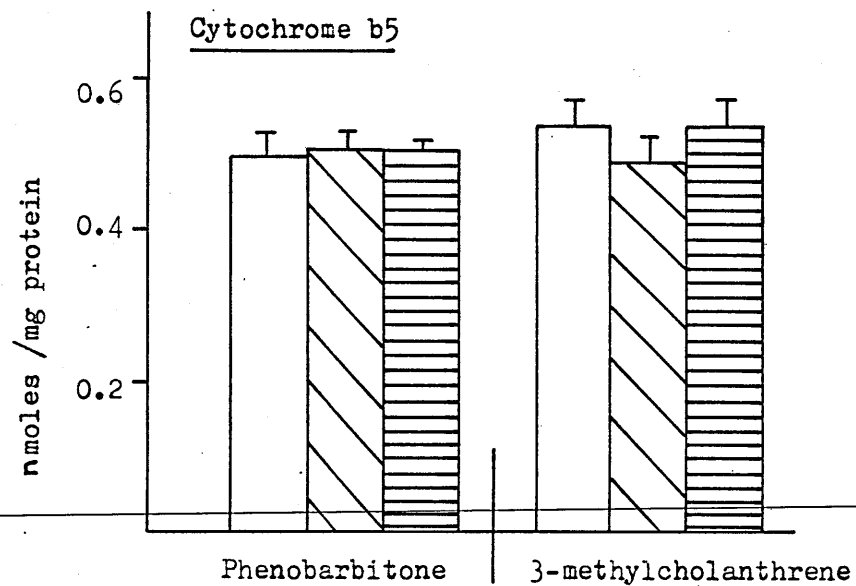
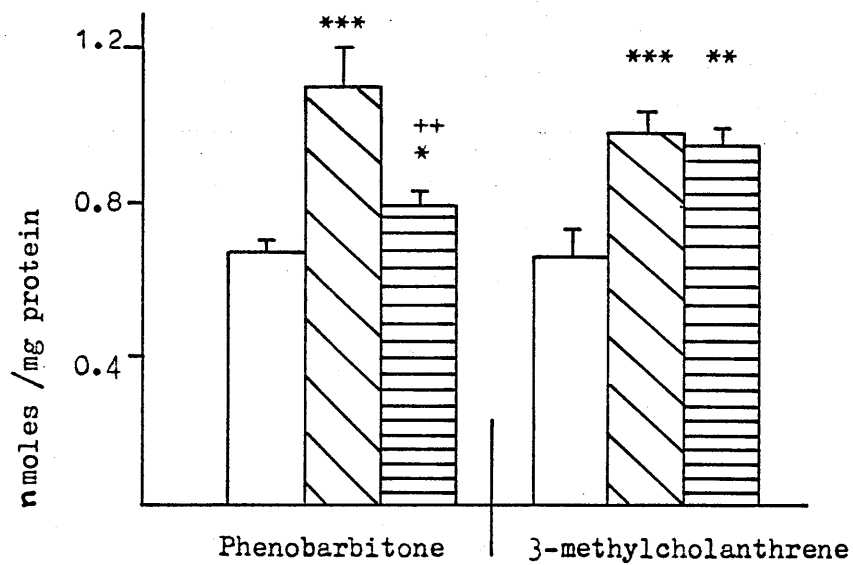


Table 3.1 Microsomal protein levels in non-pregnant, pregnant and post-partum rats

Days after conception	Microsomal protein (mg g liver)	
	Non-pregnant	Pregnant
7	20.1 $\pm$ 1.0	25.2 $\pm$ 0.8*
15	19.2 $\pm$ 1.1	21.8 $\pm$ 1.3
17	17.9 $\pm$ 1.0	25.4 $\pm$ 1.4*
20	22.5 $\pm$ 0.8	22.7 $\pm$ 1.0
		Post-partum
22 (1 day post-partum)	21.8 $\pm$ 0.75	21.0 $\pm$ 1.1
26 (5 days post-partum)	22.7 $\pm$ 1.20	24.3 $\pm$ 0.9

Results are means $\pm$ SEM (n=4 or 8). Significant differences (Students 't' test) are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$ between non-pregnant and pregnant rats.

Table 3.2 Liver biochemistry in pregnant (20 days) and non-pregnant rats

	Non-pregnant	Pregnant
DNA (mg/g liver)	1.22. +/-0.04	1.23 +/-0.08
RNA (mg/g liver)	32.6 +/-4.0	29.8 +/-2.2
Total lipid (mg/g liver)	28.5 +/-3.7	26.9 +/-3.8
Glycogen (mg/g liver)	16.2 +/-4.0	6.8 +/-2.5
Hepatocyte size (μ)	24.0 +/-0.2	25.8 +/-0.2
	n=212	n=210
Glucose-6-phosphatase (IU/g liver)	13.3 +/-0.5	8.1 +/-0.3 ***
Glutamate dehydrogenase (IU/g liver)	49.4 +/-1.6	37.8 +/-5.3
Aspartate aminotransferase (IU/g liver)	93.4 +/-2.0	122.3 +/-6.2 **
Glucokinase (IU/g liver)	1.78 +/-0.21	1.90 +/-0.34
Hexokinase (IU/g liver)	0.25 +/-0.04	0.33 +/-0.04
Glucose 6 phosphate dehydrogenase (IU/g liver)	4.23 +/-0.53	2.98 +/-0.44
Malic enzyme (IU/g liver)	2.56 +/-0.21	2.52 +/-0.72

Values expressed are mean +/- SEM (n=4 unless otherwise stated).

Significant differences (students 't' test) between non-pregnant and pregnant rats are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

3.4 Discussion

Several workers have previously noted changes in hepatic drug metabolism during pregnancy (see Chapter 1). Most studies have been carried out at one or two time points and have not attempted to examine changes over the whole period of gestation. In the present work, three hepatic microsomal transformations were measured at six time points during gestation and post-partum.

Mixed-function oxidase activity during pregnancy has been shown to depend upon the parameter to which activity was related (Figures 3.2 and 3.3). Maternal liver weight was elevated by up to 40% during pregnancy (Figure 3.1), and enzyme activity expressed per unit liver weight (Figure 3.2) was found to progressively decrease during pregnancy. This change was maximal at day 20 of gestation and 1 day post-partum but had returned to non-pregnant control levels by 5 days post-partum. The total capacity of the liver and thus the overall capability of the animal to metabolise drugs remained unchanged or even increased (Figure 3.3). This has been noted previously (Neale and Parke, 1973; Schlede and Borowski, 1974) though other investigators have reported that total hepatic mixed-function oxidase activity is decreased in late gestation (Dean and Stock, 1975).

Pregnancy-induced alterations in mixed-function oxidase activity have been attributed to a decrease in the concentration of hepatic cytochrome P450 (Guarino *et al.*, 1969a; Neale and Parke, 1973; Dean and Stock, 1975). The present investigation does not support this hypothesis as the concentration of cytochrome P450 was unchanged during pregnancy and the total hepatic content of the haemoprotein was significantly elevated above

levels found in non-pregnant control rats (Figure 3.4). This agrees with the findings of Gut et al. (1976) and Schlede and Borowski (1974). Contrary to the results presented (Figure 3.4) a number of investigators have reported a decrease in the concentration of hepatic cytochrome P450 during pregnancy (Guarino et al., 1969a; Neale and Parke, 1973; Dean and Stock, 1975; Feuer and Kardish, 1975; Tabei and Heinrichs, 1976; Mukhtar et al., 1978). The reason for these contradictory findings is not clear though in the case of Tabei and Heinrichs (1976) and Mukhtar et al. (1978) the data presented actually reveals that the concentration of cytochrome P450 is unchanged in late gestation. The decrease in cytochrome P450 levels in late pregnancy reported by Guarino et al. (1969a) is also possibly erroneous since control levels were abnormally high at this time point. In the case of Neale and Parke (1973) some of their data show decreased and some unchanged microsomal concentrations of cytochrome P450 in pregnant compared to non-pregnant rats.

~~The present work also shows that the decrease in hepatic~~
mixed-function oxidase activity during pregnancy was not related to NADPH-cytochrome c reductase activity or microsomal protein levels (Table 3.1).

Many workers have determined in vivo hexobarbital sleeping times during pregnancy. The significance of these as an indicator of in vitro hepatic mixed-function oxidase activity is questionable, particularly since the overall capacity of the liver to metabolise drugs is largely unchanged in pregnancy (Figure 3.3). Gut et al. (1976) suggested that the fate of hexobarbital administered in vivo is not determined by a change in microsomal enzyme activity, but rather by a changed drug distribution in which the foetuses act as a storage site of unmetabolised drug.

A discussion of the alterations in hepatic mixed-function oxidase activity during pregnancy must consider whether the increase in liver size found is due to hyperplasia (increase in cell number) or hypertrophy (increase in cell size). In addition, pregnancy-induced alterations in liver composition (eg. lipid and glycogen content) may also be important in quantifying any change in mixed-function oxidase activity per unit liver weight. Indicators of hepatic hypertrophy and/or hyperplasia have been discussed by Schulte-Hermann (1974). Hypertrophy is indicated by a decrease in DNA concentration, an increase in hepatic size and an increased proportion of cytoplasmic to nuclear area. Hyperplasia, in contrast, is marked by an increase in total hepatic DNA but unchanged DNA concentration, an elevation in the rate of DNA synthesis or in mitotic activity. An agreement of biochemical and histological findings is of particular importance since none of the individual procedures can prove the existence of parenchymal hyperplasia or hypertrophy or permit an estimate of the quantitative contributions of these cellular changes to the overall liver enlargement.

The appearance of the maternal liver in late pregnancy was similar to that of the non-pregnant rat (Figure 3.6). There was no evidence of lipid or glycogen accumulation or depletion histologically (Figures 3.7 and 3.8) or biochemically during pregnancy (Table 3.2), which is in agreement with the findings of Herrera et al. (1969), who reported in addition that hepatic concentrations of water and protein were unchanged in late gestation. In contrast to these results Campbell and Kosterlitz (1949) suggested that a large proportion of hepatic weight increase in the pregnant rat could be accounted for by a fractional increase in the content of water, phospholipids, protein and RNA. Reports of hepatic lipid concentrations during pregnancy have been contradictory. The concentration of

triglyceride has been reported to be increased (Smith and Walsh,1978) or decreased (Montes et al.,1978) while phospholipid is decreased (Dhami et al.,1979) or unchanged (Smith and Walsh,1975; Hererra,1969).

Liver RNA concentrations were unchanged in late gestation (Table 3.2) confirming earlier work by Ekholm et al. (1972). Contrary to this finding increased hepatic concentrations of RNA during gestation have been reported (Campbell et al.,1974; Campbell and Kosterlitz,1949).

The lack of change in hepatic morphology, DNA concentrations and hepatocyte size during pregnancy (Table 3.2) together with evidence of raised mitotic activity (Figure 3.9; Campbell et al.,1974; Dessler-Weist and Dessler, 1977) are indicative of parenchymal hyperplasia. A similar conclusion was reached by Penzes (1961) and Campbell et al. (1974) and is supported by the work of Kelly (1951) who found an increase in hepatic nucleic acid turnover during pregnancy. After parturition, the significant ~~decrease in hepatic weight appears to be due to parenchymal death and lysis~~ (Campbell et al.,1974). Maternal liver weight was still significantly elevated in the 5 day post-partum non-lactating rat (Figure 3.1) and has been reported to decline to control levels by one month after parturition (Poo et al.,1939; Schwenck and Joachim,1961). The increase in liver size during pregnancy appears to be predominantly a consequence of parenchymal hyperplasia. This implies that mixed-function oxidase activity during pregnancy is decreased as a consequence of a decrease in hepatocellular capacity to metabolise drugs and represents a real rather than an apparent change in microsomal function.

It has been suggested that pregnancy imposes a selective effect on the hepatic endoplasmic reticulum (Feuer and Kardish,1975). In the present

study a number of microsomal and non-microsomal non-drug metabolising enzymes were measured in pregnant (20 days) and non-pregnant rats (Table 3.2). It is difficult to conclude from these results that there is a selective effect of pregnancy on the microsomal enzymes as described by Feuer and Kardish (1975).

Any interpretation is complicated since an enzyme activity may alter as a response to either the metabolic requirements of pregnancy or to a change in the structure and function of a cellular component. Many non-microsomal enzymes have been shown to have an altered activity during pregnancy (Campbell et al,1972; Song and Kappas,1968; Parvez et al,1975).

Pregnancy-induced changes in mixed-function oxidase activity appear to be part of a general alteration in microsomal function. A number of other microsomal enzymes are decreased in activity during gestation. These include glucuronyltransferase (see Chapter 1, 1.2.5), glucose-6-phosphatase (Table 3.2, Feuer and Kardish,1975) and phosphatidylethanolamine S-methyltransferase (Feuer and Kardish,1975).

Alterations in enzyme activity in the endoplasmic reticulum during pregnancy may be associated with changes in membrane phospholipids. The importance of microsomal phospholipids to mixed-function oxidase activity has been discussed (see Chapter 1, 1.1.7). The results presented in Figure 3.5 show marked changes in microsomal phospholipid composition paralleling changes determined in drug metabolism during pregnancy and post-partum (Figure 3.2). Phospholipids are important in substrate binding (Narasimhula,1977) and electron transfer to cytochrome P450 (Duppel and Ullrich,1976). The lipid bilayer is probably responsible for holding the haemoprotein and NADPH-cytochrome P450 reductase in a functional complex.

Guengerich and Coon (1975) have shown that phosphatidylcholine, the predominant membrane phospholipid, lowered K_s and K_m for benzphetamine and increased ΔA_{max} and V_{max} for its metabolism. In addition, the substrate induced conformational change of cytochrome P450 may be regulated by phospholipids (Tsong and Yang, 1978). These authors investigated the molecular basis of the spectral and spin-state changes of cytochrome P450 by a temperature jump technique. It was found that the relaxation times of cytochrome P450 (which reflects conformational changes of the enzyme after substrate binding) were greater in microsomal fragments than after haemoprotein purification. This was attributed to the presence of phospholipid which significantly increased the rate of substrate induced conformational changes of the haemoprotein spin-state. It was suggested that phospholipid provides an environment for haemoprotein conformational changes to occur. This requirement for phospholipids has been reported before (Eletr et al., 1973; Fiehn and Hasselbach, 1970).

A link between microsomal phospholipids and mixed-function oxidase activity has been clearly established although the mechanism of interaction is more obscure. Alterations in drug metabolism during pregnancy are possibly as a consequence of the changes occurring in the microsomal membrane phospholipids at this time. The ratio of phosphatidylcholine to phosphatidylethanolamine is significantly decreased in gestation and 1 day post-partum (Figure 3,4) and this may be of particular importance since Strobel et al. (1970) found that phosphatidylethanolamine had an inhibitory effect on the phosphatidylcholine enhanced hydroxylation of benzphetamine. Microsomal phospholipids may also be important modulators of the ferric spin-state of cytochrome P450, a point which will be discussed in greater detail in Chapter 5.

In addition to their effect on mixed-function oxidase activity a number of studies have suggested a functional relationship between microsomal phospholipids and the enzymes glucose-6-phosphatase Nordlie,1974; Garland and Cori,1972; Dyatlovitskaya et al.,1979) and UDP-glucuronyltransferase (Vessey and Zakim,1971; Graham and Wood,1974). In guinea pig microsomes for example, Eletr et al. (1973) correlated breaks in the Arrhenius plots for glucose-6-phosphatase and UDP-glucuronyltransferase with changes in the fluidity of the microsomal membrane at 19° and 32°, thus indicating a functional dependence of these enzymes on the physical state of the membrane phospholipids.

Microsomal phospholipid levels in late pregnancy in the rat have been reported by Feuer and Kardish (1975). These authors found a significant decrease in total phospholipids and, contrary to the results of Figure 3.5, a decrease in both phosphatidylcholine and phosphatidylethanolamine. The reason for this difference is unknown. The work of this group was extended by Dharni et al. (1979a) who reported that microsomal phospholipid-fatty acids were decreased in late pregnancy, the unsaturated fatty acids being decreased to a greater extent than the saturated. Fatty acid changes were related to membrane fluidity which was suggested to influence mixed-function oxidase activity. The data presented by these authors actually reveals that the ratio of saturated to unsaturated fatty acids does not alter in late gestation.

Phosphatidylcholine is synthesised in the endoplasmic reticulum through two main pathways; (a) by direct utilisation of preformed choline via phosphorylcholine and cytidyl diphosphate choline (Kennedy and Weiss,1956) and (b) by a stepwise methylation of phosphatidylethanolamine using the methyl groups from S-adenosylmethionine (Bremer et al.,1960). The

importance of this latter pathway has been assessed and may account for 20% of phosphatidylcholine production in rat liver (Sundler and Akesson, 1975). The contribution of this pathway to phosphatidylcholine biosynthesis may be increased during choline deficiency since the activity of phosphatidylethanolamine -methyltransferase(s) is elevated (Schneider and Vance, 1978; Lombardi, 1971). Female rats use the indirect pathway more than adult male rats (Bjornstad and Bremer, 1966) as do choline deficient males, both groups having a lower content of drug metabolising enzymes as compared to adult males or choline-supplemented animals (Belina et al., 1975; Chen et al., 1972; Cooper and Feuer, 1973; Glenn and Austin, 1971; Mezey et al., 1975; Saito et al., 1975). Given that the methylation pathway becomes more active during the administration of a choline deficient diet, it has been postulated that this pathway has a potential for increasing the choline supply during times of need (Thompson, 1973). Reasoning that females use the methylation pathway more than males it was hypothesised that females have a physiological deficiency of choline (Pani et al., 1978). The changes in microsomal phospholipids seen during pregnancy (Figure 3.5) resemble those seen in a state of choline deficiency: that of decreased phosphatidylcholine and elevated phosphatidylethanolamine (Tokmakjian and Haines, 1979). Excess choline can inhibit ethanolamine phosphorylation in isolated liver preparations (Weinhold and Rathy, 1974) and it is possible that this action of choline could result in a decline in the liver phosphatidylethanolamine level. Conversely, a deficiency of choline as a source of methyl groups which are responsible for the regeneration of S-adenosylmethionine could result in elevated phosphatidylethanolamine and decreased phosphatidylcholine (Tokmakjian and Haines, 1979). Pregnancy is associated with increased fat mobilisation (Montes et al., 1978; Smith and Walsh, 1975; Herrera et al., 1969). Therefore it is not unreasonable to postulate that a relative choline deficiency in the female rat may be

accentuated during pregnancy. If this is so it is not a serious condition as there was no histological evidence of fatty degeneration in the liver (Figure 3.7) which has been found to be associated with a choline deficient diet (Koch et al., 1979; Lombardi, 1971). The effect of choline deficiency during pregnancy might be accentuated by the 10-15% decrease in phosphatidylethanolamine S-methyltransferase activity in late gestation reported by Feuer and Kardish (1975). A dietary supplement of choline throughout pregnancy produced an increase in microsomal total phospholipids and phosphatidylcholine at day 20 of gestation to the level found in non-pregnant rats fed on a normal diet (Figure 3.11).

Phosphatidylethanolamine levels in these rats were significantly decreased below control levels which resulted in an elevated phosphatidylcholine to phosphatidylethanolamine ratio. These changes were associated with an increase in mixed-function oxidase activity above the level found in pregnant rats fed on a normal diet (Figure 3.10). A choline supplemented diet given to non-pregnant rats increased microsomal phosphatidylcholine, the phosphatidylcholine to phosphatidylethanolamine ratio and mixed-function oxidase activity and decreased phosphatidylethanolamine (Figures 3.10 and 3.11). These effects in non-pregnant animals to confirm the work of Pani et al. (1978) who found similar changes in female but not male rats. The results presented in Figure 3.10 and Figure 3.11 emphasise the important relationship between microsomal phospholipid levels and mixed-function oxidase activity. The dietary supplement of choline did not affect the levels of microsomal cytochrome P450 in either pregnant or non-pregnant rats (Figure 3.12). Thus choline induced changes in mixed-function oxidase activity are not related to the level of cytochrome P450.

It is not possible to conclude that pregnancy is associated with an enhanced deficiency of choline. This, however, is worthy of further consideration particularly since Weinhold (1969) reported that pregnancy was associated with an increased incorporation of [methyl-¹⁴C]-choline into phospholipids in liver slices.

Pregnancy-associated changes in mixed-function oxidase activity can be influenced by inducing agents as well as choline. The results in Figure 3.13 and Figure 3.14 confirm previous reports that the inhibitory effect of pregnancy on drug metabolism can be decreased or eliminated by pretreatment with phenobarbitone or 3-methylcholanthrene (Neale and Parke, 1973; Kato et al., 1968a; King et al., 1963). At variance with these results, Schlede and Borowski (1974) found that phenobarbitone pretreatment induced mixed-function oxidase activity to a much greater extent in non-pregnant than pregnant (14 and 21 days) rats.

Changes in the concentration of cytochrome P450 following pretreatment with inducing agents do not necessarily follow the change in mixed-function oxidase activity. Thus cytochrome P450 is significantly elevated by phenobarbitone pretreatment in non-pregnant but not pregnant rats (Figure 3.14).

The effect of inducing agents on microsomal phospholipids in the pregnant rat has not been investigated. In the male rat, however, induction of drug metabolising enzymes in the liver by phenobarbitone injection is accompanied by a proliferation of the endoplasmic reticulum (Remmer and Merker, 1963) and increased synthesis of membrane phospholipids (Orrenius et al., 1965). Phenobarbitone also causes a redistribution of the phospholipid species in the membrane by stimulating the methylation of

phosphatidylethanolamine to phosphatidylcholine (Young et al,1971) via an increase in phosphatidylethanolamine S-methyltransferase activity (Davison and Wills,1974). Cooper and Feuer (1972) reported that phenobarbitone pretreatment significantly increased microsomal protein and phospholipids in parallel with the induction of drug metabolising enzymes. At variance with this finding it has been reported that phenobarbitone pretreatment does not affect microsomal phospholipid concentration and increases phospholipid turnover rate (Davison and Wills,1974). Pretreatment with 3-methylcholanthrene has been reported to depress both the turnover rate and the rate of formation of phospholipids (Davison and Wills,1974).

In conclusion, the results presented show that pregnancy in the rat is associated with marked alterations in hepatic mixed-function oxidase activity which appear to be related to microsomal membrane phospholipids. The extent of these changes clearly depends upon the way in which enzyme activity is expressed although there is a decrease in hepatocellular ability to metabolise drugs during gestation. The hepatic concentration of cytochrome P450 is unchanged during pregnancy. The role of choline and the effect of monooxygenase inducing agents has been discussed.

CHAPTER 4 THE INFLUENCE OF ENDOGENOUS STEROIDS ON HEPATIC DRUG

METABOLISM DURING PREGNANCY IN THE RAT

4.1 Introduction

Steroid hormones are mainly deactivated in the liver. Several enzyme systems are involved in their biotransformation which after dehydration, reduction, hydroxylation and conjugation ultimately results in the production of more polar metabolites. Cytochrome P450 in liver microsomes has been recognised as the terminal "oxygen activating" enzyme that hydroxylates several steroids in the presence of NADPH, the microsomal electron transport system and oxygen (Kuntzman et al, 1964; Conney, 1967; Conney et al, 1969; Hrycay et al, 1976).

Pregnancy is associated with a rise in the circulating levels of progesterone and oestrogens (Davis and Ryan, 1972) and it has been suggested that this is responsible for the decrease in maternal hepatic drug metabolism (Creaven and Parke, 1965; Guarino et al, 1969a; Feuer and Liscio, 1969; Soyka and Deckert, 1974; Feuer and Kardish, 1975; Feuer, 1979). Steroids may produce this effect via competitive inhibition of drug metabolism (Guarino et al, 1969a; Creaven and Parke, 1965), a combination of competitive and uncompetitive inhibition (Soyka and Deckert, 1974) or by an effect on microsomal membrane structure and function (Feuer and Kardish, 1975; Feuer, 1979). These hypotheses are based on the results of experiments which can be conveniently divided into in vitro and in vivo. The in vitro effects were determined following the addition of

steroid to the drug enzyme incubation medium prior to initiation of the enzyme reaction. The in vivo effects were assessed by determining drug metabolism following a period of animal pretreatment with the steroid.

A large proportion of the studies have involved the use of male rats. The important role of sex steroids in the manifestation of the sex difference in drug metabolism in the rat has been discussed (see Chapter 1, 1.2.10) and in view of this it would be erroneous to assume that any steroid induced effects in the male rat could be extrapolated to the female rat. Indeed, Fahim and Hall (1970) have reported that progesterone pretreatment induced mixed-function oxidase activity in female rats while an opposite effect was seen in male rats. Similarly, Defrawy et al. (1974) have shown that oestradiol pretreatment of male rats resulted in a decrease in hepatic monooxygenase activity though no effect was seen in females.

Progesterone (Juchau and Fouts, 1966; Tephly and Mannering, 1968; Soyka and Long, 1972; Tuddenberg et al., 1974; Soyka and Deckert, 1974) and its metabolites (Soyka and Long, 1973; Soyka and Deckert, 1974) have been reported to have an in vitro inhibitory effect on hepatic microsomal drug metabolism in male rats. Progesterone, at a concentration of 10^{-5} M, in the assay system produced up to 50% inhibition in drug enzyme activity as determined by a number of drug substrates, although the degree of enzyme inhibition was generally lower than this, being in the range of 0-17% for seven of the nine enzymes measured (Juchau and Fouts, 1966). A number of progesterone metabolites have been reported to be more potent inhibitors of drug metabolism than progesterone itself although in no instance was the percentage inhibition of p-nitroanisole O-demethylase greater than 25% at 10^{-5} M steroid in male rats (Soyka and Long, 1972). Pregnanolone and pregnanediolone exhibited uncompetitive kinetics in contrast to progesterone,

a competitive inhibitor. In a later study Soyka investigated the in vitro effect of a range of progestogens, oestrogens, androgens and corticosteroids on hepatic p-nitroanisole demethylation in male rats (Soyka and Deckert, 1974). Of the natural steroids tested pregnanolone and progesterone were the most potent inhibitors of drug enzyme activity (36% and 38% inhibition respectively at 10^{-5} M steroid). Oestradiol and cortisol were inactive as enzyme inhibitors while oestrone produced only 12% inhibition of enzyme activity at 10^{-5} M. Soyka and Deckert compared the inhibitory effects of steroids on p-nitroanisole demethylation in male and female rats. Of interest was the finding that the degree of inhibition produced by pregnanolone, progesterone, androsterone and oestradiol was markedly less in female than male rats. This again emphasises the importance of sex in determining steroid induced changes in drug metabolism in the rat. During pregnancy (17-20 days) Soyka and Deckert (1974) observed that the inhibitory effect of steroids was uniformly increased above non-pregnant levels.

The in vivo effects of progestogens and oestrogens on hepatic drug metabolism in the rat have been the subject of a limited number of investigations concerned primarily with contraceptive steroids. Chronic treatment of female rats with contraceptive steroids did not inhibit but increased the activity of liver microsomal enzymes (Jori et al., 1968). Progesterone itself had no effect on enzyme activity. Similar findings were reported by Juchau and Fouts (1966) and Rumke and Noordhoek (1969). Contrary to these results Freudenthal and Amerson (1974) failed to show any inductive effect of synthetic progestogens in male rats but found a weak inhibition of microsomal drug metabolism. This conclusion, based on the determination of cytochrome P450 levels and other compounds of the microsomal electron transport chain has been criticised by Briatico et al.

(1976) who reported that pretreatment with contraceptive steroids resulted in elevated mixed-function oxidase activity without a corresponding increase in cytochrome P450 levels. This effect seemed to be mediated by the progestogenic compounds while oestrogens had only a very slight effect. Fahim et al. (1971) reported that pretreatment of male and female rats with oestradiol produced a significant decrease in p-chloro-N-methyl-aniline demethylation, the effect being more pronounced in male rats. Steroids such as methyltestosterone, cortisone and spironolactone stimulated drug metabolism in female rats without a corresponding increase in the microsomal content of cytochrome P450 (Hamrick et al., 1973). Carter et al. (1974) found a significant increase in ethylmorphine N-demethylase activity in female rats following chronic treatment with a norethynodrel-mestranol mixture for up to six weeks and Jori et al. (1976) reported increased hepatic mixed-function oxidase activity in female rats following contraceptive steroid pretreatment. Sweeny and Cole (1980) found a significant decrease in mixed-function oxidase activity following ethynyl oestradiol pretreatment in male rats for 4 to 8 weeks.

The only detailed study in which the relationship between progesterone metabolites and pregnancy induced changes in drug metabolism has been investigated was undertaken by Feuer and colleagues (Feuer and Kardish, 1975; Feuer, 1979). Female rats were treated with seven daily injections (10mg/kg) of a number of 'hydroxylated' and 'reduced' progesterone metabolites. Reduced metabolites, such as 5 α -pregnane-3 β .ol-20-one and 5 α -pregnane 3 β , 20 β -diol caused a decrease in mixed-function oxidase activity, cytochrome P450 and microsomal phospholipids similar to the changes found in late gestation by these authors (Feuer and Kardish, 1975). Hydroxylated metabolites (16 α -hydroxyprogesterone) produced opposite effects. The authors have

previously determined that during pregnancy progesterone metabolism is shifted toward a greater production of reduced metabolites (Kardish and Feuer, 1972). It was argued that the pregnancy induced changes in drug metabolism were as a consequence of an altered microsomal membrane function which itself was caused by a change in the proportion of reduced to hydroxylated progesterone metabolites. Feuer and Kardish have not, however, investigated the effect of progesterone on drug metabolism in the rat and, furthermore, do not present serum profiles of the various progesterone metabolites throughout gestation.

A number of general conclusions can be drawn from the data reviewed:

(1) The in vitro inhibitory effect of a number of sex steroids on mixed-function oxidase activity has been established in male rats. The inhibition was usually less than 20%, even at high steroid concentrations (10^{-4} M to 10^{-5} M). The degree of inhibition by progesterone and oestradiol was less in female than in male rats.

(2) The in-vivo effect of progestogens and oestrogens on hepatic mixed-function oxidase activity appears to be sex-dependent. Steroid pretreatment of male rats was associated with a decrease or no change in drug enzyme activity while similar treatment in female rats produced an increase or no change. Pretreatment of female rats with progesterone metabolites has been shown, by one group of workers, to create effects on drug metabolism similar to those seen during pregnancy.

Thus the case for a steroid-mediated alteration in drug metabolism during pregnancy is inconclusive. A detailed investigation into the in vitro and in vivo effects of progesterone and oestrogens on female

mixed-function oxidase activity was undertaken to evaluate their role during pregnancy. The in vivo investigations were both acute and chronic. Chronic studies involved the administration of progesterone by intra-peritoneal (i.p) injections and by steroid-containing silicone rubber implants. It was hoped that silicone implants would maintain a constant elevated level of serum progesterone which could not be achieved by intra-peritoneal injections.

4.2 Experimental

(a) Progesterone levels in the serum and liver (10,000g supernatant and 100,000g microsomal fraction) from pregnant (20 days) and non-pregnant rats were determined by radioimmunoassay (Chapter 2, 2.2.20).

(b) The in-vitro effect of a number of steroids on drug enzyme activity in rat liver was studied (Chapter 2, 2.2.7). Each steroid (10^{-9} – 10^{-3} M) was dissolved in dimethylformamide (<0.6% assay volume) and added to the assay prior to reaction initiation. Appropriate controls were run.

(c) The chronic effect of progesterone and oestradiol on hepatic drug enzyme activity (Chapter 2, 2.2.6), cytochrome P450 (Chapter 2, 2.2.2.8) and microsomal protein (Chapter 2, 2.2.21) was determined. Groups of non-pregnant rats were injected i.p. with progesterone (10mg/kg daily for 12 days) or oestradiol (10mg/kg daily for 10 days) in corn oil and killed 24 hours later (Chapter 2, 2.2.5.3 and 2.2.5.4). Serum progesterone levels were determined by radioimmunoassay. Results were compared with control rats receiving corn oil alone.

(d) The effect of an acute dose of progesterone (40mg/kg i.p.), in corn oil one hour before death, on hepatic drug metabolism, microsomal phospholipids (Chapter 2, 2.2.13.2) and serum progesterone levels in non-pregnant rats

was investigated. Results were compared with control rats receiving corn oil alone (Chapter 2, 2.2.5.3).

(e) Silicone rubber implants containing progesterone (32mg) were surgically placed under the skin on the flank of the right leg of non-pregnant rats. Controls were sham operated. Animals were killed 7, 14 and 21 days after implantation (see Chapter 2, 2.2.5.3) and hepatic drug metabolism, microsomal phospholipids and serum progesterone levels determined.

4.3 Results

4.3.1 Hepatic and serum progesterone levels in pregnant and non-pregnant rats

Progesterone levels were determined by radioimmunoassay in liver fractions and serum from non-pregnant and pregnant (20 days) rats (Table 4.1). The concentration of progesterone in the hepatic 10,000g supernatant and 100,000g microsomal suspension was not significantly different between pregnant and non-pregnant animals. Serum progesterone concentrations were significantly elevated on day 20 of pregnancy to five times the level found in non-pregnant rats.

4.3.2 The in vitro effects of steroids on hepatic drug metabolism in female non-pregnant rats

Figure 4.1 shows the effect of four progestagens (progesterone, pregnanediol, 17 α -hydroxyprogesterone and pregnanolone) on the in vitro activity of ethylmorphine N-demethylase, aniline p-hydroxylase and p-nitrobenzoic acid reductase. Progestogen concentrations greater than 10^{-5} M were required to produce an inhibition in the activities of aniline p-hydroxylase and p-nitrobenzoic acid reductase. Ethylmorphine N-demethylase was inhibited by progestagen concentrations lower than 10^{-5} M. A progressive inhibition of ethylmorphine N-demethylase activity was seen over the concentration range used, pregnanediol being the most effective inhibitor.

In Figure 4.2. the in vitro effect of testosterone, oestradiol, oestriol and cortisol on drug enzyme activity is shown. Oestradiol did not inhibit drug enzyme activity at any concentration while the other steroids inhibited all three drug enzymes to between 55% and 90% of control levels at concentrations exceeding 10^{-5} M.

4.3.3 The effect of chronic injection (i.p) of progesterone on hepatic microsomal drug metabolism in non-pregnant female rats

Groups of non-pregnant rats were injected with progesterone (10mg/kg) i.p in corn oil for 12 days and killed on the 13th day. Hepatic drug metabolism and serum progesterone levels were compared to control rats

receiving corn oil alone (Table 4.2). Chronic dosing with progesterone resulted in a significant increase of 30% in ethylmorphine N-demethylase activity. Liver weight, body weight, aniline p-hydroxylase activity, p-nitrobenzoic acid reductase activity, cytochrome P450, microsomal protein and serum progesterone levels were not significantly altered.

4.3.4 The effect of chronic oestradiol injection (i.p) on hepatic

microsomal drug metabolism in non-pregnant female rats

Groups of non-pregnant rats were injected with oestradiol (10mg/kg) i.p. in corn oil for 10 days and killed on the 11th day. Control rats received corn oil alone. This treatment had no effect on body weight, liver weight, drug enzyme activity, cytochrome P450 or microsomal protein levels (Table 4.3).

4.3.5 The acute effect of progesterone on hepatic microsomal drug

metabolism and phospholipids in non-pregnant female rats

Groups of non-pregnant rats were dosed with progesterone (40mg/kg) i.p. in corn oil one hour before death. Control animals received corn oil alone. This pretreatment raised the serum progesterone concentration to twenty-five times the control level but had no effect on microsomal drug enzyme activity, phospholipids or cytochrome P450 (Table 4.4).

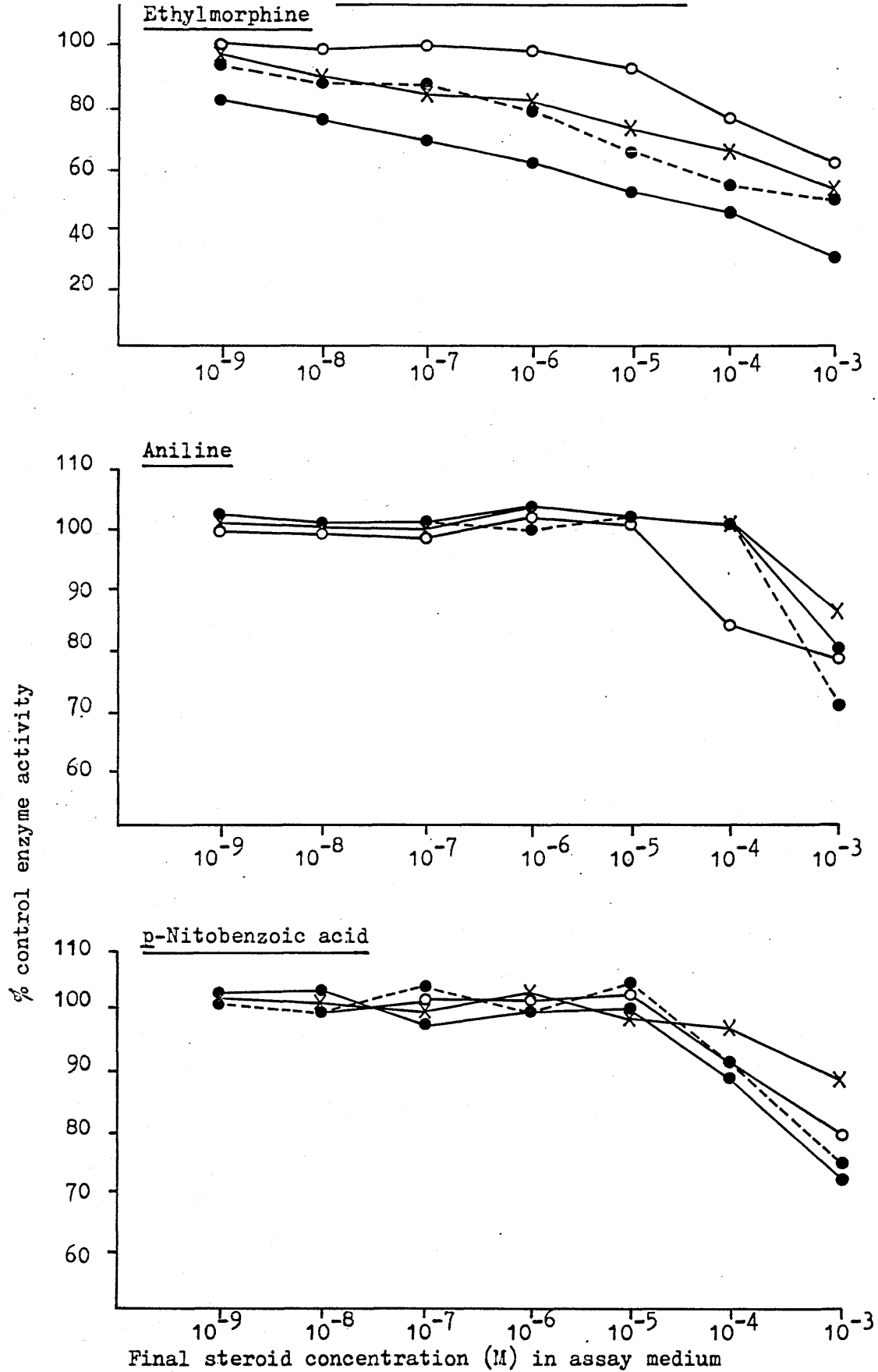
4.3.6 The effect of progesterone containing implants on hepatic

drug metabolism and phospholipids in non-pregnant female rats

The release of progesterone from the silicone implants following removal from the animals on death was measured and compared with the release from unused implants (Table 4.5). The solubility of progesterone in saline has been reported to be $48\mu\text{mol/L}$ (Heap *et al.*, 1970). It can be seen from Table 4.5 that implants removed from rats 7, 14 and 21 days after implantation released progesterone to saturate a saline solution (15mls) over three successive 24 hour periods. A similar release was determined in unused implants over 20 days. This data indicates that the implants were probably releasing progesterone at a constant rate during the period of implantation within the animal.

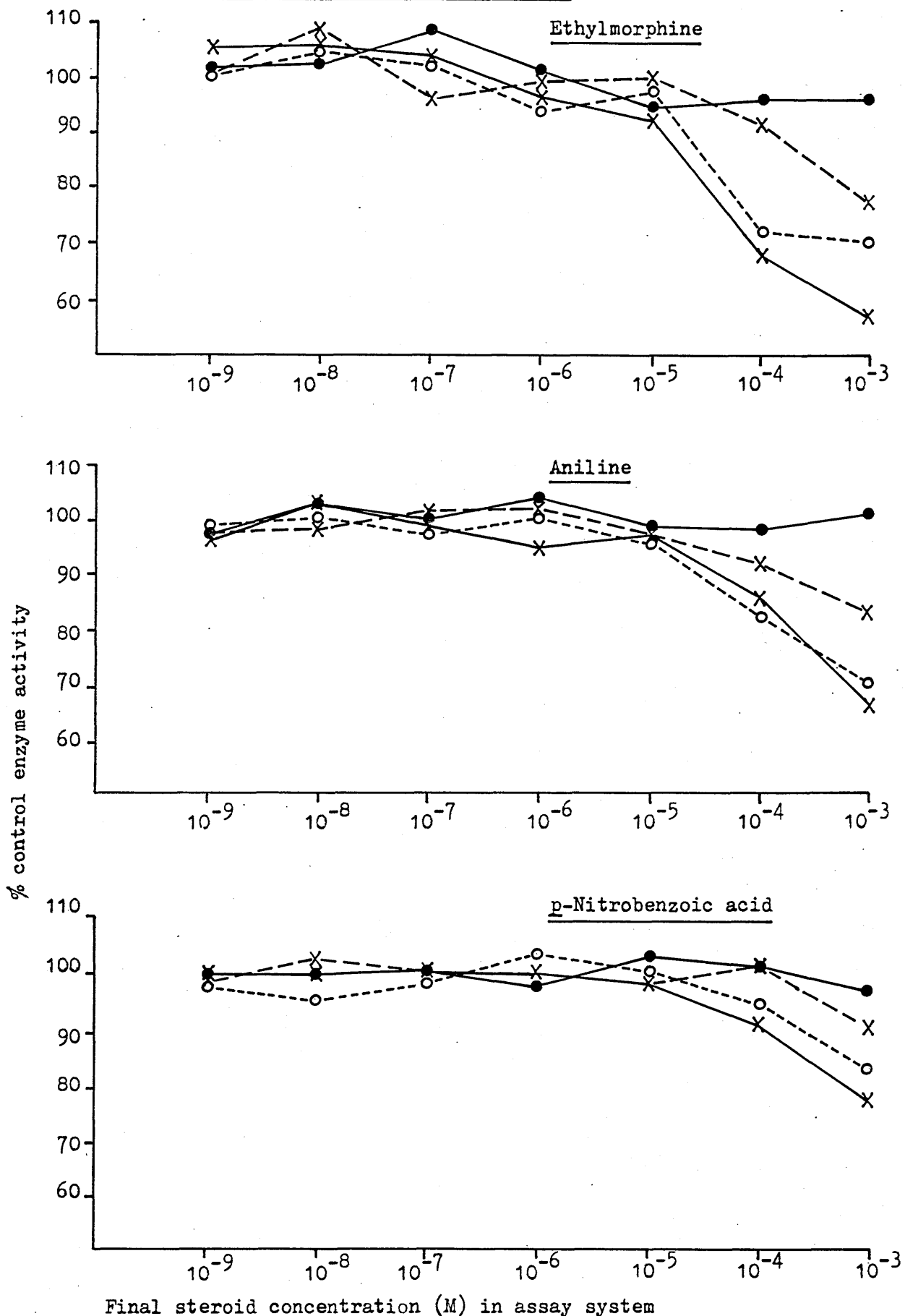
Serum progesterone levels were significantly elevated in the implanted animals to twice the level found in control rats (Figure 4.3). This was associated with significant increases in body weight 14 and 21 days after implantation and liver weight 21 days after implantation (Figure 4.5).

The activity of ethylmorphine N-demethylase was significantly elevated at 7, 14 and 21 days after implantation. The activity of *p*-nitrobenzoic acid reductase was also significantly increased 21 days after implantation (Figure 4.3). Aniline *p*-hydroxylase, cytochrome P450 and microsomal phospholipid levels were unchanged from control levels in the implanted rats (Figures 4.3 and 4.4).



Inhibition of ethylmorphine N-demethylase, aniline p-hydroxylase and p-nitrobenzoic acid reductase in hepatic 10,000g_{av} supernatants by progesterone (○—○), pregnanediolone (●—●), 17α-hydroxyprogesterone (X—X) and pregnanolone (●--●). Each point represents the mean of two experiments.

on hepatic drug metabolism in the female rat

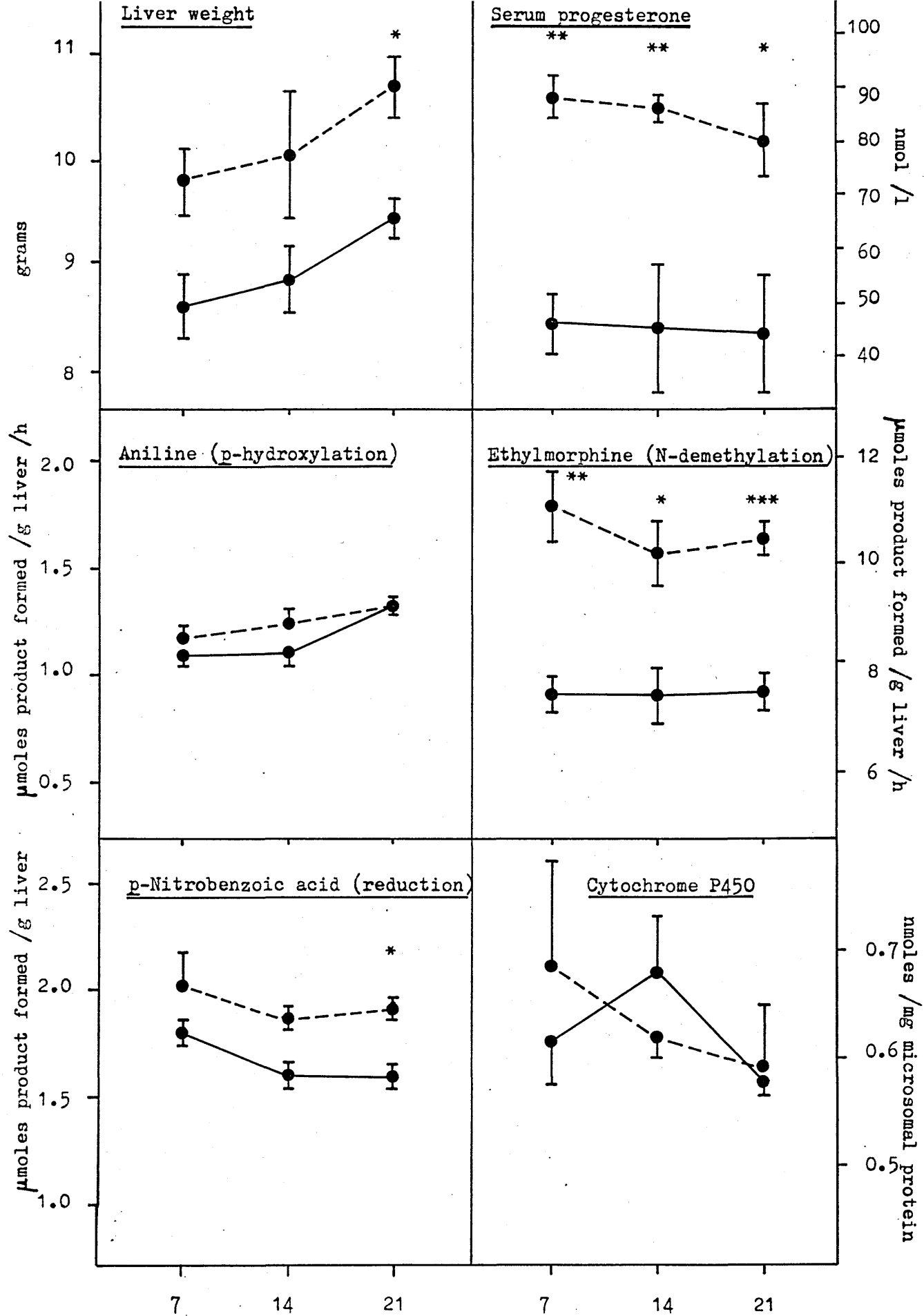


Inhibition of ethylmorphine N-demethylase, aniline p-hydroxylase and p-nitrobenzoic acid reductase in hepatic 10,000_g_{av} supernatants by testosterone (X-X), oestradiol (O-O), oestriol (●-●) and cortisol (X--X). Each point represents the mean of two experiments.

Figure 4.3 Effect of progesterone implants on hepatic drug metabolism
in the female rat

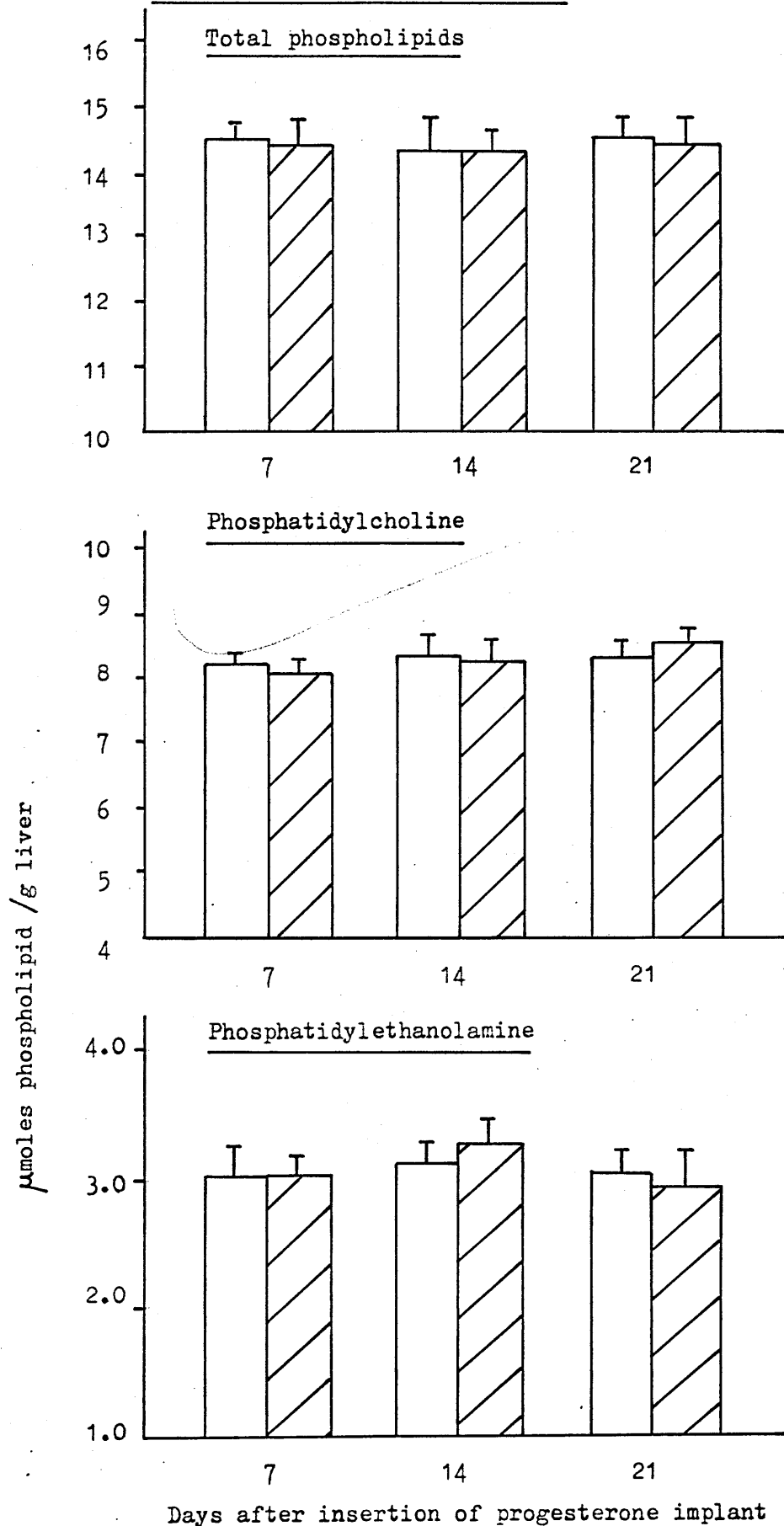
Drug metabolism, liver weight and serum progesterone levels in control
(●—●) and implant rats (○---○).

Significant differences (students 't' test) between control and treated
rats are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Days after insertion of progesterone implant

phospholipids in the female rat



Values represent mean $\pm$ SEM (n=4) for control  and implanted  animals. There were no significant differences between control and implanted animals.

Figure 4.5 The effect of progesterone containing silicone implants on body weight in female

rats 7, 14 and 21 days after implantation

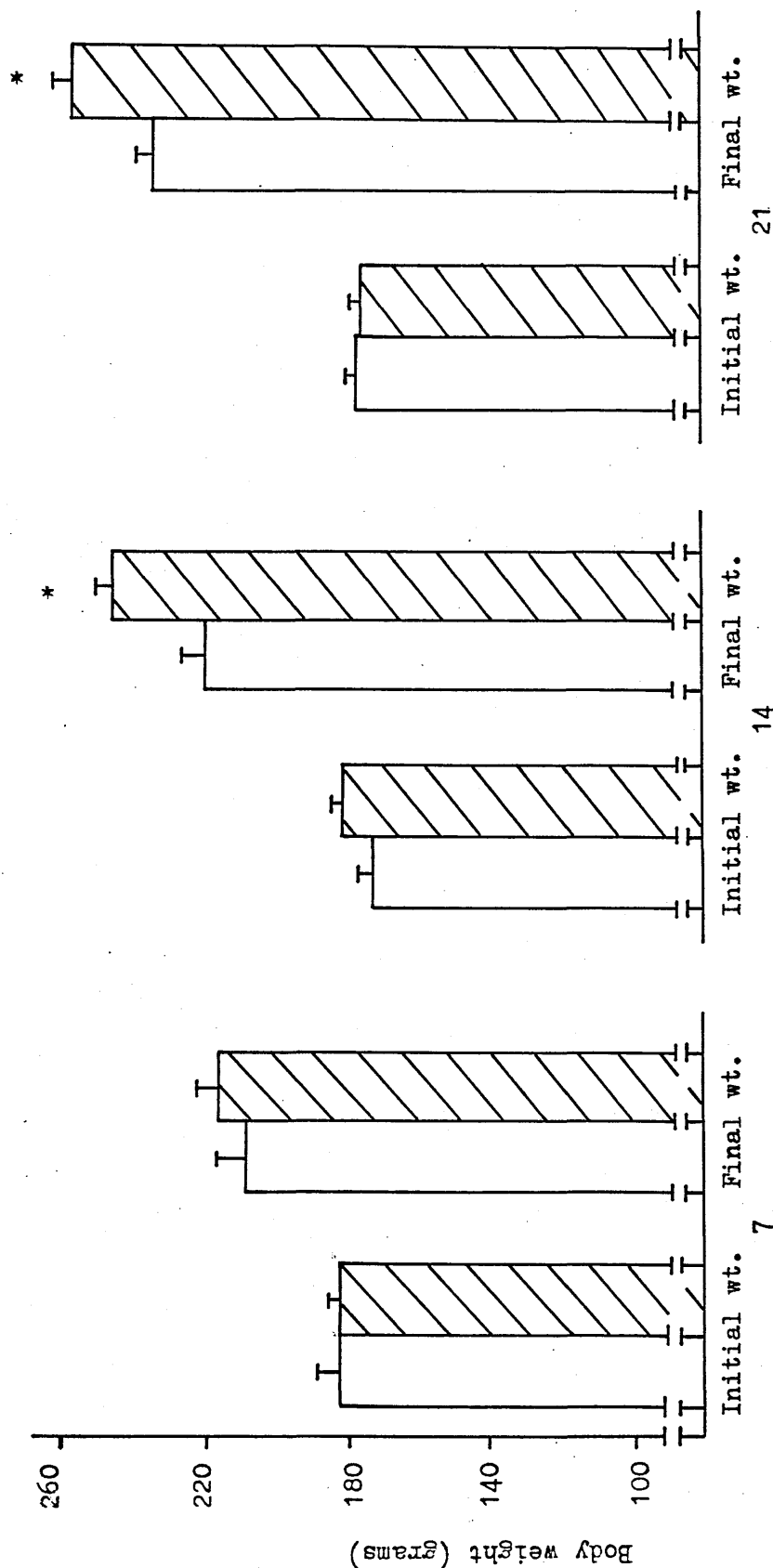


Table 4.1 Progesterone levels in liver fractions and serum from
non-pregnant and pregnant (20 days) rats

Liver fraction	Non-pregnant	Pregnant (20 days)
Progesterone pmoles /g liver		
10,000g supernatant	31.8 +/-7.0	41.6 +/-7.3
100,000g resuspended microsomal pellet	10.2 +/-3.2	14.6 +/-1.9
Progesterone nmoles /L		
Serum	48.8 +/-12.5	239.0 +/-25.0 ***

Each point represents the mean +/- SEM (n=4). Significant differences (students 't' test) are shown as *** p<0.001.

Table 4.2 The effect of progesterone (10mg /kg) i.p. for 12 days on hepatic microsomal drug metabolism in the female rat

	Control	Treated
Body weight (g) Initial	235.3 +/-5.6	235.3 +/-4.2
Final	247.2 +/-5.7	246.9 +/-6.6
Liver weight (g)	8.4 +/-0.2	9.3 +/-0.4
Aniline p-hydroxylase (μ moles /g liver /h)	1.00 +/-0.08	1.04 +/-0.07
p-Nitrobenzoic acid reductase (μ moles /g liver /h)	1.78 +/-0.04	1.73 +/-0.03
Ethylmorphine N-demethylase (μ moles /g liver /h)	9.2 +/-0.65	21.1 +/-0.7 *
Cytochrome P450 (nmoles /mg microsomal protein)	0.71 +/-0.07	0.75 +/-0.08
Microsomal protein (mg /g liver)	19.3 +/-0.8	19.2 +/-1.08
Serum progesterone (nmol /L)	55.1 +/-6.3	58.3 +/-2.5

Values represent mean +/- SEM (n=4). Significant differences (students 't' test) between control and treated animals are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4.3 The effect of oestradiol (10mg/kg) i.p. for 10 days on hepatic microsomal drug metabolism in the female rat

	Control	Treated
Body weight (g) Initial	233.8 +/-6.2	235.8 +/-4.0
Final	256.8 +/-3.7	258 +/-5.5
Liver weight (g)	10.2 +/-0.4	10.4 +/-0.3
Aniline p-hydroxylase (μ moles /g liver /h)	1.35 +/-0.05	1.37 +/-0.03
p-Nitrobenzoic acid reductase (μ moles /g liver /h)	1.77 +/-0.06	1.77 +/-0.04
Ethylmorphine N-demethylase (μ moles /g liver /h)	7.94 +/-0.25	8.79 +/-0.23
Cytochrome P450 (nmoles /mg microsomal protein)	0.63 +/-0.03	0.72 +/-0.5
Microsomal protein (mg /g liver)	19.6 +/-1.0	20.4 +/-1.0

Values represent mean +/- SEM (n=4). No significant differences were found between control and treated groups.

Table 4.4 The acute effect of progesterone (40mg /kg) i.p. 1 hour before death on hepatic microsomal drug metabolism in the female rat

	Control	Treated
Aniline p-hydroxylase (μ moles /g liver /hour)	1.35 $\pm$ 0.08	1.47 $\pm$ 0.03
Ethylmorphine N-demethylase (μ moles /g liver /hour)	9.6 $\pm$ 0.9	11.4 $\pm$ 0.3
p-Nitrobenzoic acid reductase (μ moles /g liver /hour)	1.71 $\pm$ 0.11	1.96 $\pm$ 0.09
Cytochrome P450 (nmoles /mg microsomal protein)	0.63 $\pm$ 0.04	0.70 $\pm$ 0.01
Microsomal phospholipids (μ moles /g liver):		
Total	14.20 $\pm$ 0.20	14.3 $\pm$ 0.30
Phosphatidylcholine	7.95 $\pm$ 0.32	8.15 $\pm$ 0.13
Phosphatidylethanolamine	3.05 $\pm$ 0.35	3.13 $\pm$ 0.20
Serum progesterone (nmol/L)	83.4 $\pm$ 3.0	2070.0 $\pm$ 573.0 **

Values represent mean $\pm$ SEM (n=4). Significant differences (students 't' test) between control and treated animals are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 4.5 The daily release of progesterone into saline medium from
implants

Period of implantation in the rat	Day	Progesterone (μ moles /L)
None	1	46.6 $\pm$ 1.5
	5	46.8 $\pm$ 1.8
	10	46.4 $\pm$ 0.9
	15	44.9 $\pm$ 1.3
	20	47.8 $\pm$ 1.9
7 days	1	47.4 $\pm$ 2.1
	2	46.6 $\pm$ 1.2
	3	46.4 $\pm$ 0.8
14 days	1	48.1 $\pm$ 1.1
	2	45.8 $\pm$ 1.1
	3	45.4 $\pm$ 1.5
21 days	1	46.4 $\pm$ 1.9
	2	47.6 $\pm$ 1.3
	3	49.0 $\pm$ 1.8

The daily passage of progesterone into saline (15mls) at 37° from silicone implants was determined (see Chapter 2, 2.2.18). Values represent mean $\pm$ SEM (n=4).

4.4 Discussion

Progesterone has been studied in detail in this chapter because changes in its blood concentration during gestation correlate with the changes occurring in hepatic drug metabolism. Serum levels of progesterone are elevated early in pregnancy and remain high until parturition when they fall to a nadir (Weist,1970; Davies and Ryan,1972; Sanyal,1978). Quantitatively serum progesterone concentrations during gestation are up to five hundred times higher than corresponding concentrations of oestradiol and oestrone (Fuchs,1974). In addition, serum levels of oestrogens (Shaikh,1971; Davies and Yen,1971; Fuchs,1978), corticosteroids (Ogle and Kitay,1977) and androgens (Gibori et al,1979) remain low until late gestation in the rat.

The hypothesis that pregnancy-induced changes in drug metabolism are regulated via an increase in circulating steroid levels (Creaven and Parke,1965; Guarino et al,1969a; Feuer and Liscio,1969; Soyka and Deckert,1974; Feuer and Kardish,1975; Feuer,1979) is not supported by the results presented in this chapter. Despite its high serum levels, progesterone concentration is not significantly increased in the liver during late pregnancy (Table 4.1). The in vitro investigations showed that only in the presence of unphysiologically high concentrations of progesterone was there any inhibitory effect on mixed-function oxidase activity (Figure 4.1). Similarly other steroids tested showed little or no inhibitory effect on drug enzyme activity even at high concentrations (Figures 4.1 and 4.2).

The effect on hepatic mixed-function oxidase activity of in vivo pretreatment of intact rats with progesterone depended upon the method of administration of the steroid. A large acute i.p. dose, while elevating the serum progesterone concentration to twenty-five times the control level, had no effect on hepatic microsomal mixed-function oxidase activity or phospholipids (Table 4.4). Chronic pretreatment, on the other hand, resulted in a significant increase in ethylmorphine N-demethylase (Table 4.2, Figure 4.3) and p-nitrobenzoic acid reductase (Figure 4.3) activity. These changes were not associated with alterations in microsomal cytochrome P450 (Table 4.2, Figure 4.3) or phospholipids (Figure 4.4).

Of interest was the finding that the serum progesterone level in the rats treated with silicone implants was significantly increased above the control level (Figure 4.3). In contrast to this serum progesterone levels in rats 24 hours after i.p. steroid administration were unchanged from those found in untreated rats (Table 4.2). These findings suggest that continuously raised levels of steroid can be achieved with the use of silicone implants but not by daily i.p. injection.

Chronic progesterone treatment with silicone implants was associated with a significant increase in body and liver weight (Figures 4.3 and 4.5) supporting the hypothesis that this steroid is responsible, at least in part, for the pregnancy-induced changes in liver weight (Song and Kappas, 1968; Desser-Weist and Desser, 1977).

Chronic oestradiol pretreatment (Table 4.3) had no effect on mixed-function oxidase activity or cytochrome P450.

The in vivo effects of corticosteroids and androgens on mixed-function oxidase activity have not been investigated here. Serum levels of these steroids remain low until late gestation as discussed earlier, and they appear to have little in vitro inhibitory effect on drug enzyme activity (Figure 4.2). Investigations in vivo have shown an increase in drug enzyme activity following their administration. Administration of corticosteroids was able to restore drug enzyme activity in adrenalectomised rats to normal levels (Gillette, 1963; Kato and Gillette, 1965b) and Bousquet et al. (1965) demonstrated an increased in vivo metabolism of hexobarbital after the injection of ACTH or corticosterone.

Steroids, particularly progesterone and oestradiol, are unable to produce pregnancy-associated effects on hepatic drug metabolism and are unlikely to be responsible for mediating the observed changes at this time. This conclusion is supported by an additional observation that serum progesterone levels have fallen to a nadir one day post-partum (Weist, 1970) when microsomal mixed-function oxidase activity and phospholipid levels are still significantly altered (Chapter 3, Figures 3.2, 3.3, 3.5). It is possible that the lack of effect of steroids on drug metabolism is due to their interaction with different forms of cytochrome P450 since this haemoprotein has been shown to exist in multiple forms (Guengerich, 1979) with different though overlapping substrate affinities (Haugen et al., 1975; Huang et al., 1976; Guengerich, 1977).

CHAPTER 5 CHANGES IN CYTOCHROME P450 DURING PREGNANCY:

MULTIPLE FORMS, SUBSTRATE BINDING SPECTRA AND SPIN EQUILIBRIUM

5.1 Introduction

The absolute concentration of cytochrome P450 has been shown to be unchanged during gestation and early post-partum in the rat (Chapter 3). The relationship of cytochrome P450 to mixed-function oxidase activity during pregnancy was investigated further by analysis of the electrophoretic pattern of microsomal protein with particular reference to cytochrome P450 by SDS (sodium dodecyl sulphate)-polyacrylamide gel electrophoresis. Cytochrome P450-substrate interactions were followed spectrophotometrically by the measurement of substrate binding spectra. The haem spin-state of cytochrome P450 was also determined by measuring thermally-induced spin-state transitions of the haemoprotein. A short discussion of the theoretical aspects of these techniques is presented.

5.1.1 SDS-polyacrylamide gel electrophoresis

The use of polyacrylamide gel in the electrophoretic separation of macromolecules has a number of advantages over other supporting media such as paper and cellulose acetate.

(1) The gels may be produced with a wide range of pore sizes.

- (2) They can be formed in precision bore tubes and hence give reproducible results.
- (3) They may be used with a wide range of buffers.
- (4) Their degree of adsorption and electro-osmosis is low.
- (5) They give rapid separation.
- (6) They do not absorb ultra violet light at 270nm and hence proteins separated on them may be estimated at this wavelength.
- (7) They enable macromolecules to be stained and the amount of material present determined by densitometry.

Polyacrylamide gel is prepared by co-polymerizing acrylamide ($\text{CH}_2=\text{CH}.\text{CO}.\text{NH}_2$) and N,N'-methylenebisacrylamide ($\text{CH}_2(\text{NH}.\text{CO}.\text{CH}=\text{CH}_2)_2$) with a suitable free radical catalyst accelerator such as ammonium persulphate combined with N,N,N',N'-tetramethylethylenediamine (TEMED). The porosity of the gel is determined by the relative proportion of acrylamide monomer and cross linking reagent. Gel may be prepared containing 3-30% (w/v) total acrylamide corresponding to pore sizes of 0.2 and 0.5nm respectively. The lower percentage gels have a larger pore size and thereby offer less resistance to the passage of large molecules.

The use of sodium dodecyl sulphate as a detergent in electrophoresis systems was developed by Shapiro et al. (1967) and extended by Weber and Osborn (1969). Membrane disruption and protein denaturation and solubilization occur after treatment with SDS and a sulphydryl reducing agent. The resulting polypeptide chains can be separated on the basis of molecular weight alone, though this depends on three assumptions (Maddy, 1976):

(1) That the binding of SDS to a polypeptide disrupts all but the covalent interactions within chains and between chains and that the interactions between proteins and lipids are destroyed. It has been reported that not all membrane proteins are solubilized by SDS (Maddy, 1976) and furthermore that some lipid may be associated with proteins becoming dissolved in the SDS (Dunn et al., 1975).

(2) That the binding of the detergent per unit length of polypeptide is constant and sufficient to abolish charge differences between polypeptides. Variations in SDS binding to different proteins (Maddy, 1976; Grefarth and Reynolds, 1974) and to different extents in any one protein (Robinson and Tanford, 1975) have been reported.

(3) That conformational changes between polypeptides are destroyed such that frictional drag during passage through the gel is a function of molecular weight alone.

The resolution of different proteins may be improved by the use of discontinuous (disc) electrophoresis, in which an upper large pore stacking gel is used to concentrate the sample prior to its entry into the lower running gel. In this system the pH of the buffer in the upper reservoir is lower than the buffer saturating the stacking gel and this allows the sample to be concentrated between highly mobile leading ions (Cl^-) and less mobile trailing ions (glycinate).

Electrophoresis systems containing SDS for the resolution of membrane proteins are of great use when the resolution of the maximum number of polypeptide bands is the main objective. Since SDS treatment largely destroys enzyme activity this method is of limited use when the retention

of enzyme activity is desired.

Further details on the principles and practice of electrophoresis are to be found in Williams and Wilson (1975).

5.1.2 Substrate induced cytochrome P450 binding spectra and haemoprotein spin-state

Narasimhulu et al (1965) observed the formation of a spectral transition in the Soret region of cytochrome P450 upon addition of a steroid to a suspension of adrenal corticol microsomes. Less than one year later two types of spectral change were described when drugs were added to liver microsomes (Remmer et al, 1966; Schenkman et al, 1967; Imai and Sato, 1966). The first spectral change, termed 'type 1' was characterised by an absorption maximum at 385-390nm and a minimum at 420nm; the second spectral change, 'type 2', was shown to possess an absorption maxima at 425-435nm and minima at 390-400nm (Schenkman et al, 1967). The third spectral transition, initially called 'modified type 2' displayed a mirror image of the 'type 1' spectral change and was subsequently described as 'reverse type 1' (Schenkman et al, 1973). All three spectral transitions are induced following substrate addition to a liver microsomal suspension and may be followed by spectrophotometry or more conveniently by difference spectrophotometry. The spectral binding affinity (K_s) and the maximum binding capacity (ΔA_{max}) can be calculated from a double reciprocal plot of absorbance change against substrate concentration analogous to the calculation of the kinetic constants K_m and V_{max} from a Lineweaver-Burke plot.

Mason and co-workers (1965) described an electron paramagnetic resonance (EPR) spectrum in liver microsomes which resembled low-spin ferrihaemoproteins and suggested that the EPR signal was related to cytochrome P450. Cammer et al. (1966) reported similar findings and in addition showed that both 'type 1' and 'type 2' substrates affected the low-spin g_x and g_z components of the first derivative EPR spectrum in opposite directions. For example, in the presence of aniline ('type 2' substrate) the g_x signal was shifted to lower field; the opposite occurred in the presence of hexobarbital, a 'type 1' substrate (Cammer et al., 1966). A conversion of the low-spin ferric haemoprotein to the high-spin form following addition of camphor was reported in the Pseudomonas putida cytochrome P450 system by Tsai et al. (1970) and Peterson (1971). The spin state of ferric cytochrome P450 can thus be modulated by substrate addition, shifting from low- to high-spin with 'type 1' compounds, high to low-spin with 'reverse type 1' compounds or forming a new low-spin species with 'type 2' compounds. Similar spin state changes occurred with adrenal cortex mitochondrial cytochrome P450 (Whysner et al., 1970). In addition both Hilderbrandt et al. (1968) and Jefcoate and Gaylor (1969) reported that in liver microsomes from 3-methylcholanthrene treated animals there was an EPR signal at about $g=6$ indicative of high-spin P450. Kumaki et al. (1978) have subsequently attempted to correlate the observed spectral changes ('type 1', 'type 2' and 'reverse type 1') with the absolute spin state of the iron.

The low-spin to high-spin transition associated with a 'type 1' substrate is thought to arise from obstruction of the binding of the iron sixth ligand which is probably a water molecule (White and Coon, 1980). With the release of the water ligand the iron moves out of the plane of the porphyrin towards the fifth (thiolate) ligand. This ligand obstruction

phenomenon is probably also manifested in the variation of carbon monoxide bonding geometry with and without substrate as detected in the infrared spectrum of ferrous carbonyl P-450cam by O'Keeffe et al. (1978). Upon a change of the coordination sphere from approximately octahedral (hexacoordinate) to square pyramidal (pentacoordinate) the energetic ordering of the ferric d-orbital changes and the electron pairing pattern shifts from low-spin ($s=1/2$) to high-spin ($s=5/2$). The high-spin iron has a larger radius and can no longer fit inside the central hole of the porphrin (White and Coon, 1980). It is now apparent that the high-spin form of all types of cytochrome P450 is pentacoordinate and that the equilibria between low and high-spin forms is a manifestation of the binding equilibria of the sixth ligand (White and Coon, 1980; Ebel et al., 1978; Orme-Johnson et al., 1979).

Recently Sligar (1976) showed that cytochrome P450cam in the presence or absence of substrate contained a mixture of high-spin and low-spin haem iron which was temperature sensitive. In related studies Rein et al. (1979) reported the presence of a substrate-induced high-spin/low-spin equilibrium which was temperature dependent. At the same time Pierson and Cinti (1977) found that the microsomal 'type 1' and 'reverse type 1' spectral changes could be attained by an alteration of temperature without the addition of substrate. Furthermore it was shown by both Rein et al. (1977) and Pierson and Cinti (1977) that the substrate-induced types '1' and '2' spectral changes were affected by temperature.

A relatively simple method for determining the spin state of cytochrome P450 based on thermally induced spin state transitions has been developed by Sligar (1976). The results obtained by this method are in agreement with the haemoprotein spin state determinations by high

temperature Mossbauer and electron paramagnetic resonance spectroscopy results (Sligar, 1976). A method slightly modified from that of Sligar (1976) has been used to determine the absolute spin state of microsomal cytochrome P450 in pregnant and non-pregnant animals. A detailed description of the analysis is presented.

In the simplest two-state model at any physiological temperature the ferric P450 molecule can be considered to exist as a mixture of high-spin (total spin, $s=5/2$) or low-spin ($s=1/2$) forms corresponding to the ordering of the five d-orbital electrons. To each of the high- and low-spin forms there corresponds a unique optical spectrum. Thus, at any wavelength the total absorbance A , $=E_{HS}[P-450_{HS}] + E_{LS}[P450_{LS}]$ where E_{HS} and E_{LS} refer to the extinction coefficients and $[P450_{HS}]$ and $[P450_{LS}]$ to the concentrations of high- and low-spin forms respectively. In addition, at all wavelengths there exists a maximum (A_{max}) and a minimum (A_{min}) value of absorbance which corresponds to the limiting cases when the P450 molecule is in either the complete low-spin or complete high-spin state. When cytochrome P450 exists as a mixture of spin states the absorbance will fall between the limiting values A_{max} and A_{min} with the distance from the upper and lower limits being directly proportional to the high- and low-spin fractions. For example at 420nm the fraction of low-spin material is given by the ratio $(A - A_{min}) / (A_{max} - A_{min})$ while the proportion of high-spin is $(A_{max} - A) / (A_{max} - A_{min})$. Defining an equilibrium constant for the process $P450_{LS} \rightleftharpoons P450_{HS}$ by $K = [P450_{HS}] / [P450_{LS}]$ yields the following expression for K :

$$K = A_{max} - A / A - A_{min}$$

If this process is then assumed to follow standard Vant Hoff behaviour the following can then be written:

$$K = A_{max} - A / A - A_{min} = e^{\Delta G / RT} = e^{\Delta S / R - \Delta H / RT}$$

or

$$\ln K = \ln A_{\max} - A / A - A_{\min} = \Delta S / R - \Delta H / RT$$

which completely describes the variation in spin state equilibrium constant with temperature. In the analysis, changes in the wavelength couple 390 minus 420nm were recorded. These represent the wavelengths used to determine the low-spin to high-spin transition of a 'type 1' spectrum. Since the values $A_{\max}$ and $A_{\min}$ cannot be known a priori by any experimental means they are used as parameters in a regression fit to the variation in observed absorbance value as a function of temperature. Using the correlation coefficient as a convergence parameter for the least squares straight line fit of $\ln[(A_{\max} - A) / (A - A_{\min})]$ vs. $1/RT$ one can determine the values $A_{\max}$ and $A_{\min}$ which give the best fit to the observed absorbances as well as the slope ΔH and the intercept $\Delta S/R$. The computer analysis was simplified by assuming an extinction coefficient for the wavelength couple 390 minus 420nm of $126\text{mM}^{-1}\text{cm}^{-1}$ (Cinti et al., 1979; Gibson et al., 1980a).

5.2 Experimental

(a) Microsomal proteins from non-pregnant and pregnant (20 days) rats were separated by SDS-polyacrylamide gel electrophoresis as described in Chapter 2 (2.2.14). Microsomal protein ($10\mu\text{g}$ or $20\mu\text{g}$) was applied to the stacking gel together with a series of standard proteins of known weight.

(b) Substrate-induced difference spectra for microsomal cytochrome P450 from non-pregnant and pregnant (15 and 20 days) rats have been determined (Chapter 2, 2.2.11). The following substrates were used; aniline, ethylmorphine, progesterone and 17α -hydroxyprogesterone. Spectral binding affinities (K_s) and the maximum spectral change ($\Delta A_{\max}$) were calculated from the difference spectra.

(c) Thermally induced spin state transitions were determined in non-pregnant and pregnant (20 days) rats. The data was fitted to a Vant Hoff plot by the method described (Chapter 2, 2.2.12). Haemoprotein spin-state, ΔH , ΔS and ΔG were calculated from this plot.

5.3 Results

5.3.1 SDS-polyacrylamide gel electrophoresis of microsomal

proteins from non-pregnant and pregnant (20 days) rats

The body and liver weights of the rats together with microsomal protein and cytochrome P450 are shown in Table 5.1. Body weight and liver weight were significantly increased in the pregnant animals (Table 5.1) and were similar to the increases reported in Chapter 3 (Figure 3.1). Microsomal levels of protein and cytochrome P450 were unchanged.

The electrophoretic patterns of the microsomal proteins from non-pregnant and pregnant animals are shown in Figures 5.1 and 5.2. No difference was seen in the pattern of microsomal proteins between non-pregnant and pregnant animals. A change in the type(s) of cytochrome P450 present would be expected to occur in the molecular weight region 45,000 - 60,000 (Guengerich, 1979). In Figure 5.1 this region is heavily stained and it is difficult to distinguish individual protein bands. This problem is overcome in Figure 5.2 by the application of less protein (10 μ g) to the gel. However no difference in protein bands was observed in this

region between pregnant and non-pregnant rats.

5.3.2 Spectral binding affinities (K_s) and the maximum spectral change

(ΔA_{max}) for four substrates in non-pregnant and pregnant rats

Substrate-induced difference spectra for microsomal cytochrome P450 have been determined. Ethylmorphine (type 1 substrate) produced a difference spectra with an absorption maxima at 385nm and minima at 415nm. Aniline (type 2 substrate) produced an absorption maxima at 430nm and minima at 390-395nm. Progesterone and 17 α -hydroxyprogesterone (reverse type 1 substrates) produced absorption maxima at 415-420nm and minima at 385nm (Figure 5.3). The spectral binding affinities (K_s) and the maximum absorbance changes (ΔA_{max}) are shown in Tables 5.2 and 5.3 respectively. These parameters were calculated from the difference between the absorbance maxima and minima for each substrate. The spectral binding affinity represents the substrate concentration at which the absorbance change is half maximal.

During pregnancy the spectral binding affinities (K_s) for the four substrates used did not change from the values determined in control non-pregnant rats (Table 5.2). The K_s values for the four different substrates used vary considerably. The concentration of progesterone and 17 α -hydroxyprogesterone required to produce a half maximal absorbance change is up to one thousand times less than the equivalent concentration of ethylmorphine or aniline. In Table 5.3 the ΔA_{max} values have been calculated from the same data used for Table 5.2. A significant decrease in the ΔA_{max} for aniline in the 20 day pregnant rat was found. The ΔA_{max}

for ethylmorphine, progesterone and 17α -hydroxyprogesterone was unchanged during gestation. The ΔA_{max} for ethylmorphine was less than half of the value for the other three substrates. This is reflected in the maximum absorbance changes shown in Figure 5.1.

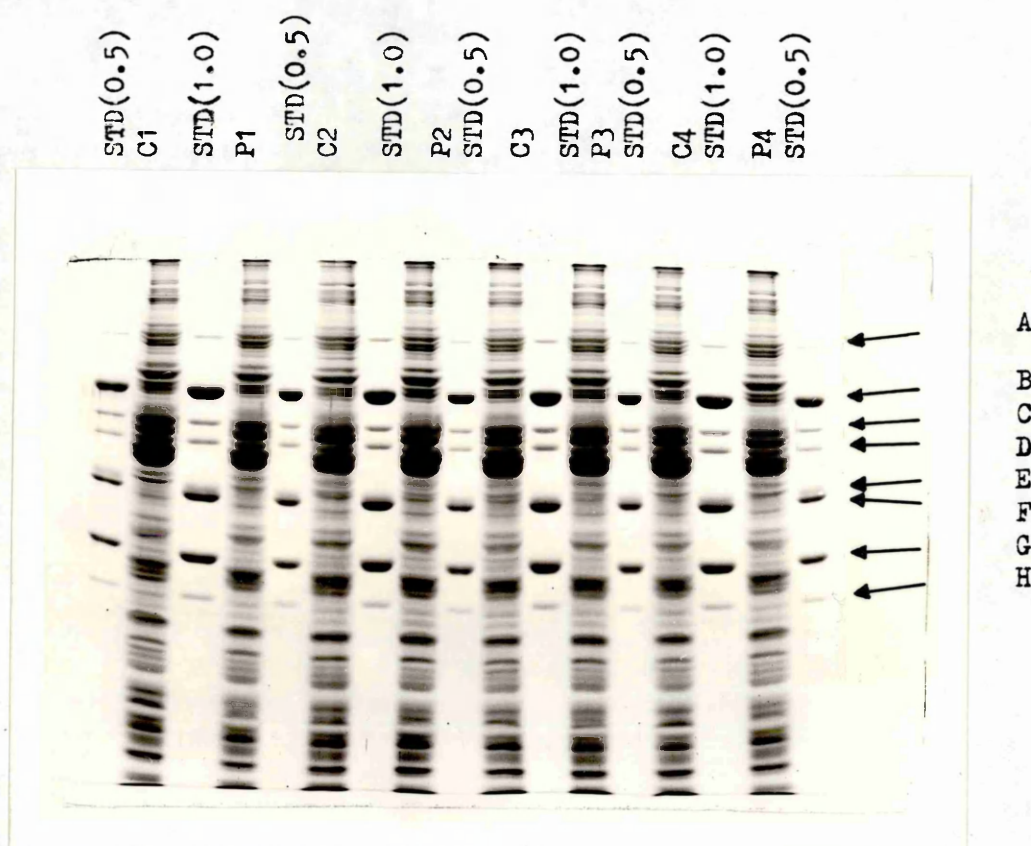
5.3.3 Microsomal cytochrome P450 spin-state equilibria in microsomes

from non-pregnant and pregnant (20 days) animals

The equilibrium spin-state of microsomal cytochrome P450 was determined in non-pregnant and pregnant (20 days) rats. Body weights and liver weights for the pregnant (20 days) and non-pregnant rats together with the concentrations of microsomal protein and cytochrome P450 are shown in Table 5.1. Thermally induced absorbance changes between 390 and 420nm were fitted to Vant Hoff equation and this is shown in Figure 5.4. This analysis allows the values of ΔS , ΔH , ΔG and K to be calculated (Table 5.4).

The equilibrium constant (K) was significantly lower in the pregnant compared with the non-pregnant animals over the temperature range used (Figure 5.4). The proportion of high-spin cytochrome P450 present was calculated from K and these are shown in Table 5.4 at 20° and 37° . Pregnancy was associated with a significant decrease in the high-spin form of cytochrome P450 and an increase in low-spin cytochrome P450. Associated with this change were significant alterations in ΔH , ΔS and ΔG during pregnancy.

Figure 5.1 SDS-polyacrylamide gel electrophoresis of microsomal proteins from non-pregnant and pregnant (20 days) rats

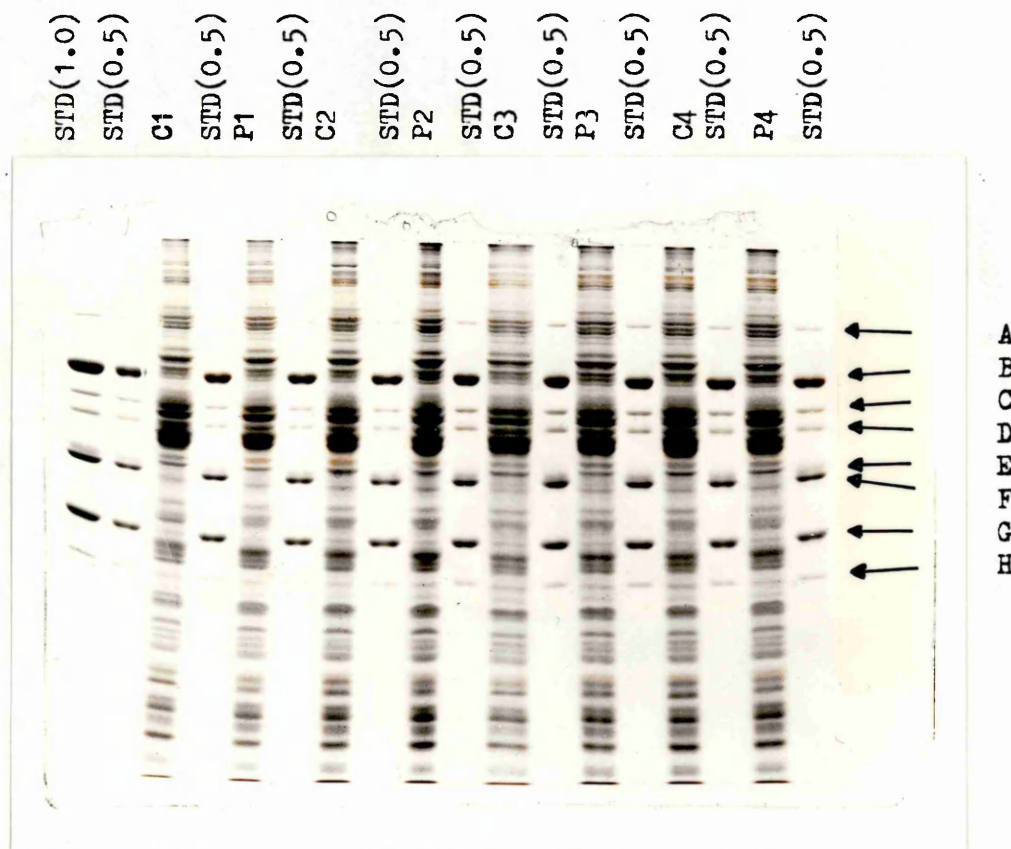


Protein standards:

A	Phosphorylase a	Mol.wt. 92,500
B	Bovine serum albumin	Mol.wt. 68,000
C	Catalase	Mol.wt. 60,000
D	Glutamate dehydrogenase	Mol.wt. 53,000
E	Chicken egg albumin	Mol.wt. 43,000
F	Creatinine phosphokinase	Mol.wt. 40,000
G	Lactate dehydrogenase	Mol.wt. 36,000
H	Cytochrome c	Mol.wt. 11,700

Microsomal protein (20 μ g) from non-pregnant (C1-C4) and pregnant (P1-P4) rats was separated together with a standard protein mixture (0.5 μ g standard mixture: STD (0.5) and 1.0 μ g standard mixture: STD (0.5)). Electrophoresis conditions are described in Chapter 2 (2.2.14).

Figure 5.2 SDS-polyacrylamide gel electrophoresis of microsomal proteins from non-pregnant and pregnant (20days) rats

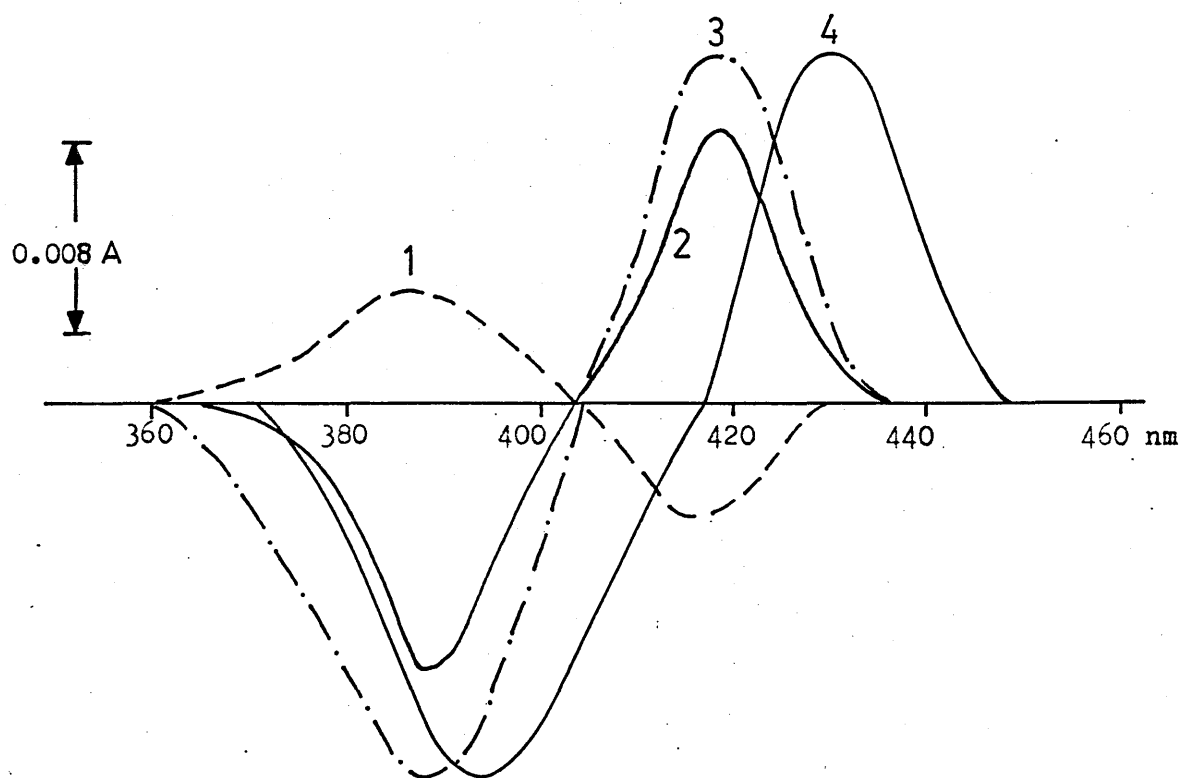


Protein standards

- A Phosphorylase a Mol.wt 92,500
- B Bovine serum albumin Mol.wt. 68,000
- C Catalase Mol.wt. 60,000
- D Glutamate dehydrogenase Mol.wt. 53,000
- E Chicken egg albumin Mol.wt 48,000
- F Creatinine phosphokinase Mol.wt. 40,000
- G Lactate dehydrogenase Mol.wt. 36,000
- H Cytochrome c Mol.wt. 11,700

Microsomal protein (10 μ g) from non-pregnant (C1-C4) and pregnant (P1-P4) rats was separated together with a standard protein mixture (0.5 μ g standard mixture : STD (0.5) and 1.0 μ g standard mixture STD (1.0)). Electrophoresis conditions are described in Chapter 2 (2.2.14).

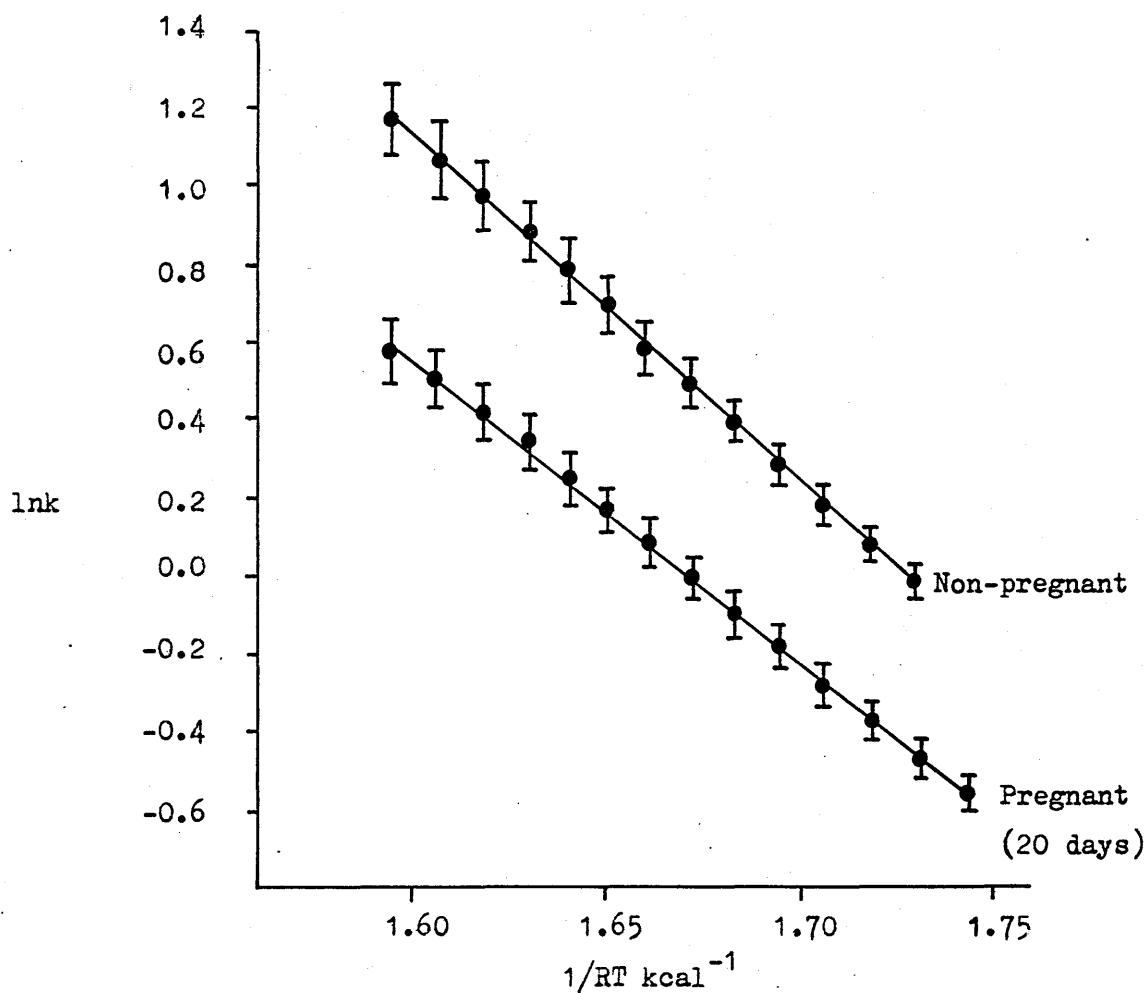
Figure 5.3 Substrate induced cytochrome P450 difference spectra
in the presence of saturated amounts of substrate.



- 1 Ethylmorphine (type '1' substrate)
- 2 Progesterone (reverse type '1' substrate)
- 3 17 α -hydroxyprogesterone (reverse type '1' substrate)
- 4 Aniline (type '2' substrate)

Substrate induced difference spectra were obtained according to the method described (see Chapter 2, 2.2.11).

Figure 5.4 Thermally induced spin state transition in hepatic microsomal cytochrome P450 from pregnant (20 days) and non-pregnant rats.



Thermally induced spin state transitions were fitted to a Vant Hoff equation by the procedure described in Chapter 2 (2.2.12).

Each point represents the mean $\pm$ SEM (n=4). Non-pregnant and pregnant values are significantly different at all points ($p < 0.01$).

Table 5.1 Body and liver weights, and microsomal contents of protein and cytochrome P450 from non-pregnant and pregnant (20 days) rats used in electrophoretic analysis of microsomal proteins

	Non-pregnant	Pregnant
Body weight (g)	218.9 +/-3.0	288.6 +/-6.0 ***
Liver weight (g)	7.63 +/-0.32	11.86 +/-0.41 ***
Microsomal protein (mg /g liver)	18.0 +/-0.4	18.6 +/-0.8
Cytochrome P450 (nmoles /mg microsomal protein)	0.76 +/-0.08	0.78 +/-0.08

Values represent mean +/- SEM (n=4). Significant differences (students 't' test) are shown as * p<0.05, ** p<0.01, *** p<0.001.

Table 5.2 Cytochrome P450 spectral binding affinities (Ks) in control and pregnant rats

	Control	15 day pregnant	20 day pregnant
Ethylmorphine (μM) (Type '1')	16.7 $\pm$ -2.3	14.3 $\pm$ -0.8	17.1 $\pm$ -2.6
Aniline (mM) (Type '2')	0.42 $\pm$ -0.09	0.48 $\pm$ -0.11	0.41 $\pm$ -0.05
Progesterone (μM) (Reverse type '1')	0.44 $\pm$ -0.16	0.42 $\pm$ -0.08	0.49 $\pm$ -0.13
17 α -Hydroxyprogesterone (μM) (Reverse type '1')	1.14 $\pm$ -0.15	1.15 $\pm$ -0.15	1.50 $\pm$ -0.10
Cytochrome P450 (nmoles mg microsomal protein)	0.78 $\pm$ -0.05	0.74 $\pm$ -0.03	0.77 $\pm$ -0.03

Each value represents the mean $\pm$ SEM (n=4). There were no statistically significant differences between control and pregnant animals.

Table 5.3 Substrate induced maximum absorbance changes ΔA_{max} in non-pregnant and pregnant rats

Substrate	ΔA_{max} ($\times 10^3$) /nmole cytochrome P450		
	Non-pregnant	15 days pregnant	20 days pregnant
Ethylmorphine	5.5 $\pm$ 0.6	5.9 $\pm$ 0.2	5.4 $\pm$ 0.5
Aniline	17.3 $\pm$ 1.7	18.3 $\pm$ 3.6	11.8 $\pm$ 0.6 *
Progesterone	12.3 $\pm$ 1.7	13.6 $\pm$ 1.7	10.0 $\pm$ 1.3
17 α -Hydroxy-progesterone	15.1 $\pm$ 1.5	16.0 $\pm$ 2.6	13.4 $\pm$ 0.7

Each value represents the mean $\pm$ SEM (n=4). Significant differences (students 't' test) between non-pregnant and pregnant rats are shown as

* $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Table 5.4 Spin content and thermodynamic analysis of microsomal cytochrome P450 from non-pregnant and pregnant (20 days) rats

	Non-pregnant	Pregnant (a)
%High spin P450		
20°	61.3 +/-2.0	43.2 +/-1.6 **
37°	73.6 +/-1.7	54.5 +/-1.1 **
Equilibrium constant (K)		
20°	1.20 +/-0.05	0.76 +/-0.05 **
37°	2.82 +/-0.26	1.61 +/-0.12 **
ΔH (kcal)	9.05 +/-0.05	7.83 +/-0.41
ΔS (e.u)	31.3 +/-1.8	26.2 +/-1.4
ΔG (kcal /mol) 37°	-0.64 +/-0.05	-0.29 +/-0.05

a : based on the equilibrium constant $K = (P450_{HS}) / (P450_{LS})$. Values represent mean +/- SEM (n=4). Significant differences (students 't' test) are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

5.4 Discussion

5.4.1 The value of SDS-polyacrylamide gel electrophoresis in detecting multiple forms of microsomal cytochrome P450 during gestation

Evidence for the existence of multiple forms of cytochrome P450 comes from electrophoretic mobility studies (Welton and Aust, 1974; Haugen et al., 1975; Ryan et al., 1975; Hashimoto and Imai, 1976; Guengerich, 1979). Using SDS-polyacrylamide gel electrophoresis Welton and Aust (1974) resolved three haemoproteins with molecular weights of 45,000, 50,000 and 53,000 in rat liver microsomes. The haemoprotein of 50,000 molecular weight predominated in control microsomes while phenobarbitone and 3-methylcholanthrene pretreatment induced the 45,000 and 53,000 molecular weight bands respectively. These findings were confirmed by Alvares and Siekevitz (1973) who also reported that all three bands were induced by polychlorinated biphenyls. Haugen et al. (1976) demonstrated the potential of the same approach by analysing the responses of mouse liver microsomal membrane polypeptides to various xenobiotics including pregnenolone-16 α -carbonitrile (PCN). The induction of novel microsomal membrane polypeptides following 2-acetylaminofluorene (Cameron et al., 1976) ethanol (Ohnishi and Leiber, 1977) and isosafrole (Dickins et al., 1978) administration has been reported. Recently, Sharma et al. (1979) have described twelve different patterns of induction following the treatment of rats with phenobarbitone, 3-methylcholanthrene, polychlorinated biphenyls, acetylaminofluorene, safrole, PCN or ethanol either alone or in various combinations.

Caution is required in interpreting the results of SDS-polyacrylamide gel electrophoresis of microsomal proteins which have been stained for protein with Coomassie blue. This arises because various microsomal proteins which are not related to cytochrome P450 have molecular weights in the 45,000 to 60,000 region. These include epoxide hydrolase (Bentley and Oesch, 1975; Lu et al., 1975), UDP-glucuronyl transferases (Bock et al., 1977) and stearyl CoA desaturase (Strittmatter et al., 1974).

SDS-polyacrylamide gel electrophoresis of microsomal protein from pregnant and non-pregnant animals revealed no observable difference in the quantitative or qualitative pattern of polypeptides (Figures 5.1 and 5.2). This technique, although giving a good indication, is not definitive for detecting small changes in polypeptides, particularly haemoproteins. It is unlikely that small changes in microsomal proteins could be detected by this method. Furthermore evidence has been presented that different forms of cytochrome P450 can appear to have identical mobilities (Philpot and Arinc, 1976; Thomas et al., 1976; Guengerich, 1977; Haugen et al., 1977). A number of other methods should be used in conjunction with SDS-polyacrylamide gel electrophoresis to establish whether or not an altered spectrum of cytochrome P450 haemoproteins exists during gestation. These should include haemoprotein purification, immunochemical techniques, peptide mapping and amino acid compositional studies (Guengerich, 1979).

The gels shown in Figure 5.1 and Figure 5.2 were stained for protein with Coomassie blue. A technique which involves staining for residual haem peroxidase activity has been used to locate haemoproteins on gels (Welton and Aust, 1974; Haugen et al., 1975). This method is based on the observation that cytochrome P450 on conversion to cytochrome P420 by SDS retains peroxidase activity (Hryciak and O'Brien, 1971, a, b). This method was

not used in this study because of the difficulties involved and limitations in its use (for a detailed discussion of these see Fennell (1980)).

5.4.2 The significance of haemoprotein spin state changes during pregnancy in the rat

During pregnancy a significant decrease was found in the microsomal content of high-spin cytochrome P450 in the absence of exogenous substrates (Table 5.4). The significance of this in mixed-function oxidase activity is probably associated with the rate limiting step in monooxygenation. A number of investigations have reported that the reduction of ferricytochrome P450 by NADPH-cytochrome P450 reductase is the rate limiting step for some drugs (Björkhem, 1977). The kinetics of reduction by NADPH via the reductase can be conveniently studied by rapid mixing of anaerobic, carbon-monoxide saturated solutions of the reductase-P450 complex and of NADPH in a stopped-flow spectrophotometer. Using this technique Gigon *et al.* (1968) showed that ethylmorphine, hexobarbital, aminopyrine and imipramine, all of which cause 'type 1' spectral changes, markedly enhanced the rate of reduction of cytochrome P450. In contrast aniline, nicotinamide and 2,4-dichloro-6-phenylphenoxyethylamine which cause 'type 2' spectral changes, decreased the rate of haemoprotein reduction. The substrate-induced enhancement of cytochrome P450 reduction by NADPH was found to be closely related to the rate of hydroxylation. The rate of cytochrome P450 reduction in the presence of substrate minus the rate in the absence of substrate corresponded to the rate of hydroxylation under several different conditions. This observation has been subsequently confirmed (Gigon *et al.*, 1969; Diehl *et al.*, 1970; Gillette and Sasame, 1970;

Gillette,1971; Holtzman and Rumack,1973), though the rate limiting step appears to change following phenobarbitone induction (Miwa et al.,1978).

Studies on the activation energies of a number of reactions have shown that for aminopyrine demethylation the activation energy of the overall enzyme reaction was very close to the energy of activation of the ferricytochrome P450 reductase reaction in the presence of substrate, suggesting that this step is rate limiting (Schenkman,1971). This, however, was found not to be the case for ethylmorphine N-demethylation (Schenkman,1971; Holtzman and Carr,1972).

Investigations on the inhibitory effect of deuterated water on monooxygenase activity have revealed that the degree of inhibition of ethylmorphine N-demethylase by deuterated water was correlated with the degree of inhibition of the substrate-induced reduction of cytochrome P450, suggesting that this step was rate limiting (Holtzmann and Carr,1970). Similar findings have been reported by Björkhem (1972). High ionic strength is known to stimulate the rate of reduction of cytochrome c by purified NADPH-cytochrome c reductase preparations (Philips and Langdon,1962). In accordance with the suggestion that the reductase step is rate limiting Estabrook et al. (1971) found that the rate of N-demethylation of ethylmorphine was increased at high ionic strength of the buffer medium. A similar effect was reported by Björkhem and Danielson (1973).

Microsomal hydroxylations can be supported in the absence of NADPH and O₂ by organic hydroperoxides. This effectively bypasses the NADPH-cytochrome P450 reductase step. If this step is rate limiting a faster rate of hydroxylation would be expected. This has been found to be

the case for the 6 β -, 7 α - and 16 α hydroxylations of androstenedione (Hrycay et al.,1976).

As mentioned earlier, the rate of electron transfer from NADPH-cytochrome P450 reductase to ferricytochrome P450 is altered by the presence of substrates: various 'type 1' substrates (which increase the high-spin form of the cytochrome) increase the rate of reduction of ferricytochrome P450 and 'type 2' substrates (which increase the low-spin form) inhibit the reduction rate (Schenkman,1968; Gigon et al.,1968). Ristau et al. (1978) measured the dissociation constants for the low and high-spin substrate protein complexes and the high-spin/low-spin equilibrium constants in the presence and absence of substrate. The substrate benzphetamine binds nearly five times more tightly to the high-spin than to the low-spin enzyme and consequently in the presence of benzphetamine the high-spin/low-spin equilibrium is "pulled" towards the high-spin.

The substrate-induced change in electron spin state is associated with a shift towards a more positive value in the equilibrium redox potential of the cytochrome for both bacterial (Sligar and Gunsalus,1976; Sligar,1976) and hepatic (Sligar et al.,1979) systems. This shift in redox potential might increase the rate of electron flow from NADPH-cytochrome P450 reductase to cytochrome P450. The rate constants for this process have been measured in bacterial systems and the substrate-induced change in redox potential of cytochrome P450 was reflected by a modulation of the forward rate constant for electron transfer into the haemoprotein (Sligar,1976).

A model emerges where, during substrate binding to cytochrome P450 and its subsequent reduction by NADPH-cytochrome P450 reductase, three interacting equilibria are present: (1) substrate binding, (2) oxidation-reduction of the haem group, and (3) interconversion between high and low-spin P450 states. It would appear that the binding of 'type 1' substrates to hepatic cytochrome P450 could increase the rate of electron flow from NADPH-reductase to cytochrome P450 by modulating the spin equilibrium of the electron accepting cytochrome. As this redox transfer process is rate limiting for some substrates the haem iron spin equilibrium could be of major importance in the regulation of monooxygenase activity (Sligar et al., 1979). This suggests that the endogenous spin state of the haemoprotein in the microsomal membrane may have an important regulatory effect on the rate of drug metabolism. This is in agreement with the finding (Table 5.4) that in late pregnancy there was a significant reduction in the high-spin form of hepatic microsomal cytochrome P450 compared with the non-pregnant state. This change was associated with a significant decrease in mixed-function oxidase activity, as discussed in Chapter 3. Haemoprotein spin state changes are accompanied by significant alterations in the enthalpy (ΔH), the entropy (ΔS) and the free energy (ΔG) of the system during gestation. The values for the thermodynamic parameters obtained from non-pregnant rats were similar to values reported by Cinti et al. (1979) in male rats (%high-spin at 37° = 78.5, ΔH = 14.7 kcal/mol and $\Delta G(20^{\circ})$ = -0.043 kcal/mol).

It would thus appear that the decreased content of high-spin cytochrome P450 in the endoplasmic reticulum during gestation might be an important factor in mediating alterations in mixed-function oxidase activity by modulation of the redox potential of the haemoprotein.

It is now important to consider how spin state changes in cytochrome P450 are controlled. Progesterone caused a 'reverse type 1' spectral change on interaction with cytochrome P450 (Figure 5.3) and may shift the haemoprotein spin state equilibrium towards the low-spin state (Kumaki et al., 1980). The microsomal concentration of progesterone, however, was not significantly different in pregnant compared to non-pregnant rats (Chapter 4, Table 4.1). Furthermore, it has been shown (Chapter 4) that high concentrations of progesterone and other steroids administered in vitro or in vivo have little effect on mixed-function oxidase activity.

Spin state changes might alternatively be controlled by alterations in microsomal lipids. Purified soluble cytochrome P450 from both bacteria (Sligar, 1976; Sligar and Gunsalus, 1976; Sligar et al., 1979) and rat liver (Cinti et al., 1979; Gibson et al., 1980a; Sligar et al., 1979) sources, in the absence of added substrate, have been found to exist predominantly in low-spin configuration. In contrast, unpurified microsomal P450 in the absence of added substrate, contains large amounts of high-spin cytochrome P450 (>50% at 20°) (Cinti et al., 1979; Gibson et al., 1980a). Gibson et al. (1980a,b) showed that microsomal lipids were responsible for holding the haemoprotein in the high-spin form and Ruckpaul et al. (1980) have reported that the addition of phospholipids to purified cytochrome P450 resulted in an increase in the high-spin form of the haemoprotein. In contrast Gibson et al. (1980a,b) found that the free fatty-acid fraction was responsible for a low-spin to high-spin transition of the haem iron of cytochrome P450. Phospholipids caused, if anything, a slight high-spin to low-spin transition (Gibson et al., 1980a; Tsong and Yang, 1979). It is possible that the altered microsomal phospholipid composition which has been shown to occur during pregnancy (Chapter 3) is responsible for the decrease in the high-spin form of cytochrome P450 (Table 5.4). Phospholipids may therefore

cause a decrease in mixed-function oxidase activity in one or both of two ways. Firstly, changes in phospholipids may alter the membrane environment of the haemoprotein (discussed in Chapter 3) without affecting the spin equilibrium or, secondly, phospholipids may have a direct effect on the proportion of cytochrome P450 in the high-spin state.

In view of the observed changes found in haemoprotein spin state during pregnancy the lack of change in ΔA_{max} for ethylmorphine, progesterone and 17α -hydroxyprogesterone (Table 5.3) is somewhat surprising. Kumaki et al. (1978) suggested that ΔA_{max} depended on the spin content of the haemoprotein; the greater the amount of endogenous high-spin P450 the lower was the ΔA_{max} produced by 'type 1' substrates added in vitro, and the greater was the ΔA_{max} produced by 'reverse type 1' and 'type 2' compounds. Conversely, the lower the amount of endogenous high-spin P450 the greater was the ΔA_{max} produced by 'type 1' substrates added in vitro, and the lower was the ΔA_{max} produced by 'reverse type 1' and 'type 2' compounds. The decrease in ΔA_{max} for aniline during pregnancy (Table 5.3) might therefore be explained by the increase in the endogenous low-spin form of cytochrome P450. The lack of change in ΔA_{max} for the substrates ethylmorphine, progesterone and 17α -hydroxyprogesterone is possibly because only a portion of the cytochrome P450 in microsomal preparation will undergo a low-spin to high-spin transition upon the addition of substrates (Ullrich and Duppel, 1975; Ebel et al., 1977). This may be related to the presence of multiple forms of cytochrome P450 with different but overlapping affinities for different substrates (Estabrook et al., 1973; Werringloer and Estabrook, 1975; Ullrich and Kremers, 1977). Thus a small change in ΔA_{max} produced by a substrate binding to part of the total haemoprotein population might remain undetected.

The lack of marked alterations in K_s and ΔA_{max} during pregnancy (Tables 5.2 and 5.3) support the results obtained from SDS-polyacrylamide gel electrophoresis which indicated that the types of cytochrome P450 do not alter substantially during gestation.

Results from this chapter indicate that the most important factors controlling alterations in mixed-function oxidase activity during pregnancy are microsomal phospholipids and haemoprotein spin equilibrium.

CHAPTER 6 THE ROLE OF PLACENTAL LACTOGEN IN DRUG METABOLISM DURING

PREGNANCY IN THE RAT

6.1 Introduction

Pregnancy is associated with significant alterations in hepatic microsomal mixed-function oxidase activity which is related to changes in membrane phospholipids and haemoprotein spin state (Chapters 3 and 5). The high levels of circulating steroids, particularly progesterone, during pregnancy do not appear to be responsible for these changes (Chapter 4).

The increase in liver size during gestation is due to parenchymal hyperplasia (Chapter 3). It has been established that microsomal drug metabolism is lower than normal in many conditions characterised by the rapid growth of liver cells (Fouts, 1963; Wilson and Froham, 1974), for example in the foetus and neonate (Fouts and Adamson, 1959; Jondorf et al., 1959; Fouts and Hart, 1965), after partial hepatectomy (Fouts et al., 1961) and in hepatic tumours (Adamson and Fouts, 1961; Kato et al., 1968b). In all of these conditions the presence of increased growth promoting factors has been reported. An increase in growth promoting factors such as growth hormone might be responsible for the inhibitory effect of pregnancy on drug metabolism.

It is well established that pituitary growth hormone causes a decrease in drug metabolism in male rats (Wilson, 1968a, 1969a, 1971, 1973a,b; Wilson and Spelsberg, 1976; Kramer et al., 1975, 1978). The administration of growth

hormone to male rats lowered the level of hepatic cytochrome P450 and decreased the rates of metabolism of various substrates (Wilson, 1971, 1973a,b; Kramer et al., 1975, 1978). A similar effect was seen after implantation of a growth hormone producing tumour (Wilson, 1968a,b), injection of a tumour homogenate (Wilson, 1968c, 1969b,c) or after growth hormone injections to castrated (Wilson, 1971; Kramer et al., 1978), hypophysectomised (Kramer et al., 1975) or hypophysectomised / adrenalectomised (Wilson and Spelsberg, 1976) male rats. Growth hormone had no effect on oxidative drug metabolism in castrated (Kramer et al., 1978) or normal (Kramer et al., 1975) female rats.

Growth hormone would appear to be an important factor in mediating the sex differences in mixed-function oxidase activity in rats. The effect of gonadal hormones on hepatic drug and steroid metabolism in rats has been well characterised (see Chapter 1, 1.1.9; Kato, 1974). In general androgens increase the activity of cytochrome P450 containing oxidative enzymes but decrease the rate of reductive steroid metabolism whereas oestrogens exert the opposite effect. Colby et al. (1973) demonstrated that the effects of both testosterone and oestradiol on hepatic corticosteroid metabolism were not demonstrable in hypophysectomised animals, suggesting mediation by or permissive effects of pituitary factors. Subsequently other investigators have reported a similar pituitary requirement for the development and maintenance of sex dependent patterns in hepatic steroid metabolism (Denef, 1974; Gustafsson and Stenberg, 1974; Lax et al., 1974; Kramer et al., 1975, 1979). It has been proposed that the pituitary mediator of oestrogen effects on drug metabolism is growth hormone (Kramer et al., 1979). The effects of exogenous (Wilson, 1969a, 1973a,b) or endogenous (Wilson and Frohman, 1974) sources of growth hormone on microsomal drug metabolism have been found to be essentially identical to those of

oestradiol (Kramer and Colby,1976; Kramer et al.,1978). The effects of the two hormones are not additive (Kramer et al.,1978) suggesting a similar mechanism of action. Furthermore oestrogen administration increases plasma growth hormone levels (Frantz and Rabkin,1965; Lloyd et al.,1971).

A sex difference exists in the concentration of hepatic growth hormone receptors in the rat. Liver membrane preparations from mature female rats had three times the capacity to bind human growth hormone than similar preparations from male rats (Herington et al.,1976a). The difference in receptor levels developed at puberty (Posner et al.,1974a) at the same time as the development of the sex difference in drug metabolism (Kato,1974). Hepatic growth hormone receptors are induced by oestrogens (Posner et al.,1974b; Kelly et al.,1975; Herington et al.,1976a,b) an effect inhibited by testosterone (Furuhashi and Fang,1979).

A role for growth hormone in producing the alterations in drug metabolism during pregnancy is unlikely since the plasma concentrations of this hormone are low in gestation in the rat (Schatch and Reichlin,1966), sheep (Basset et al.,1970), cow (Ingalls et al.,1973), human (Verma et al.,1971) and monkey (Mintz et al.,1969). However, a hormone of placental origin which has structural and functional similarities to growth hormone is placental lactogen (Sherwood et al.,1980).

In the human, placental lactogen has been extensively studied (for reviews see Chatterjee and Munro,1977; Villee,1979) since it was first recognised by Josimovich and Maclaren (1962). Studies on placental lactogen in species other than man are less extensive. A placental lactogen analogous to that found in the human has been described in the plasma of the rat, mouse, hamster, dog, guinea pig, rabbit, sheep, cow,

goat, chinchilla, baboon and rhesus monkey (Shiu et al.,1973; Shome and Friesen,1971; Talmantes,1975; Kohmoto and Bern,1970; Buttle et al.,1972). The lactogen present in rat plasma has been identified as rat placental lactogen by radioreceptor assay (Kelly et al.,1975).

The plasma level of placental lactogen in the various mammals studied begins to rise at or before mid pregnancy and either remains elevated until term (hamster,goat, sheep,monkey and human) or declines gradually reaching peak concentrations just after mid-pregnancy (guinea pig) or has two peaks of activity (mouse and rat) (Kelly et al.,1976). The serum of the maternal rat shows two peaks, one between days 11 and 13 and the second between days 17 and 21 of gestation (Buttle et al.,1972). In contrast to the widespread occurrence of placental lactogen in mammalian pregnancy, a peptide hormone analogous to human chorionic gonadotrophin has only been positively identified in the rhesus monkey, chimpanzee and baboon though its presence has been suggested in the rat and rabbit (Chatterjee and Munro,1977).

Human placental lactogen appears to have three major effects during pregnancy. These have been established in experimental animals and are all directed toward maternal metabolism. Firstly it is luteotrophic, as evidenced by its stimulation of progesterone and oestrogen secretion by the corpora lutea of the hypophysectomised pseudopregnant rat, an effect that is potentiated by the administration of human placental lactogen and human chorionic gonadotrophin together (Josimovich,1968). Thus human placental lactogen secretion during the second trimester of pregnancy may supplement the stimulant action on the corpus luteum of human chorionic gonadotrophin which is diminishing at this time. However, attempts to demonstrate such an action in pregnant women have not been successful (Stock et al.,1971).

Secondly placental lactogen is so named because of its capacity to stimulate milk production in rabbit (Josimovich and Maclaren, 1962; Josimovich and Brande, 1964; Friesen, 1966) and simian mammary gland (Beck, 1972) and to stimulate the pigeon crop (Josimovich and Maclaren, 1962) analogous to the action of pituitary prolactin. Human placental lactogen and prolactin compete for receptors on mammary gland cell membranes (Shiu et al., 1973) but their relative roles in mammary gland development are unknown. Human placental lactogen may be more important since it is present at term in concentrations 35 times greater than prolactin (Hwang et al., 1971; Friesen, 1971). Leader (1975) has postulated that the primary action of human placental lactogen is to stimulate the development of the mammary gland during pregnancy without causing milk secretion. At birth withdrawal of human placental lactogen then results in milk secretion under the active stimulus of prolactin of pituitary origin.

Finally the actions of human placental lactogen on maternal metabolism analogous to the activities of pituitary growth hormone are implicit in the alternative names; chorionic growth hormone prolactin and chorionic somatomammotrophin. The lactogenic activity of human placental lactogen on a weight basis is similar to that of human growth hormone (Handwerger et al., 1972). The somat otrophic activity of human placental lactogen is less than 1% of human growth hormone though the circulating concentration of human placental lactogen is 1000 times that of growth hormone at term pregnancy (Sherwood et al., 1980).

In various tests on animals (Josimovich and Maclaren, 1962; Florini et al., 1966; Josimovich, 1966; Friesen, 1965;) human placental lactogen showed a distinct but smaller growth promoting activity than human growth hormone. Grumbach et al. (1968) suggested that a major function of human placental

lactogen is to so alter maternal metabolism that adequate supplies of glucose, amino acids and minerals are made available to the foetus during pregnancy. In the mother there are no gross changes in carbohydrate or lipid metabolism during the first trimester, but in the last trimester there is a diminished responsiveness to insulin, impaired glucose tolerance and mobilisation of lipid stores (Picard et al.,1968; Fairweather,1971).

Prolonged infusion of human placental lactogen into normal or hypopituitary subjects resulted in increased free fatty acid levels and impaired tolerance for glucose despite an increase in plasma insulin levels following the glucose administration (Beck and Daughaday,1967; Samaan et al.,1968). However, changes in blood sugar response are disputed by Josimovich and Mintz (1968) and the alterations in free fatty acid levels were not observed by Kalkhoff et al. (1969).

Studies with animals treated with human placental lactogen confirm the increased secretion of insulin (Martin and Friesen,1969; Lopez-Quijada and Blazquez,1971) and the release of free fatty acids from adipose tissue (Turtle and Kipnis,1967; Genazzani et al.,1969; Felber et al.,1972; Mochizuki et al.,1975). The benefits to the foetus of this mobilization of substrate are illustrated by experiments in which human placental lactogen was given to pregnant rats resulting in increased foetal weight, glycogen, protein and lipid content (Mochizuki et al.,1973). No such response was observed when human placental lactogen was administered directly to the foetus.

Human placental lactogen is preferentially synthesised in the syncytiotrophoblast of the placenta on membrane-bound polyribosomes and greater amounts are synthesised by the 40 week than 20 week placenta

(Chatterjee et al,1976; Chatterjee and Munro,1977). Friesen et al. (1969) concluded that human placental lactogen was the major protein synthesised by the placenta near term accounting for as much as 10% of the protein released from slices of human full term placenta.

Heterogeneity of human placental lactogen has been demonstrated (Belleville et al,1975 Chatterjee et al,1977) which may reflect a precursor form (Cox et al,1976). Szczesna and Boime (1976) have shown that m-RNA from full term placenta directs the synthesis of a protein that is larger (mol.wt.25,000) than human placental lactogen (mol.wt.22,000). Cleavage of this precursor molecule occurs on ribosome-bound nascent peptide chains. The human placental lactogen molecule is a single chain polypeptide of 191 amino acids containing two intramolecular disulphide bonds (Chatterjee and Munro,1977; Sherwood et al,1980). Structurally human placental lactogen bears a marked resemblance to human growth hormone, about 80% of the amino acid residues being identical in the two molecules (Sherwood et al,1980). Observation of the internal sequence homologies among pituitary growth hormone, prolactin and placental lactogen suggests that these polypeptide hormones may have evolved by genetic reduplication from a smaller ancestral peptide (Niall et al,1971; Sherwood et al,1980).

The regulatory factors in human placental lactogen synthesis are unknown. In general the amount of human placental lactogen formed is related to placental size, but other factors such as ionic environment may also contribute (Choy and Watkins,1976). Evidence that there may be control mechanisms specific for the synthesis or release of human placental lactogen is provided by the observation that placental slices incubated in vitro rapidly lose their ability to synthesise this hormone (Gaspard and Franchimont,1972). Human placental lactogen secretion may be regulated by

a placental factor analogous to the hypothalamic factor controlling growth hormone release from the pituitary gland. Other releasing factors have been described for the placenta (De Palatis et al, 1980; Gibbons et al, 1975; Khodr and Siler-Khodr, 1978).

Reports have appeared on the purification and characterisation of rat placental lactogen (Kelly et al, 1975; Robertson and Friesen, 1975; Linkie and Niswender, 1973). It has been reported to have mammatropic (Matthies, 1967; Kinzey, 1968; Cohen and Gala, 1969; Shani et al, 1970; Anderson, 1975), luteotrophic (Matthies, 1967; Linkie and Niswender, 1971, 1973; Anderson, 1975) and sommatotrophic (Contopolous and Simpson, 1957) activity.

Human and rat placental lactogens show an immunological similarity. Rabbit antisera to human placental lactogen cross reacts with rat placental extracts (Leake and Burt, 1969; Gusdon et al, 1970) and binds to the rat placenta in vivo (Gusdon et al, 1975). In addition passive or active immunization of pregnant rats with human placental lactogen has deleterious effects on pregnancy and lactation (El Tomi et al, 1970, 1971) though neutralization of endogenous prolactin by anti-human placental lactogen antibodies might account for some of these effects.

In this chapter the effect of placental lactogen on hepatic mixed-function oxidase activity, microsomal phospholipids and haemoprotein spin state will be investigated in non-pregnant rats and the results compared to the situation present during pregnancy. Non-pregnant rats will be administered (i.p) with either homogenised placentae from pregnant rats (13 days) or purified human placental lactogen. These techniques have been used previously to investigate the physiological effects of placental

lactogen in rats (Blank et al.,1977; Linkie and Niswender,1973).

6.2 Experimental

(a) The effect of in vivo pretreatment with rat placental homogenates on hepatic drug metabolism was investigated. Placentae from pregnant (13 days) rats were homogenised in saline. Non-pregnant rats were injected (i.p) for six days with placental homogenates (2 placental equivalents / day) and killed on the seventh day (Chapter 2, 2.2.5.7). Control non-pregnant received saline alone. The effect of this treatment on hepatic drug enzyme activity (Chapter 2, 2.2.6), cytochrome P450 (Chapter 2, 2.2.8), cytochrome b5 (Chapter 2, 2.2.9), microsomal phospholipids (Chapter 2, 2.2.13), microsomal protein (Chapter 2, 2.2.21) and haemoprotein spin state (Chapter 2, 2.2.12) was determined.

(b) The effect of in vivo pretreatment with human placental lactogen on in vitro hepatic drug metabolism, microsomal phospholipids and haemoprotein spin state was investigated. Non-pregnant female rats received human placental lactogen (0.22mg in saline-lactose solution /rat /day i.p) for six days and were killed on the seventh day. Control rats received saline-lactose solution alone (Chapter 2, 2.2.2.6).

6.3 Results

6.3.1 The effects of in vivo pretreatment with placental homogenates on in vitro hepatic mixed-function oxidase activity, microsomal phospholipids and haemoprotein spin state in the female rat

In vivo pretreatment with rat placental homogenates had no effect on body weight, liver weight, microsomal protein, cytochrome P450 and cytochrome b5 compared with control rats (Table 6.1). The three drug metabolising enzymes aniline p-hydroxylase, ethylmorphine N-demethylase and p-nitrobenzoic acid reductase were significantly decreased in treated rats to 75-85% of control levels (Figure 6.1). Changes in mixed-function oxidase activity were accompanied by a significant decrease in microsomal phosphatidylcholine and the phosphatidylcholine to phosphatidylethanolamine ratio and an increase in phosphatidylethanolamine in treated compared with control rats (Figure 6.2). Total phospholipids were unchanged.

The relationship between the microsomal cytochrome P450 spin equilibrium constant (K) and temperature in treated and control rats is shown in a Van't Hoff plot (Figure 6.3). Thermodynamic and spin state data have been calculated from this plot and are shown in Table 6.2. Pretreatment of rats with placental homogenates produced a significant decrease in the spin equilibrium constant (K) and high spin form of cytochrome P450 at 20° and 37°. Of the thermodynamic parameters ΔG was significantly decreased by pretreatment with placental homogenates while ΔH and ΔS were unchanged.

6.3.2 The effect of in vivo pretreatment with human placental lactogen

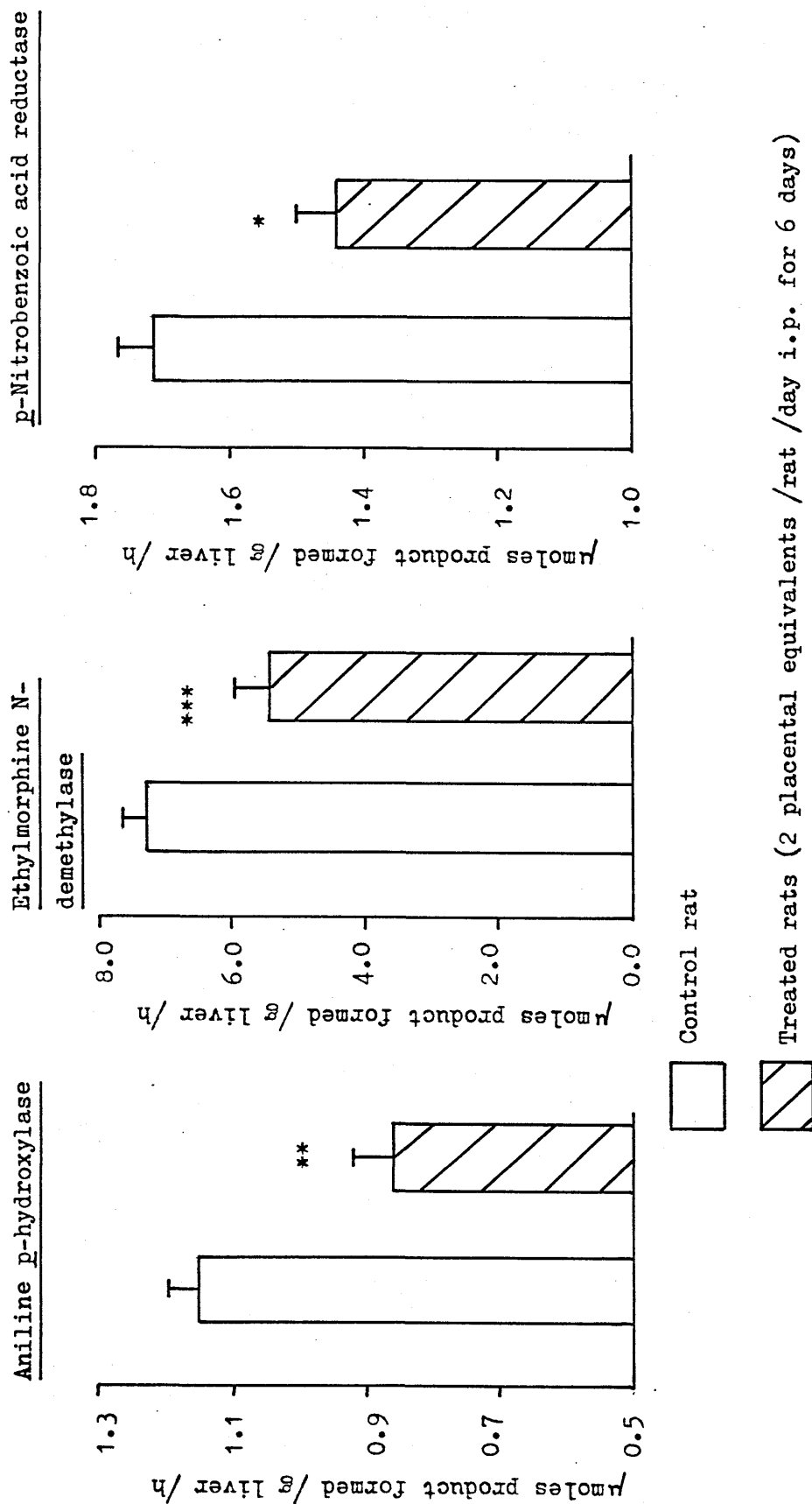
on in vitro hepatic mixed-function oxidase activity, microsomal phospholipids and haemoprotein spin state in the female rat

In vivo pretreatment with human placental lactogen had no effect on body weight, liver weight, microsomal protein, cytochrome P450 and cytochrome b5 (Table 6.3). The three drug enzymes aniline p-hydroxylase, ethylmorphine N-demethylase and p-nitrobenzoic acid reductase were decreased in treated compared with control rats but this was only statistically significant in the case of aniline p-hydroxylase (Figure 6.4).

Microsomal phosphatidylcholine was significantly decreased in the treated animals while total phospholipids, phosphatidylethanolamine and the phosphatidylcholine to phosphatidylethanolamine ratio were unchanged (Figure 6.5).

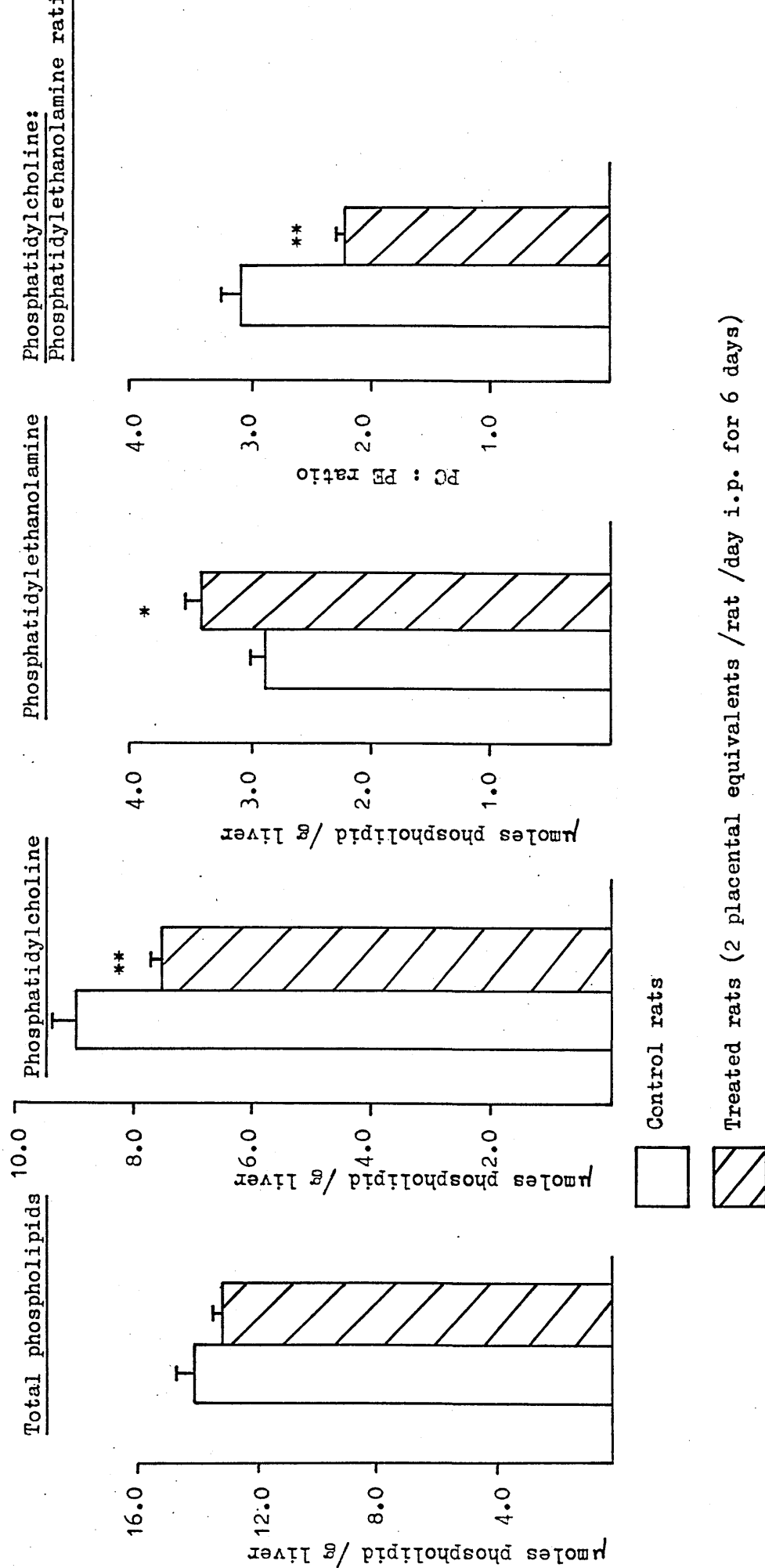
The relationship between the microsomal cytochrome P450 spin state equilibrium constant (K) and temperature in treated and control rats is shown in a Van't Hoff plot (Figure 6.6). Thermodynamic and spin state data have been calculated from this plot (Table 6.4). A significant decrease was seen in the spin equilibrium constant and in the percentage high-spin form of cytochrome P450 at 20° in treated compared with control rats. The thermodynamic parameters ΔS , ΔG and ΔH were unchanged by human placental lactogen pretreatment.

Figure 6.1 Effect of in vivo pretreatment with rat placental homogenate on hepatic mixed-function oxidase activity in the female rat



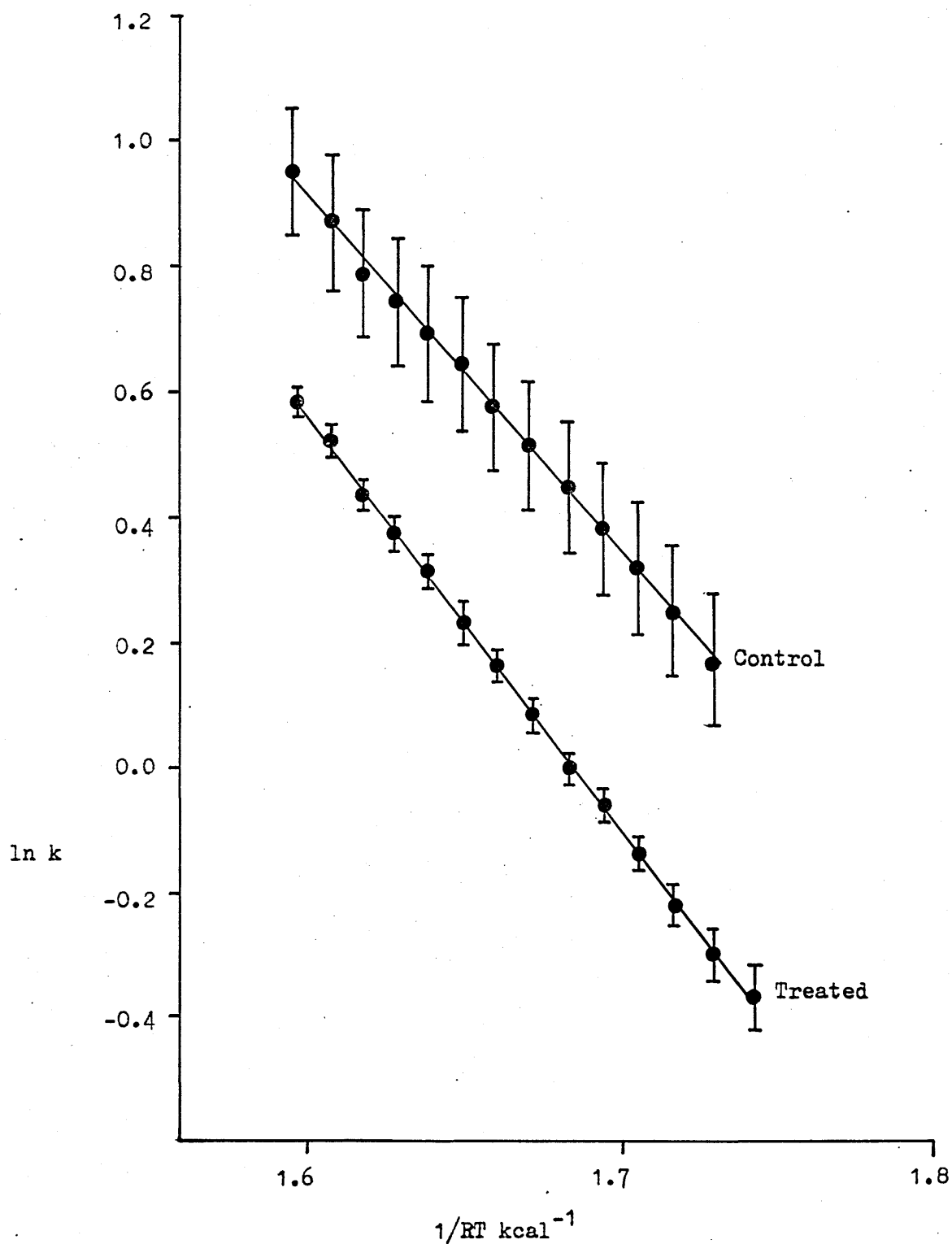
Values represent mean $\pm$ SEM (n=4). Significant differences (students 't' test) are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6.2 Effect of in vivo pretreatment with rat placental homogenate on hepatic microsomal phospholipids in the female rat



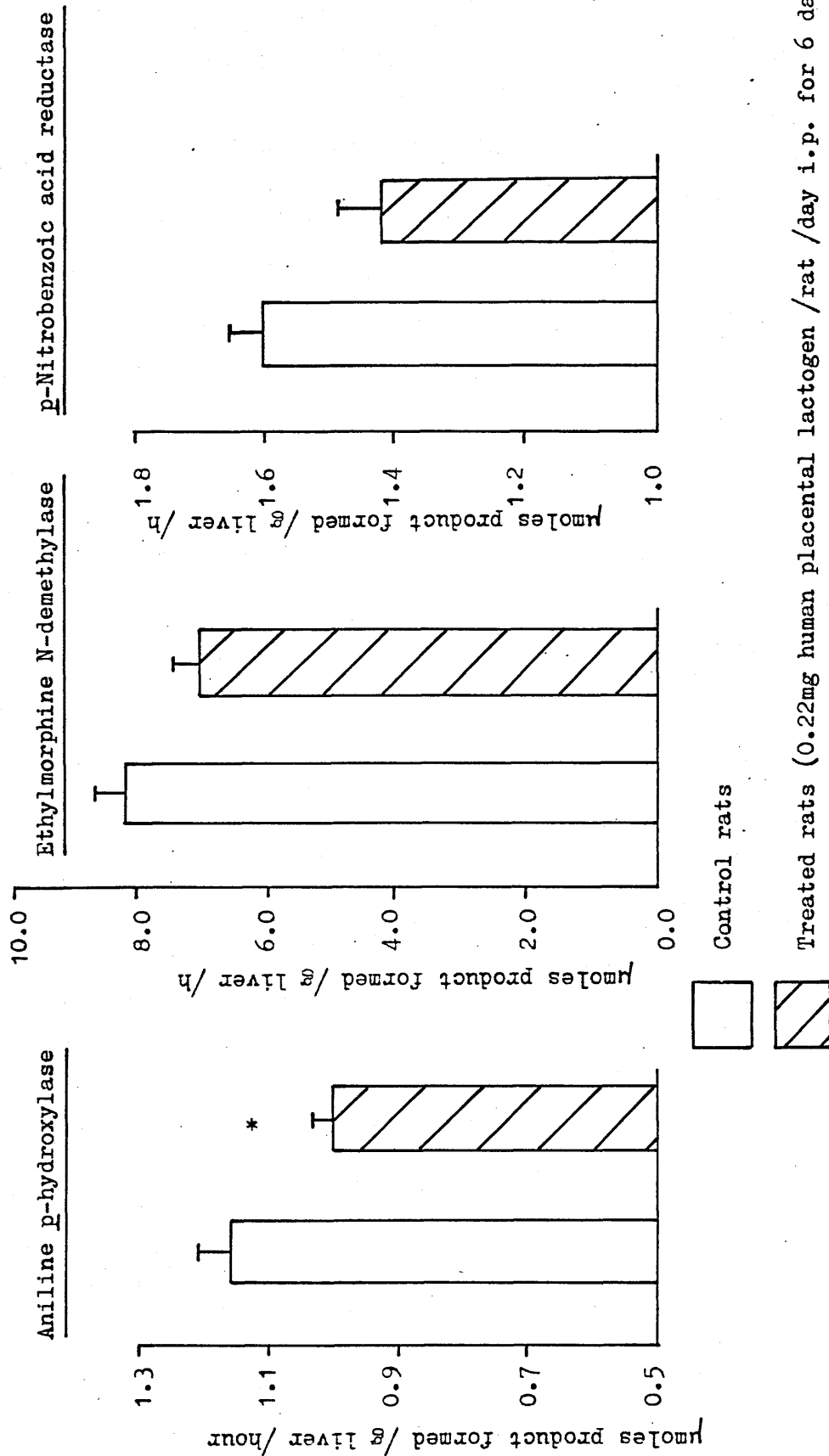
Values represent mean $\pm$ SEM (n=4). Significant differences (students 't' test) are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6.3 Effect of in vivo pretreatment with rat placental homogenate on the thermally induced spin state transition of hepatic microsomal cytochrome P450 in female rats



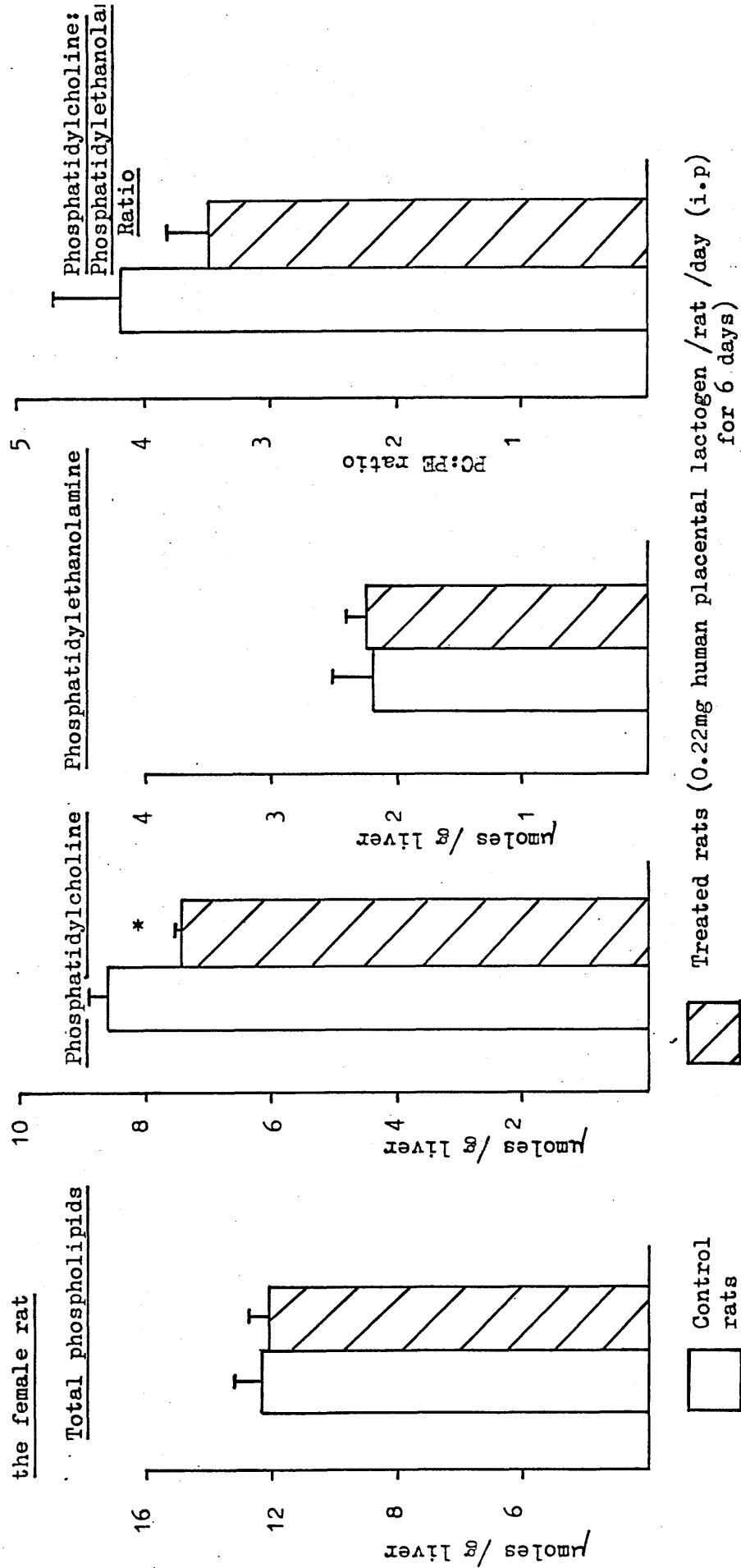
Thermally induced spin state transitions were fitted to a Vant Hoff equation by the procedure described in Chapter 2 (2.2.12). Each point represents the mean $\pm$ SEM (n=4). Control and treated values are significantly different at all points ($p < 0.05$).

Figure 6.4 Effect of in vivo pretreatment with human placental lactogen on hepatic mixed-function oxidase activity in the female rat

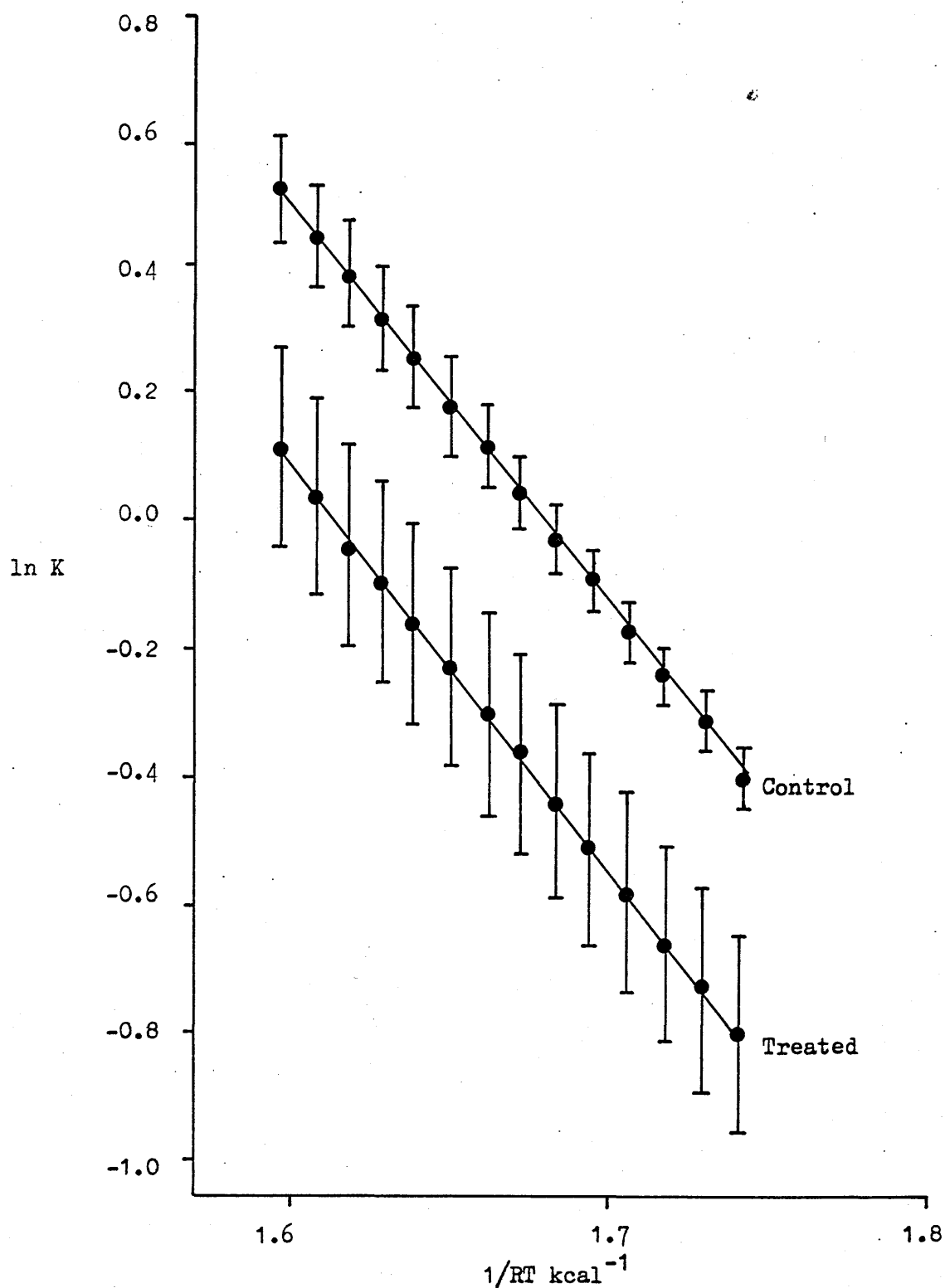


Values represent mean $\pm$ SEM (n=4). Significant differences (students 't' test) are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.

Figure 6.5 Effect of in vivo pretreatment with human placental lactogen on hepatic microsomal phospholipids in the female rat



Values represent mean $\pm$ SEM (n=4). Significant differences (students 't' test) are shown as * $p < 0.05$, ** $p < 0.01$, *** $p < 0.001$.



Thermally induced spin state transitions were fitted to a Vant Hoff equation by the procedure described in Chapter 2 (2.2.12). Each point represents the mean $\pm$ SEM ($n=4$). Control and treated values were not significantly different at $p < 0.05$.

Table 6.1 The effect of in vivo pretreatment with rat placental homogenate on rat body weight, liver weight, hepatic microsomal protein, cytochrome P450 and cytochrome b5

	Control	Treated (a)
Body weight (g)		
Initial	207.5 $\pm$ 3.3	202.0 $\pm$ 3.4
Final	216.3 $\pm$ 3.8	212.8 $\pm$ 4.2
Liver weight (g)	8.46 $\pm$ 0.50	8.70 $\pm$ 0.35
Microsomal protein (mg /g liver)	22.8 $\pm$ 1.3	20.7 $\pm$ 0.8
Cytochrome P450 (nmoles /mg microsomal protein)	0.89 $\pm$ 0.03	0.74 $\pm$ 0.06
Cytochrome b5 (nmoles /mg microsomal protein)	0.64 $\pm$ 0.01	0.77 $\pm$ 0.06

Values represent mean $\pm$ SEM (n=4). No statistical differences were found between control and treated rats.

(a) : Treated rats received 2 placental equivalents /day (i.p.) for six days and were killed on the seventh.

Table 6.2 Effect of in vivo pretreatment with rat placental homogenate on the spin content and thermodynamic parameters of microsomal cytochrome P450 in female rats

	Control	Treated (b)
%High-spin P450		
20°	57.5 +/-2.8	46.5 +/-0.7 *
37°	69.5 +/-2.5	61.5 +/-0.6 *
Spin equilibrium constant (K) (a)		
20°	1.39 +/-0.17	0.87 +/-0.05 *
37°	2.36 +/-0.32	1.59 +/-0.05 *
ΔH (kcal)	6.04 +/-0.31	6.63 +/-0.05
ΔS (e.u)	23.7 +/-1.2	23.7 +/-1.6
ΔG (kcal /mol) at 37°	-1.33 +/-0.20	-0.74 +/-0.05 *

Values represent mean +/- SEM (n=4). Significant differences (students 't' test) are shown as * p<0.05, ** p<0.01, *** p<0.001.

a : Based on the equilibrium constant $K = (P450_{HS}) / (P450_{LS})$

b : Treated rats received 2 placental equivalents /rat /day (i.p.) for 6 days.

Table 6.3 The effect of in vivo pretreatment with human placental lactogen on rat body weight, liver weight, hepatic microsomal protein, cytochrome P450 and cytochrome b5

	Control	Treated
Body weight (g)		
Initial	215.3 +/-4.5	213.3 +/-2.8
Final	227.5 +/-6.5	227.0 +/-4.8
Liver weight (g)	8.9 +/-0.6	8.96 +/-0.36
Microsomal protein (mg /liver)	20.7 +/-0.8	20.8 +/-0.6
Cytochrome P450 (nmoles /mg microsomal protein)	0.79 +/-0.05	0.79 +/-0.05
Cytochrome b5 (nmoles /mg microsomal protein)	0.60 +/-0.03	0.55 +/-0.04

Values represent mean +/- SEM (n=4). No significant differences were found between control and treated rats.

a : Treated rats received 0.22 mg human placental lactogen /rat /day (i.p.) for six days and killed on the seventh.

Table 6.4 Effect of in vivo pretreatment with human placental lactogen on the spin content and thermodynamic parameters of microsomal cytochrome P450 in female rats

	Control	Treated b
%High-spin P450		
20°	45.6 +/-1.2	36.1 +/-3.6 *
37°	60.1 +/-2.05	50.3 +/-3.6
Spin equilibrium constant (K) a		
20°	0.85 +/-0.04	0.58 +/-0.10 *
37°	1.53 +/-0.12	1.04 +/-0.16
ΔH (kcal)	6.03 +/-0.45	6.15 +/-0.05
ΔS (e.u.)	20.3 +/-1.5	19.8 +/-0.2
ΔG at 37° (kcal /mol)	-0.26 +/-0.05	0.003 +/-0.100

Values represent mean +/- SEM (n=4). Significant differences (students 't' test) are shown as * p<0.05, ** p<0.01, *** p<0.001.

a : Based on the equilibrium constant $K = (P450_{HS}) / (P450_{LS})$

b : Treated rats received 0.22mg human placental lactogen /rat /day (i.p.) for six days.

6.4 Discussion

Treatment of non-pregnant rats with rat placental homogenates produced decreases in hepatic mixed-function oxidase activity (Figure 6.1), changes in microsomal phospholipids (Figure 6.2) and haemoprotein spin state (Table 6.2) similar to those seen during pregnancy (Chapters 3 and 5). Levels of cytochrome P450, b5 and microsomal protein were unchanged analogous to the situation found during pregnancy (Table 6.1).

The content of LH, FSH, progesterone and prolactin in rat placenta (12 to 16 days) is very low or undetectable (Linkie and Niswender, 1973) and it is unlikely that the effect of placental injections on microsomal mixed-function oxidase activity and membrane composition is due to these hormones. The presence of a factor with LH-like activity, analogous to human chorionic gonadotrophin, has been reported (Cheng, 1975; Bambra and Gombe, 1978) and it has been suggested that at least two luteotrophic factors may be present in crude placental homogenates: placental lactogen and chorionic gonadotrophin (Blank et al., 1977).

Rat placental lactogen is probably the most important constituent of placentae that affects drug metabolism since the injection of purified human placental lactogen produced similar effects on mixed-function oxidase activity (Figure 6.4), microsomal phospholipids (Figure 6.5) and haemoprotein spin state (Table 6.4) to those seen after placental injections or during pregnancy. Drug enzyme activities were decreased by human placental lactogen pretreatment though this was only significant for aniline p-hydroxylase. A larger dose of human placental lactogen might produce a more significant effect on mixed-function oxidase activity

(Wilson, 1973a).

The mechanism by which rat placental lactogen may alter hepatic mixed-function oxidase activity and microsomal membrane composition is unknown. The structural and functional similarity between placental lactogen and growth hormone (Sherwood et al., 1980) suggests, however, that the action of placental lactogen may be similar to that of growth hormone. Growth hormone has been reported to have at least three actions on microsomal drug metabolism: an effect on (1) transcription (2) the rate of reduction of ferricytochrome P450 and (3) on membrane phospholipids. These are possibly mediated via hepatic growth hormone receptors. A discussion of the mechanisms of growth hormone action and the relationship of this to placental lactogen follows.

A physical interaction between a hormone molecule and a specific binding site (receptor) on the surface membrane of target cells is the initial step in the mechanism of action of protein and polypeptide hormones (Roth, 1973). For growth hormone however, a direct link between receptor binding and biological response has not been demonstrated. Studies with ¹²⁵I-labelled human growth hormone have demonstrated the existence of specific binding sites in cultured human lymphocytes (Lesnaiak et al., 1973, 1974) and in microsomal membranes prepared from livers of rabbits (Tsushima and Friesen, 1973; Herington et al., 1974; Gerasimo et al., 1979) and rats (Posner et al., 1974b; Herington et al., 1976a,b; Veith et al., 1975). Studies have been carried out on the physiological regulation of liver binding sites (Herington et al., 1976a,b; Posner et al., 1974a) their ontogenesis (Kelly et al., 1974) and species variation (Posner et al., 1974b). Of interest to the present work was the finding that during pregnancy in the rat (Kelly et al., 1974; Herington et al., 1976a) and rabbit (Gerasimo et

al,1979) the growth hormone receptor content (expressed as the binding capacity of hepatic membranes for human growth hormone) was approximately double that found in non-pregnant animals. In the rat these receptors interact mainly with lactogenic hormones such as placental lactogen, prolactin and primate growth hormone (Posner et al,1974b). This finding was based on earlier reports of binding specificity (Posner et al,1974a; Herington et al,1976a) where there was a lack of competition by growth hormone from species other than man and a significant but variable competition by various prolactin preparations. The physiological regulation of growth hormone binding sites has been studied. Marked increases in binding sites were induced by oestrogen administration and high prolactin or growth hormone concentrations. Marked decreases were induced by hypophysectomy or testosterone administration (Herington et al,1975,1976a; Posner et al,1974a). Progesterone and human placental lactogen did not effect the receptor levels (Herington et al,1976a).

It is well established that growth hormone markedly affects the protein synthesizing activity of its target cells (Korner,1965). When the hormone is administered in vivo, an enhancement of protein synthesis is observed in vitro or in vivo with isolated ribosomal fractions. However when growth hormone is added in vitro to isolated fractions there is no effect on protein synthesis. These results support the hypothesis that growth hormone initially acts in areas of the cell other than those where protein synthesis takes place. By using inhibitors of protein and RNA synthesis Russel et al (1970) demonstrated that growth hormone induced increases in ornithine decarboxylase activity in rat liver were dependent primarily on RNA synthesis. Further, this action of growth hormone on gene transcription occurred within 2 hours after the injection of growth hormone into adult or weanling rats. These results together with the finding that

growth hormone induced synthesis of total cell protein can be inhibited with appropriate actinomycin D treatment (Tata,1970) support the idea that growth hormone manifests its action on various enzymes through the alteration of gene transcription. Further studies have shown that growth hormone stimulation of RNA involves all species of RNA but most significantly ribosomal RNA (Talwar et al,1964; Salaman et al,1972; Ip and Brossard,1971; Tata,1970). Other studies have indicated that growth hormone treatment increases the activity of nucleolar polymerase I (which synthesises ribosomal RNA) with no observable effects on nuclear polymerase II (synthesising DNA-like RNA) (Janne and Raina,1969; Smuckler and Tata,1971) or on DNA restriction in the chromatin (Guptar and Talwar,1968; Breuer and Florini,1966). Spelsberg and Wilson (1976) reported that there was no correlation between the transcriptional response to growth hormone and the activities of the mixed-function oxidase system. These authors concluded that the induction of RNA polymerase I activity and derepression of DNA by growth hormone probably represents the selective synthesis of protein involved in liver growth rather than drug metabolism.

Wilson (1973a) reported that the decrease in liver metabolism of the 'type I' substrate hexobarbital following growth hormone treatment in male rats was related more closely to a decrease in the reduction of ferricytochrome P450 by NADPH-cytochrome P450 reductase than to the change in cytochrome P450 concentration. This finding is of interest in view of the importance of the haemoprotein spin equilibrium in determining the rate of ferricytochrome P450 reduction (Chapter 5). Thus alterations in microsomal mixed-function oxidase activity during gestation (Chapter 3) or following rat placentae (Figure 6.1) and human placental lactogen (Figure 6.4) administration may be related to the effect of placental lactogen on haemoprotein spin state (Table 6.4) and hence the rate of ferricytochrome

P450 reduction.

The important relationship between drug metabolism and microsomal phospholipids during pregnancy has been discussed (Chapter 3). Pretreatment of non-pregnant rats with rat placenta or human placental lactogen produced similar effects on microsomal phospholipids (Figures 6.2 and 6.5). Growth hormone in vivo and in vitro produced a conformational change in the phospholipids of hepatic plasma membranes (Rubin et al, 1973; Postel-Vinay et al, 1974). In addition growth hormone affects phospholipid synthesis increasing the incorporation of ^{32}P into liver phospholipids in vivo and phosphorylcholine into choline glycerophosphatides in the homogenates from the livers of growth hormone treated rats (Leal and Greenbaum, 1961). The incorporation of radioactivity from [methyl- ^{14}C]-choline, [1,2- ^{14}C]-ethanolamine and [methyl- ^{14}C]-methionine into phospholipids was increased in liver slices from pregnant rats compared with slices from non-pregnant rats (Weinhold, 1969).

In conclusion, evidence has been presented which suggests that the alterations in microsomal mixed-function oxidase activity during pregnancy in the rat is due to the presence of high levels of circulating rat placental lactogen. This effect may be mediated via changes in microsomal phospholipids and haemoprotein spin state. In addition transcriptional changes may occur which result in a selective synthesis of proteins concerned with liver growth rather than drug metabolism. The biological activity of placental lactogen is possibly associated with its binding to the hepatic microsomal growth hormone receptor which is elevated during gestation.

CHAPTER 7 GENERAL DISCUSSION

Changes in hepatic drug metabolism during pregnancy in the rat have been characterised and the role of steroids and other hormonal factors investigated. The results presented suggest that elevated levels of circulating placental lactogen cause a significant decrease in maternal hepatic mixed-function oxidase activity when expressed per gram liver. This effect is probably mediated via changes in microsomal phospholipids and/or the cytochrome P450 spin equilibrium. Changes in drug metabolism are not related to alterations in the concentration of cytochrome P450. The rise in circulating steroids, particularly progesterone, during gestation does not appear to be an important modulator of mixed-function oxidase activity. It is possible, however, that the gradual rise in circulating oestrogens has an indirect action on drug metabolism by inducing hepatic growth hormone receptors. These receptors are possibly important mediators of placental lactogen action.

The pregnancy associated alterations in drug metabolism may represent part of a biological control mechanism which ensures that the maternal liver adapts during pregnancy, enabling the mother to cope with the metabolic requirements of the developing foetus. The growth of the liver is seen as a response to these requirements. A selective proliferation of systems other than those concerned with the metabolism of drugs results in a progressive decrease in mixed-function oxidase activity when expressed per gram liver. The total capacity of the liver to metabolise drugs, however, remains unchanged or increased. This 'level' of drug metabolism is possibly all that is required to satisfy the requirements of pregnancy. Furthermore, an increase in drug metabolism in proportion to the increase

in liver weight could have a detrimental effect if it resulted in the increased metabolism and excretion (and hence decrease in blood levels) of monooxygenase substrates such as progesterone. High circulating levels of progesterone are required to maintain pregnancy. This view is possibly simplistic since pregnancy seems to be associated with a general alteration in microsomal membrane composition and metabolism.

Many areas of further investigation arise from the results of this thesis. Of particular interest is the postulated interaction between microsomal phospholipids and haemoprotein spin state which may have broader implications in the field of drug metabolism particularly if such interactions are hormonally controlled. The role of microsomal phospholipids could be investigated using a purified cytochrome P450 system. Microsomal phospholipids purified from pregnant and non-pregnant rats could be added to cytochrome P450 and NADPH-cytochrome P450 reductase from non-pregnant and pregnant rats respectively. Changes in mixed-function oxidase activity and haemoprotein spin state could then be determined. This 'cross-over' experiment might establish more clearly the role of membrane phospholipids in drug metabolism. In addition the influence of pregnancy and membrane phospholipids on the rate of ferricytochrome P450 reduction and on the forward and reverse rate constants for the low-spin to high-spin transition should be investigated. The importance of subtle changes in microsomal membrane composition and haemoprotein spin state on alternative pathways of xenobiotic metabolism is unknown. The role of cytochrome P450 in the metabolic activation of environmental toxicants and carcinogens such as aflatoxin B₁, 2-acetamidofluorene and dimethylnitrosamine is well established (Parke, 1980). The role of the microsomal membrane and haemoprotein spin state in these activation reactions is less clear. One may speculate that

alterations in the microsomal membrane environment and/or the haem spin equilibrium could result in the metabolic activation of a previously harmless drug resulting in toxic changes or cancer. Such alterations may be produced by changes in nutritional status or abnormally high levels of circulating hormones. The activities of many of the enzymes which metabolise drugs have been shown to be dependent on nutritional status (Parke, 1978). Studies on laboratory animals have shown that starvation or dietary deficiencies in protein, lipids, vitamins and essential minerals generally lead to a progressive impairment of drug metabolism. When metabolism leads primarily to the deactivation of drugs and chemicals, starvation and dietary deficiency will result in increased pharmacological activity. Conversely, it would appear that when metabolism leads to activation, tissue necrosis and carcinogenesis, dietary deficiency or starvation should decrease toxicity.

Abnormally high levels of circulating placental lactogen, growth hormone and other polypeptide hormones have been reported to be produced by ectopic hormone producing neoplasias. Many other pathological states may result in a rise in circulating hormone levels (eg. growth hormone in acromegaly). A detailed characterisation of the effect of these hormones on hepatic drug metabolism is necessary in order to evaluate possible responses to applied drug therapy.

The low level of foetal drug metabolism has been discussed (Chapter 1). It has been reported that in the human foetal circulation there are negligible levels of placental lactogen but elevated levels of growth hormone (Grumbach et al., 1968). The growth hormone is of foetal origin and there is little placental transfer of either growth hormone or placental lactogen. The elevated levels of growth hormone may be responsible, at

least in part, for the depressed rate of foetal drug metabolism. The mechanism of this action might be similar to that in the adult (see Chapter 5).

Purified rat placental lactogen is not available commercially though purification procedures have been described (Linkie and Niswender, 1973; Kelly et al., 1975) with rat placental lactogen. The effect of dosing on drug metabolism, phospholipids, haemoprotein spin state and ferricytochrome P450 reduction could then be studied. The potential role of the pituitary and ovaries in mediating the action of placental lactogen could be investigated by comparison with hypophysectomised and ovariectomised animals.

The significance of the findings in this thesis to the human is difficult to evaluate. Pharmacokinetic studies during pregnancy are equivocal and influenced by many physiological changes occurring at this time (see Chapter 1). Any evaluation is further hindered by a lack of data on species differences in hepatic drug metabolism during pregnancy. Mixed-function oxidase activity has been investigated predominantly in the rat and rabbit (see Chapter 1). Species differences in drug metabolism are well characterised (for review see Kato, 1979) and it would be important to establish whether similar differences existed during gestation.

The work presented in this thesis has highlighted the importance of factors other than the concentration of cytochrome P450 in the regulation of hepatic mixed function oxidase activity. Furthermore an important connection has been established between polypeptide hormones and the hepatic mixed-function oxidase system. The pregnant rat would seem to offer a convenient system in which to study interactions between

circulating hormones, microsomal membrane composition and drug metabolism.

A full investigation of this system would be an important requirement for a detailed characterisation and understanding of the mixed-function oxidase system.

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