THE INFLUENCE OF PREGNANCY AND ORAL CONTRACEPTIVE STEROIDS ON DRUG METABOLISM

by

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Abstract

Methods for the determination of the hydroxylation of biphenyl in rat liver and the glucuronidation of 4-methylumbelliferone in rat and rabbit liver preparations have been developed by the modification of existing methods.

Using these methods, the activities of biphenyl-hydroxylase and 4-methylumbelliferone glucuronyl transferase and p-nitrobenzoic acid nitroreductase and cytochrome P450 have been determined in normal and pregnant rats. When expressed per gram of liver, biphenyl-4-hydroxylase, 4-methylumbelliferone glucuronyl transferase and cytochrome P450 were found to be decreased in the livers of 19-20 day, but not 15-16 day, pregnant rats. However, liver weight also increased so that the total liver content of these parameters was similar to that of non-pregnant animals; total microsomal protein and nitroreductase activity were increased. Pregnancy did not prevent the induction of these parameters with phenobarbitone.

None of these parameters is altered in the full-term pregnant rabbit, although the hydroxylation of coumarin is decreased.

The action of hexobarbitone in full-term pregnant rats is prolonged, confirming the in vitro studies.

The effects of pregnancy on hepatic microsomal enzymes could not be reproduced by pretreatment with progesterone or oestradiol, alone or combined, though both of these endogenous steroids, and also oral contraceptive steroids, inhibited the hydroxylation and nitroreduction in vitro. Pretreatment with high doses of chlormadinone, norgestrel, norethynodrel, ethynodiol or mestranol for eighteen days, did not alter any of the liver parameters, though a single dose of norethynodrel
caused inhibition of biphenyl-4-hydroxylation at one and twenty-four hours after dosing. Eighteen days treatment with ethynodiol and mestranol combined, increased the hydroxylation. Long-term oral administration of a low dose of chlormadinone or ethynodiol, but not norethynodrel, decreased the hydroxylation but none of the other parameters. Pretreatment of rats with oral contraceptive steroids, except chlormadinone, decreased the duration of action of hexobarbitone in vivo.

A method for the determination of the urinary metabolites of phenacetin by gas chromatography has been developed by modification of previous methods. The pattern of excretion in pregnant and non-pregnant rats is similar. Humans on the "pill" possibly excrete more unchanged phenacetin and less conjugated N-acetyl-p-aminophenol than controls.
To my parents

and

Lorraine
ACKNOWLEDGMENTS

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The true worth of an experimenter consists in his pursuing not only what he seeks in his experiment, but also what he did not seek.

CLAUDE BERNARD
# CONTENTS

<table>
<thead>
<tr>
<th>CHAPTER I</th>
<th>Introduction</th>
<th>Page 8</th>
</tr>
</thead>
<tbody>
<tr>
<td>CHAPTER II</td>
<td>Materials and Methods</td>
<td>53</td>
</tr>
<tr>
<td>CHAPTER III</td>
<td>Reappraisal of the Method for the Determination of Biphenyl Hydroxylation in Rat Liver Preparations</td>
<td>67</td>
</tr>
<tr>
<td>CHAPTER IV</td>
<td>Development and Investigation of 4-Methylumbelliferone Glucuronyl Transferase Determination in Rat and Rabbit Liver Preparations</td>
<td>87</td>
</tr>
<tr>
<td>CHAPTER V</td>
<td>Effects of Pregnancy on Hepatic Drug metabolizing Enzymes in Rats and Rabbits.</td>
<td>111</td>
</tr>
<tr>
<td>CHAPTER VI</td>
<td>Effects of Progestogens, Oestrogens and Oral Contraceptive Steroids on Hepatic Drug-metabolizing Enzymes</td>
<td>152</td>
</tr>
<tr>
<td>CHAPTER VII</td>
<td>Effects of Pregnancy and Oral Contraceptive Steroids on the Pharmacological Activity of Hexobarbitone</td>
<td>194</td>
</tr>
<tr>
<td>CHAPTER VIII</td>
<td>In Vivo Metabolism of Phenacetin in the Rat and Human</td>
<td>219</td>
</tr>
<tr>
<td>CHAPTER IX</td>
<td>General Discussion</td>
<td>235</td>
</tr>
<tr>
<td>References</td>
<td></td>
<td>245</td>
</tr>
</tbody>
</table>
CHAPTER I

INTRODUCTION
A. General Principles of Drug Metabolism

Drugs are compounds that are generally of little or no nutritive value to the body, since they cannot be utilized for the production of energy or for the build up of tissue. They are "foreign" to the major metabolic pathways of the body, which are concerned with the metabolism of nutrients, and are therefore classed as "foreign compounds". They are distinguished from other foreign compounds by the fact that they produce a pharmacological effect, but as far as the body is concerned they are treated the same as all other foreign compounds. Since these compounds are alien to the body it is necessary for the animal to eliminate them as rapidly as possible, otherwise if allowed to remain they will accumulate and eventually lead to the poisoning of the animal. The body thus possesses a defence system which transforms these nutrients (as they are otherwise called) so that they can be readily excreted. The term "drug metabolism" is applied to these transformations that occur. The site of these transformations is generally the liver, and as a result of them the activity and/or toxicity of a compound may be altered.

Apart from drugs, there are present in our environment today many foreign compounds which can get into the body by various means. These include food additives, such as preservatives, antioxidants, sweeteners, flavours and colours, cosmetics, detergents, pesticides and industrial chemicals. As far as the body and its defence system are concerned, drugs and these other classes of compound are treated similarly, and therefore the principles of drug metabolism can be applied to most foreign substances. Moreover, the
administration of one compound may affect the metabolism of another (Fouts, 1964), so it is important to know the metabolic interactions of these different classes of compound.

The study of drug metabolism and of factors affecting it are therefore important since they help to understand "what the body does to the drug" as opposed to pharmacology which is concerned with "what the drug does to the body". (Williams, 1967). Much information on this subject prior to 1958 has been covered by Williams, R.T., in his book "Detoxication Mechanisms" (1959), while more recent information is covered by Parke, D.V. in his book "The Biochemistry of Foreign Compounds" (1968a). There have also been numerous reviews on the subject, (Williams, 1960, 1964, 1965: Maynert, 1961: Parke, 1962, 1968b; Boyland and Booth, 1962: Shuster, 1964: Williams and Parke, 1964).

**Metabolic reactions of Drugs and Foreign Compounds**

Metabolic reactions of drugs and foreign compounds have been classified into two types - Phase I and Phase II (Williams, 1960). In the first the compound undergoes one or more of a variety of oxidations, reductions or hydrolyses which result in the introduction or unmasking of functional groups which increase the polarity of the compound and therefore render it more readily excretable. These groups often act as a centre for the second phase of metabolism in which a synthetic step occurs so that the compound is conjugated with an endogenous molecule. The products of the second phase, so-called conjugation products, are usually water soluble acids which are easily excreted. The majority of phase two conjugations involve glucuronic and sulphuric acids, but conjugation with amino acids and methyl or other alkyl groups also occurs.
The majority of compounds are metabolised by both phases of reaction, for example benzene is chiefly metabolized as follows:

\[
\text{Benzene} \xrightarrow{\text{oxidation}} \text{Phenol} \xrightarrow{\text{conjugation}} \text{Phenyl glucuronide}
\]

When a compound already possesses a polar group it often only undergoes phase two metabolism. Benzoic acid is therefore primarily conjugated with glycine and glucuronic acid to form hippuric acid and benzoyleglucuronide respectively and only undergoes oxidation to give hydroxybenzoic acid to a small extent.

These transformations can influence the pharmacological activity and the toxicity of the compounds. Phase one metabolism may activate or deactivate a drug, or alter its toxicity so that it becomes either more toxic (intoxication) or less toxic (detoxication). Phase two metabolism generally results in deactivation of a drug and usually a decrease in toxicity. Examples of some of these processes are shown below:

\[
\begin{align*}
\text{Phenacetin} & \rightarrow p-\text{Acetamido} & \rightarrow p-\text{Acetamidophenol} \\
\text{Activation} & \text{Deactivation} & \text{Glucononide} \\
\text{Phenobarbitone} & \rightarrow \text{Hydroxyphenobarbitone} & \rightarrow \text{Hydroxyphenobarbitone glucuronide} \\
\text{Activation} & \text{Deactivation} & \text{Inactive excretory product}
\end{align*}
\]

However, phase two metabolism can lead to toxic metabolites and such a process is termed "lethal synthesis" (Peters 1952).
Microsomal Drug-metabolizing Enzymes

The metabolic changes described above are, in general, brought about by enzymes located in the endoplasmic reticulum of the liver, and, to a lesser extent, other organs such as the lungs, gastrointestinal tract and kidneys. These enzymes are referred to as "microsomal" enzymes and they play a major role in the oxidation and reduction of foreign compounds. There are also other enzymes associated with the metabolism of foreign compounds which are not associated with endoplasmic reticulum. These "non-microsomal" enzymes, however, have only a minor role in drug metabolism. A few foreign compounds may also be metabolized by some of the normal enzymes of intermediary metabolism such as alcohol dehydrogenase and xanthine oxidase.

The endoplasmic reticulum is a lipoprotein, tubular network extending throughout the whole of the cytoplasm of a cell, and comprises two major components, the rough endoplasmic reticulum (RER) and the smooth endoplasmic reticulum (SER). The surface of the former is studded with ribosomes which are sites of protein synthesis: the latter has no ribosomes. Many, but not all, drug-metabolizing enzyme systems are preferentially concentrated in the smooth endoplasmic reticulum (Orrenius and Ernster, 1964; Gram and Fouts 1967).

Solubilization of the microsomal drug-metabolizing enzyme system, necessary for a comprehensive study of its characteristics and components, has proved exceptionally difficult. However, it is possible to undertake a preliminary study of the characteristics of these systems using homogenized tissue. Centrifugation of homogenized tissue in isotonic solution at 10,000 x g for ten minutes deposits cell debris, mitochondria and nuclei, leaving the endoplasmic reticulum which is now broken up into small vesicles and therefore called
microsomes, in the supernatant. Further centrifugation of this supernatant at 105,000 x g for one hour deposits the "microsomes", and leaves the soluble fraction as supernatant (Mitoma et al. 1956). Dallner (1963) has described a method for separating the RER and SER by centrifugation. Since the microsomal enzymes have a requirement for certain factors (see later) present in this soluble fraction, investigations of the enzyme activity are usually carried out on the 10,000 x g supernatant fraction.

The microsomal enzymes do not metabolize endogenous compounds such as phenylalanine, L-tryptophan and kynurenine (Mitoma et al. 1956) which are hydroxylated by specific enzymes in other parts of the cell. They generally metabolize lipid-soluble foreign compounds (Gaudette and Brodie 1959) forming more polar compounds, but the latter type compounds themselves can also be metabolized by these enzymes (Mazel and Henderson 1965). They catalyze reactions involving both oxidation and reduction.

**Drug Oxidations**

All drug oxidations, and that covers a wide range of reactions, may be classed as hydroxylations (Parke 1968a).

**Aromatic hydroxylation**

\[
R - C_6H_5 + [O] \rightarrow R - C_6H_4OH
\]

**Acyclic oxidation**

\[
R - CH_3 + [O] \rightarrow R - CH_2OH
\]

**N - dealkylation**

\[
R - NHCH_3 + [O] \rightarrow R - NHCH_2OH \rightarrow RNH_2 + HCHO
\]

**O - dealkylation**

\[
R - O - CH_3 + [O] \rightarrow R - OCH_2OH \rightarrow ROH + HCHO
\]

**Deamination**

\[
R - CHNH_2 + [O] \rightarrow R - C(OH)NH_2 \rightarrow R - CO + NH_3
\]

**Sulphoxidation**

\[
R - S - R + [O] \rightarrow R - S^+(OH)-R \rightarrow R - S - R + H^+
\]
All these reactions have the paradoxical requirement for both reduced coenzyme NADPH₂ and oxygen (Brodie et al 1958). Experiments with ^18O₂ and H₂¹⁸O have shown that the oxygen of the hydroxyl group introduced into the foreign compound is derived from molecular oxygen and not from water (Mason 1957; Hayaishi 1962). It is for this reason that the microsomal hydroxylating enzymes have been termed "mixed function oxidases", (Mason 1957; 1965).

It has also been shown that the liver microsomes contain NADPH₂-dependent enzyme systems which hydroxylate various steroid hormones (Conney and Klutch 1963; Kuntzman et al 1964). Many similarities exist between these enzyme systems and those responsible for drug oxidations (Conney et al 1966; 1968; Tephly and Manerling 1968), and it has therefore been suggested that these reactions are mediated through the same enzymes, and that steroid hormones are naturally occurring substrates for drug-metabolizing enzymes in liver microsomes (Kuntzman et al 1964).

These findings led Gillette (1962) to suggest that microsomal hydroxylation occurs by a coupled redox reaction in which NADPH₂ reduces a coenzyme which then combines with oxygen to form "active oxygen". This finally reacts with the foreign compound in the presence of various hydroxylases to give the hydroxylated compound. The postulated sequence is shown below:

1. \[ \text{NADPH} + \text{H}^+ + \text{A} \rightarrow \text{AH}_2 + \text{NADP} \]
2. \[ \text{AH}_2 + \text{O}_2 \rightarrow \text{"Active oxygen"} \]
3. \[ \text{"Active oxygen" + drug} \rightarrow \text{Oxidized drug} + \text{A + H}_2\text{O} \]

where A represents a reducible coenzyme.
Cytochrome P450

Gillette (1962) suggested that the reducible coenzyme "A" was a microsomal flavoprotein. Later Omura and Sato (1962; 1964a) suggested that it was a haemoprotein and this was confirmed by Omura et al (1965) who named the haemoprotein "cytochrome P450". This cytochrome was so-called because of its anomalous spectral behaviour in combination with carbon monoxide (CO). In such combination cytochrome P450 in the reduced form gives a Soret peak at 450 nm. Such a CO-binding pigment had been first observed in liver microsomes in 1958 (Klingberg; Garfinkel).

The concept of the involvement of cytochrome P450 in the liver microsomal hydroxylating process is based on the demonstration that the process is inhibited by CO (Orrenius et al 1964) and reactivated by light with an action spectrum exhibiting a maximum at 450 nm (Cooper et al 1965). In addition, the cytochrome P450 content of liver microsomes increases parallel to the increase in hydroxylating activity of liver microsomes upon treatment of animals with barbiturates or carcinogens (Orrenius and Ernster 1964; Remmer and Merker 1965; Ernster and Orrenius 1965).

Although the electron transport system of microsomal mixed function oxidases has proved too labile for the solubilization or separation (Omura and Sato 1964b) of its components, the steroid hydroxylating system of beef adrenal cortex mitochondria has been separated into a flavoprotein NADPH$_2$ diaphorase, a non-haem iron protein and cytochrome P450 (Omura et al 1966). The authors therefore suggested the following electron transport system for hepatic microsomal hydroxylations (Omura et al 1965):—
Kato (1966) accumulated much evidence in support of this idea.

Remmer et al (1966) showed, however, that it was the oxidised form of cytochrome P450 that forms the complex with the drug, suggesting that P450 first reacts with drugs and is then reduced by NADPH₂. Thus the present view is as follows (Brodie 1967):

Drug + P450 (ox) \[\rightarrow\] Drug - P450 (ox) + NADPH₂ \[\rightarrow\] Drug - P450 (red) + NADP⁺ + H⁺ + H₂O

Thus cytochrome P450 is a key component of the microsomal hydroxylase system. A great deal of attention has been focussed on cytochrome P450 in the last five years so it is worth considering it in more detail.

The haemoprotein referred to as cytochrome P450 on the basis of its reaction with CO also combines in the reduced form with ethyl isocyanide (Omura and Sato 1962; 1964; Imai and Sato 1966) to give Soret peaks at 430 and 455 nm. This led Imai and Sato (1966) to suggest that cytochrome P450 existed in two forms in microsomes which were in pH-dependent equilibrium. The equilibrium between the two forms is also affected by ionic strength (Imai and Sato 1968). Treatment of rats with phenobarbitone or the polycyclic hydrocarbon, 3-methylcholanthrene (3-MC) increases the apparent concentration of
cytochrome P450 present in liver microsomes (Remmer and Merker 1965; Ernster and Orrenius 1965). Using ethyl isocyanide as ligand differences were shown in the induction by phenobarbitone and 3-MC, and it was suggested that 3-MC treatment causes the formation of a new haemoprotein called P450 (Sladek and Mannering 1966; Alvares et al 1967). In further support of this idea it was found that the haemoprotein induced by phenobarbitone is not the same as that induced by 3-MC, when CO was used as ligand. Thus in the 3-MC treated microsomes there was a shift in absorption maximum from 450 nm to 448 nm (Alvares et al 1967; Kuntzman et al 1968). Recently treatment with 3-MC of male rats has been shown to change the Michaelis constant for the hydroxylation of 3,4-benzpyrene, and it is possible that this effect is due to a greater affinity of 3,4-benzpyrene for the haemoprotein preferentially formed in 3-MC-treated rats, (Alvares et al 1968; Gurtoo et al 1968).

The above considerations posed the question of whether a single cytochrome P450 existed in two or more spectrally distinguishable forms, or whether two or more haemoproteins of the type of cytochrome P450 are present in microsomes. These could be induced by various agents and could be specific for substrates metabolized in hydroxylation reactions.

Much of the early work was carried out on microsomal suspensions using the CO and ethyl isocyanide difference spectra. Attempts have been made to isolate cytochrome P450 in the active form using various techniques, but all have resulted in its conversion to a spectrally modified form called "cytochrome P420" (Omura and Sato 1964 b; Mason et al 1965) which has, however, been solubilized and partly purified (Omura and Sato 1964 b). However, various techniques have been developed for measuring the
absolute spectrum of cytochrome P450 (Kinoshita and Horie 1967; Remmer et al 1968; Miyake et al 1968; Nishibayashi and Sato 1968) and the results obtained have been used to provide further data as to whether cytochrome P450 is a single haemoprotein or not (Hildebrandt et al 1968; Schenkman et al 1969). The absorption spectrum of oxidised P450 has been shown to possess a peak at 420 nm and the reduced P450 peak at 408 nm.

In investigations of the absolute spectrum of cytochrome P450 in animals pretreated with phenobarbitone or 3-MC, different types of spectrum are obtained (Hildebrandt et al 1968; Schenkman et al 1969). Hildebrandt et al (1968) suggest that two spectrally distinct haemoproteins exist which are interconvertible forms of a single haemoprotein. Schenkman et al (1969) suggest that one of these forms is the native haemoprotein, the other the haemoprotein-substrate-complex. However, Kuntzman et al (1968) have shown that 3-MC added in vitro, or given to a rat immediately prior to killing, does not bring about the spectral change caused by 3-MC mentioned above, and therefore the binding of 3-MC to the haemoprotein is not responsible for the changes.

The addition of drugs to liver microsomal preparations causes changes in the absorption spectrum of oxidised cytochrome P450. Remmer et al (1966) and Imai and Sato (1966 a) showed that drugs could be classified into two groups on the basis of the change that they brought about and they called them type I and type II. The type I spectral change, characterized by a difference spectrum with a trough at 420 nm and a peak at 385 - 390 nm, is caused by the addition of compounds such as hexobarbitone, aminopyrine and phenobarbitone to liver microsomes, while the type II
is characterized by a peak at 430 nm and a trough at 390 nm and is brought about by compounds such as aniline and nicotinamide. Explanations of these two types of binding can be given to fit either theory as to the number of microsomal haemoproteins that exist, (Mannering et al 1969). Thus two binding sites could exist on a single haemoprotein, the different drugs combining preferentially with one site or the other, or two haemoproteins could exist which combine selectively with different drugs.

The problem of whether cytochrome P450 is one haemoprotein or more has not, therefore, been solved. Two schools of thought appear to exist. Kuntzman and co-workers who suggest the existence of more than one cytochrome responsible for the oxidation of drugs as opposed to Remmer and co-workers who suggest that only one cytochrome exists. As to which school of thought is correct will probably have to await solubilization and characterization of the haemoprotein(s) found in normal, phenobarbitone and 3-MC treated animals (Kuntzman 1969).

Microsomal Reductions

In addition to the oxidative enzymes, the endoplasmic reticulum of the liver contains enzymes that reduce drugs. These enzymes catalyze the reduction of aromatic nitro and azo compounds to amines (Fouts and Brodie 1957; Fouts et al 1957). The system responsible for the reduction of nitro groups can use either NADH₂ or NADPH₂ as electron donor, and is active under anaerobic conditions, but virtually inactive in air (Fouts and Brodie 1957). The nitro-reductase system is a flavoprotein, having FAD as its prosthetic group, and it was originally suggested that microsomal enzymes, e.g. NADPH₂-cytochrome c reductase or NADH₂-cytochrome b₅ reductase, reduce FAD to FADH₂ which
then reduce the nitro group non-enzymically
(Kamm and Gillette, 1963). Recently (Gillette et al 1968) it has been suggested, however, that the reduction of p-nitrobenzoic acid to p-aminobenzoic acid by liver microsomes is mediated by cytochrome P450.

Other Metabolic Pathways

Drugs and foreign compounds also undergo various non-microsomal metabolic transformations. These include non-microsomal enzyme systems such as are present in mitochondria, e.g. amine oxidases, and in the soluble fraction, e.g. alcohol dehydrogenases. There are also enzymes in the blood and in the intestinal microflora. There are, finally, many metabolic transformations that occur for which the enzymes and their locations are as yet unknown. A consideration of these enzyme systems is of little importance in the present context but mention of their existence serves to complete this section on metabolic transformations (see Parke 1968a).

Conjugation Reactions

Conjugation with glucuronic acid is probably the most important conjugation mechanism and this is also brought about by enzymes located on the endoplasmic reticulum, principally of the liver (Dutton and Storey 1954). The formation of glucuronides is a two-stage process involving first the biosynthesis of the coenzyme donor, UDPGA, and secondly the transfer of the glucuronyl moiety from UDPGA to the aglycone. The glucuronylation has been well defined and is shown below:

\[
\begin{align*}
\text{UDPG} + 2 \text{NAD} & \rightarrow \text{UDPG} + 2 \text{NADH}_2 \\
\text{UDPGA} + \text{ROH} & \rightarrow \text{RO}_6\text{H}_9\text{O}_6^+ \text{UDP} \\
\text{dehydrogenase} & \\
\text{Glucuronyl Transferase} & \\
\end{align*}
\]
The first stage, involving oxidation of UDPG, is brought about by enzymes located in the supernatant fraction of the cell (Strominger et al. 1957), but the glucuronyl transferases (otherwise known as transglucuronylases) are microsomal enzymes. Unlike the enzymes involved in phase I metabolism, those involved in phase II metabolism, including glucuronyl transferases, are responsible for conjugating endogenous substrates (e.g. bilirubin, thyroxine) as well as foreign compounds. These enzymes occur mainly in the liver, and to a lesser extent in the kidney, gastrointestinal tract and the skin, and there is probably a multiplicity of liver microsomal glucuronyl transferases.

Glucuronides are classified as O-glucuronides, N-glucuronides or S-glucuronides, formed from oxygen, nitrogen and sulphur containing compounds respectively. O-glucuronides are formed from phenols and alcohols (ether-type) and carboxylic acids (ester-type); N-glucuronides from amines, and S-glucuronides from thiols. Much information about glucuronides and the enzymes responsible for their synthesis is given in "Glucuronic acid, Free and Combined" (Dutton 1966).

The other types of conjugation reactions are sulphate conjugation, methylation, acetylation, peptide conjugation and glutathione conjugation. These reactions are not catalyzed specifically by microsomal enzymes and are in general relatively minor routes of metabolism compared with glucuronide conjugation. They are therefore not mentioned in detail here.

Factors affecting drug metabolism

The activity of the hepatic microsomal drug-metabolizing enzymes can be affected by a variety of factors resulting in altered metabolism of a compound and thereby possibly altered activity or toxicity (Conney and Burns 1962; Fouts 1963; Parke 1968b). Increased enzyme
activity resulting from stimulation leads to more rapid metabolism which can lead to either an increased or decreased duration of action depending on whether phase I metabolism activates or deactivates the drug. Alternatively decreased enzyme activity resulting from inhibition will produce the opposite effect. Phase II metabolism always deactivates drugs so that stimulation or inhibition of this phase leads to decreased or increased duration of action respectively. The factors that affect drug metabolism may be genetic, physiological or environmental in origin. Such factors include species, strain, age, sex, the nutritional state of the animal, disease, stress, ingestion of other foreign compounds and pregnancy.

Species - the difference in drug metabolism between species is well known and is of particular importance to the pharmacologist who wishes to extrapolate data from animals to man. It is now common practice to investigate the metabolism of any new compound in a number of animal species before it is considered for human use. As a result the metabolic data of a wide variety of foreign compounds are known in quite a large variety of species, and the knowledge has been profitably used in the design of new drugs. Some species differences in drug metabolism are qualitative, for example the enzyme responsible for the 7-hydroxylation of coumarin is found in the liver of the rabbit, guinea pig, coypou, cat, hamster and hen, but is absent from the liver of rat, mouse and ferret (Creaven et al., 1965a). However, detoxication by different mechanisms is rare, and therefore most species differences in the metabolism of drugs are quantitative. Species differences are also known in the effects of other factors on drug metabolism. For reviews on species differences see Williams (1959 and 1964).
**Strain** - heredity seems to play an important role in determining rates of drug metabolism. Many investigators have shown differences in drug metabolism in different strains of a given species (Quinn et al 1958; Cram et al 1965; Vesell 1968).

**Age** - the activity of drug-metabolizing enzymes is markedly affected by age (Kato et al 1964a). Newborn mice, guinea pigs, rabbits and rats lack microsomal enzymes, including cytochrome P450 (Kato 1966), which oxidatively metabolize drugs. In rats these enzymes increase progressively up to the age of thirty days and then gradually decrease. Of particular interest is the observation of Creaven et al (1965b) that young rabbits and young rats were able to hydroxylate biphenyl in the ortho position as well as the para, but adult animals of both species were only capable of para-hydroxylation. Conjugation reactions are also low in young animals. In mouse, rabbit, guinea-pig and man glucuronyl transferase activity of the liver is low at birth and gradually increases to adult levels depending on the species and substrates (Dutton 1963). However, in rats the glucuronyl transferase activity towards certain substrates is as high in the newborn as in the adult liver (Dutton et al 1964).

**Sex** - it has long been known that the duration and intensity of drug action is often greater in the adult female rat than in the adult male (Holck et al 1937). Quinn et al (1958) showed this difference was due to higher drug-metabolizing enzyme activity in the male rat liver than the female. Later workers have found similar differences (Kato et al 1962; Schenkman et al 1967). However, not all metabolic reactions show a sex difference. Kato and Gillette (1965) have shown that although the aliphatic hydroxylation of hexobarbitone and pentobarbitone and the N-demethylation of aminopyrine and morphine show sex differences,
the aromatic hydroxylation of aniline and zoxazolamine show virtually none. It was suggested that the sex differences were due to a balance between male and female sex hormones since they appear only at puberty and may be abolished by castration. Moreover, administration of androgens to female rats increases the activities of the microsomal enzymes, while in males the opposite effect is brought about by oestrogens (Quinn et al 1958; Murphy and Dubois 1958). These sex differences in the rat are perhaps not surprising since the sex hormones are probably endogenous substrates for the microsomal drug-metabolizing enzymes (Kuntzman et al 1964). The influence of sex hormones, particularly female ones will be discussed in more detail in section B. The sex differences in drug-metabolizing enzymes seem to be confined mainly to the rat since no such differences have been observed in guinea-pigs, rabbits, cats and dogs. There does seem to be a sex difference in mice with respect to pentobarbitone and hexobarbitone metabolism but the effect is the opposite to that observed in the rat, (Westfall et al 1964; Novick et al 1966).

Nutrition - the nutritional status of an animal can markedly affect the activity of its hepatic microsomal enzymes. Metabolism of compounds which show a sex dependence such as hexobarbitone and aminopyrine, is impaired by starvation in male rats, while aniline metabolism, which is independent of sex, is enhanced. In female rats, though, starvation causes an increase in all drug-metabolizing enzymes (Kato and Gillette 1965). Refeeding of these animals with a standard diet or sucrose diet leads to a marked decrease in enzyme activity (Kato 1967). Rats fed on a low protein diet show decreased hepatic drug-metabolizing activities (Kato et al 1968) and a decrease in microsomal cytochrome P450 content (Marshall and McLean 1969). Similarly a diet
deficient in calcium leads to diminished enzyme activity (Dingell et al 1966).

Disease - the duration of action of carisoprodol, zoxazolamine and pentobarbitone are increased in male and female rats bearing abdominal carcinosarcomas (Kato et al 1968 a) and this has been shown to be due to decreased liver microsomal enzyme activity and cytochrome P450 (Kato et al 1968 b). The activity of microsomal enzymes in various hepatic tumours is also impaired or absent (Adamson and Fouts 1961; Gram et al 1968). In the situations reported rapid liver growth is taking place. Similarly in the newborn animal where there is rapid growth taking place the microsomal enzymes are lower in activity. It therefore seems that low levels of drug-metabolizing enzymes are found in situations of rapid growth (Conney 1967).

Stress - adverse environmental conditions, i.e. stresses, have been shown to affect drug metabolism (Franklin 1969). Exposure to cold appears to increase microsomal metabolism; thus the hydroxylation of acetanilide by rat liver microsomes is almost doubled on exposure to cold, while that of biphenyl is also increased. However, Kalsner and Kunig (1969) have shown that exposure of rats to 5°C for up to seven weeks does not alter hexobarbitone metabolism, whereas similar exposure at 25°C increases hexobarbitone metabolism.

Hepatic microsomal drug metabolism also follows a circadian rhythm. Thus Radzialowski and Bousquet (1968) showed that the oxidative metabolism of amino-pyrene, p-nitroanisole and hexobarbitone was maximal in male rats at 0200 hours and minimal at 1400 hours. Plasma corticosterone levels were also shown to follow circadian rhythm but the maximum and minimum occurred at 1400 hours and 0200 hours respectively, i.e. twelve hours out of phase with the metabolism.
Other Foreign Compounds - simultaneous administration of more than one foreign compound or drug can lead to either decreased or increased metabolism of one or both of those compounds depending on whether the compounds inhibit or stimulate drug-metabolizing enzymes. These aspects have therefore received much attention during the last decade or so because of the great bearings on drug action that they confer.

The stimulatory effect of foreign compounds on liver microsomal enzyme activity was first observed by Brown, Miller and Miller (1954) after administration of polycyclic hydrocarbons to rats and mice. The studies were developed further by Conney et al (1956, 1957 a & b) and the activation was shown to involve an increase in synthesis of drug-metabolizing enzymes. Later, two independent groups of workers showed that phenobarbitone and a number of other common drugs also acted as inducers of drug-metabolizing enzymes (Conney & Burns 1959; Remmer 1959). Many other compounds have since been shown to induce drug-metabolizing enzymes, e.g. phenylbutazone, the barbiturates, chlorinated insecticides and many carcinogenic polycyclic hydrocarbons (see Conney 1967). The amount of work done has been sizeable and several reviews have been published (Ernster and Orrenius 1965; Remmer 1962; Conney 1967; Kuntzman 1969).

Inducers of drug-metabolizing enzymes are of two types exemplified by phenobarbitone and methylcholanthrene. Phenobarbitone and similar compounds effect a relatively non-specific induction of microsomal enzymes, whereas polycyclic hydrocarbons produce a more specified induction (Conney 1967). A fine example of the differential stimulation by polycyclic hydrocarbons and phenobarbitone is afforded by the observation (Creaven and Parke 1966) that in rats and mice 3,4-benzopyrene stimulates the 2-hydroxylation of biphenyl but not its 4-hydroxylation, whereas phenobarbitone causes a large increase in
4-hydroxylation but only a small increase in 2-hydroxylation. Pretreatment of rats with phenobarbitone causes parallel increases in drug-metabolizing activity and cytochrome P450 (Remmer and Merker 1965; Ernster and Orrenius 1965). However, 3-MC induces the formation of a microsomal haemoprotein with spectral characteristics different from those observed in liver microsomes obtained from control or phenobarbitone treated rats, (Alvares et al 1967; Kuntzman et al 1968). Further differences between the effects of phenobarbitone and 3-MC on cytochrome P450 have already been discussed.

The changes that accompany drug-induced microsomal enzyme synthesis have been well reviewed by Orrenius et al (1968). The mechanism of the induction of microsomal enzymes by foreign compounds, however, is not definitely known. Parke (1968a) has suggested that activation of the enzymes which metabolize foreign compounds may be due to induction of one or more genetic systems by de-repression of an operator gene(s). By combining with the repressor substance the activator would cause de-repression and thus stimulation of messenger RNA synthesis and induction of the enzyme system (see fig. 1). How the different modes of stimulation of phenobarbitone and 3-MC occurs has not been suggested, but since that produced by both is abolished by simultaneous administration of actinomycin D (Orrenius et al 1965, Gelboin and Blackburn 1964) it would seem that activation of the microsomal enzyme systems occurs at the level of messenger-RNA synthesis in both cases (see Parke 1968a).
Possible mechanism for the induction of the hepatic microsomal drug-metabolizing enzymes.

* These proteins could be mixed function oxidases or components of the electron transport chain involved in microsomal hydroxylation.

An observation of extreme practical importance was made by Ferguson (1966). He found that mice kept on red cedar chip bedding exhibited significant reductions in sleeping time when administered hexobarbitone or pentobarbitone sodium. Vessell (1967) showed that this effect was produced by a factor in the cedarwood which induced liver microsomal enzymes responsible for metabolizing hexobarbitone, aniline and ethylmorphine. Wade et al (1968) have since shown that the constituents of cedarwood oil, cedrol and cedrene, are inducers of drug-metabolizing enzymes.

Several drugs and foreign compounds are known which inhibit the microsomal metabolism of foreign compounds.
e.g. β-diethylaminoethyldiphenylpropylacetate hydrochloride (SKF 525-A), 2,4-dichloro-6-phenylphenoxymethyl ethyldiethylamine hydrobromide (Lilly 1947), 2,4-dichloro-6-phenylphenoxymethylmine (DPEA), iproniazid, imipramine and glutethimide. Not all microsomal reactions are inhibited, however. SKF 525-A, which is typical of these inhibitors (Gillette 1963), for example, inhibits the in vitro oxidation of barbiturates, the demethylation of ethylmorphine (Anders and Mannering 1966), hydroxylation of aniline and glucuronide formation (Ikeda et al 1968), but not the hydroxylation of acetanilide (Pouts and Brodie 1955), the sulphoxidation of chlorpromazine (Gillette and Kamm 1960) or the reduction of nitro- and azo-compounds (Gillette 1963). These facts would suggest that a variety of inhibitory mechanisms may exist. However, none of these mechanisms is fully understood.

It has been shown that SKF 525-A inhibits the N-demethylation of ethylmorphine in rat liver microsomes competitively, suggesting that SKF 525-A combines with the active site of the demethylase as an alternative substrate (Anders and Mannering 1966). In contrast Ikeda et al (1968) showed that the inhibition of the hydroxylation of aniline and of the glucuronidation of o-aminophenol by SKF 525-A was non-competitive. Remmer et al (1968) have shown that SKF 525-A and DEEA have a much greater affinity than have aniline, hexobarbitone or aminopyrine for cytochrome P450, and they therefore suggest that this explains their inhibitory actions. Rubin et al (1964) have shown that many compounds which are substrates, for hepatic microsomal enzymes are competitive inhibitors of each other's metabolism in vitro, while certain drugs which are not metabolized did not act as inhibitors. The inhibition is presumably due to the substrates competing for cytochrome P450. However,
these compounds, unlike SKF 525-A and the other inhibitors mentioned above, do not act as inhibitors of drug metabolism when given in vivo.

Many compounds which initially inhibit hepatic microsomal enzyme systems when given in vivo exhibit a stimulating phase later (Kato et al 1964). Thus prolonged administration of SKF 525-A induces microsomal enzyme activity by the synthesis of new enzyme protein, but both the old and the new enzymes are inhibited by a further dose of SKF 525-A (Anders and Mannering 1966b). Similarly the steroid norethynodrel has been shown to produce such a biphasic effect on hexobarbital metabolism (Juchau and Fouts 1966). The time for reversal of action to occur varies from drug to drug (Kato et al 1964), so that with potent activators like glutethimide reversal occurs after a shorter period. Thus, the terms "stimulation" and "inhibition" are really only relative, determined by the time interval after pretreatment.

The inhibitory and stimulatory effects of foreign compounds on the metabolism of other foreign compounds often results in changes in toxicity and intensity of pharmacological activity. The action of a drug may thus be affected by the administration of another drug simultaneously. This can either lead to synergism or tolerance. In the former a synergist is given along with a drug, and depending on whether metabolism activates or deactivates the drug, the synergist stimulates or inhibits its metabolism so that its action is prolonged. The latter, i.e. tolerance, can be produced where the action of a drug is reduced, due to increased metabolism, either by another compound or by the drug itself.

Enzymic considerations

I mentioned earlier that the role of cytochrome P450 is now firmly established as the terminal oxidase
in drug oxidations by liver microsomes. Is the total amount of cytochrome P450 then the rate limiting step of a reaction? If so, in view of all the differences described above, one would expect to find large variations in the cytochrome P450 content of different species, strains etc. Several workers have correlated changes in cytochrome P450 content with changes in hydroxylase activity (Orrenius and Ernster 1964; Orrenius et al 1965; Remmer and Merker 1965). However all the variations and differences in drug metabolism described above cannot be explained on the grounds of varying amounts of cytochrome P450 (Remmer et al 1968; Kuntzman 1969), since the amount of P450 does not vary very much. How then can they be explained? Schenkman et al (1967) have shown that the sex difference in hexobarbitone and aminopyrine oxidation in rat liver is due to a difference in substrate affinity for the cytochrome P450 and to a greater maximum extent of binding of substrate by the microsomes. However, the small sex difference in aniline hydroxylation is due to the 20% difference in cytochrome P450 content. Remmer et al (1968) have extended this idea to cover even wider differences in drug metabolism. However, Davies et al (1969) were unable to find any correlation between the maximum rates of ethylmorphine demethylation by liver microsomes from various species with magnitude of the type I spectral change caused by ethylmorphine and therefore concluded that at least in the case of ethylmorphine the affinity and binding of the drug to cytochrome P450 was not the rate limiting step. They found that the demethylation was most closely related to the rate of reduction of cytochrome P450. This was not the case though with the sex difference in rats, and Gigon et al (1969) have since shown that the limiting step may well be the rate of reduction of the cytochrome P450-substrate complex.
Thus, many factors can influence the metabolism of drugs by microsomal enzymes, and since this in turn can profoundly influence the duration of action and even pharmacological action of a drug, an understanding of them is extremely important. There are, however, two important omissions from the above consideration of the factors affecting drug metabolism. They are the effect of pregnancy and the effect of oral contraceptives. As these topics form the subject of this thesis they are considered more fully in sections B and C of this chapter.
B. The Effect of Pregnancy on Drug Metabolism

It has been shown in the previous section that many factors can influence the metabolism of drugs resulting in altered durations of action or in toxicity. Several reports have appeared over the last fifteen years which suggest that during pregnancy the metabolism of drugs is inhibited, although there do not seem to be many reports in the literature of increased pharmacological effect of drugs during pregnancy, at least in humans. However, Crawford and Rudofsky (1966) have commented that there is in informed clinical circles a slowly-growing impression that pregnancy in the human imposes a modification in the woman's response to drugs, although they do not give any evidence for this.

It is known that pregnancy can influence the activity of certain enzymes which are 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; Pulkinnen and Willman, 1968). Other serum enzymes have been shown to decrease in activity during pregnancy in women; acetylcholinesterase, lactic dehydrogenase and succinic dehydrogenase are all thus affected (Vasiliu et al, 1967). The enzyme system responsible for conjugating bromsulphthalein 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).

During pregnancy there is a large increase in steroid hormones - oestrogens and progestogens - circulating in the blood (Brown, 1956; Short, 1961; Eto et al, 1962; Nicol et al, 1964; Grota and Eik-nes, 1967; Hashimoto et al, 1968; Yannone et al, 1968). Several studies have shown that these steroids are metabolized by NADPH₂-dependent enzyme systems of liver microsomes.
(Conney, 1967) which have many similarities with those that oxidise drugs from which it may be inferred that steroid hormones are naturally occurring substrates for drug-metabolizing enzymes in liver microsomes (Murphy and Dubois 1958; Kuntzman et al 1964, 1965; Tephly and Mannerling 1968; Conney et al 1968; Lange and Thun 1969). If this is the case then it would not be surprising if steroids, and therefore pregnancy, affect drug metabolism.

Conjugations

Cessi (1952) discovered that the conjugation of o-aminophenol with glucuronic acid in the pregnant guinea-pig is about 50% of that in the non-pregnant animal. This could be attributed to the fact that the in vitro conjugation in the liver and kidney was decreased in the pregnant animal. Later Lathe and Walker (1958) showed that conjugation of bilirubin with glucuronic acid by rat liver slices was inhibited by serum from pregnant women and new-born infants, but not by "normal" human serum. The conjugation by rabbit and monkey liver slices was also inhibited, but to a smaller extent. On the suggestion that this inhibition might be due to high steroid levels in the blood, Lathe and Walker (1958) investigated the effect of adding various steroids to liver slices. In the rat (but not rabbit or monkey) liver slices, conjugation of bilirubin was inhibited by pregnane-3α,20α-diol, progesterone and pregnanolone but not by testosterone, cortisone or oestrogens. However, neither pregnant serum nor any of the steroids inhibited conjugation of o-aminophenol with glucuronic acid in liver slices. Although these authors found an inhibitory effect of various steroids or glucuronide conjugation they did not identify the inhibitor present in the "pregnant" serum. This was achieved by Hsia et al (1960) who showed that the
inhibition was primarily due to pregnane-3α-20α-diol and also allo-pregnanetriol and pregnanolone. Several other physiologically important progestational steroids were also shown to inhibit phenolphthalein conjugation by rat liver microsomes (Hsia et al. 1960). These authors, unlike Lathe and Walker (1958), obtained inhibition of glucuronide conjugation by rat liver microsomes by progesterone using o-aminophenol, phenolphthalein and bilirubin as substrates.

Later work by Hsia et al. (1963) has shown that progesterone, pregnanediol and other progestogens decrease the activity of guinea-pig liver glucuronyl transferase in vitro using o-aminophenol, p-nitrophenol and 4-methylumbelliferone as aglycones. Oestriol and testosterone produced a similar effect, but neither oestradiol nor oestrone had any effect. Pregnanediol was the most potent of the inhibitors.

Recently Lauritzen and Lehmann (1967) have shown that administration of oestriol, pregnanediol or cortisone, but not progesterone, to new-born infants, increased the level of unconjugated bilirubin in the blood serum. A similar effect on the suckling infant is produced when the hormones are given to the nursing mother but in this case progesterone does raise the blood bilirubin level. Arias and Gartner (1964) demonstrated that the presence of pregnane-3α, 20β-diol in mothers' milk led to hyperbilirubinaemia and that administration of it to neonates caused raised serum bilirubin levels.

Some of the above information was concerned with evaluating the cause of hyperbilirubinaemia in the new-born infant, but the evidence does seem to suggest that steroid hormones, particularly progestogens, are capable of producing profound effects on glucuronide formation. It would not be surprising, therefore, if
the inhibition of glucuronide formation during pregnancy was attributable to high blood levels of progestogens. However, several conflicting reports have appeared. Pulkkinen and Rauramo (1963) failed to find any inhibition of guinea-pig glucuronyl transferase by the addition of serum from pregnant women to microsomal preparations of the liver. Prolonged administration of progesterone or oestradiol to female dogs dosed with salicylamide does not affect the blood level of the glucuronide (Rauramo et al 1963 a) although women given salicylamide during pregnancy have lower blood levels of the glucuronide than normal (Rauramo et al 1963 b). Hartiala et al (1963) noted, however, that not only is the blood level of salicylamide glucuronide decreased in pregnancy in rabbits, but so is the blood level of the salicylamide itself, and they therefore suggest that there is an altered resorption as well as an altered detoxication function during pregnancy. Lessel and Cliffe (1964), studying the metabolism of aspirin, noted a similar decrease in salicylate concentrations in the blood during pregnancy in rabbits, but not in rats, and suggested that this was due to an increased rate of metabolism brought about by the maternal hormones and cited the work of Booth and Gillette (1962) who showed that some steroid hormones increase the activity of drug-metabolizing enzymes. In fact the steroids used by Booth and Gillette were male sex hormones and the effect was demonstrated in rat liver.

Creaven and Parke (1965) have shown that in pregnant rats glucuronide conjugation of dl-borneol in vivo is lower than in non-pregnant rats. This they attributed to the high level of progestogen present in the blood during pregnancy since they showed that pretreatment of non-pregnant animals with progesterone led to decreased in vivo conjugation of dl-borneol and salicylamide in
rats and rabbits respectively. They also demonstrated that the \(\alpha\)-aminophenol glucuronyl transferase activity of pregnant female rabbit liver microsomes was about two-thirds that of non-pregnant rabbits.

Sulphate conjugation of \(p\)-nitrophenol is lowered during pregnancy in the rat (Pulkkinen 1966). This author suggests this is possibly due to the high levels of oestrogen, since prolonged administration of oestradiol to a rat leads to an inhibition of \(\alpha\)-aminophenyl glucuronide formation in liver slices. Inscoe and Axelrod (1960) had previously shown that oestradiol administration to male rats decreased conjugation of \(\alpha\)-aminophenol with glucuronic acid.

All the above considerations have been concerned with conjugation reactions and have shown that the effect of pregnancy on these reactions, although a little confused by the many variable factors used, e.g. different species, different substrates, appears to be one of inhibition, brought about by inhibition of the glucuronyl transferases of the liver by oestrogenic and/or progestogenic steroid hormones.

**Metabolic Transformations**

The hydroxylation of biphenyl and of coumarin and the metabolism of phenacetin and of aminopyrine by rabbit liver microsomal preparations are all reduced to approximately half the normal values during late pregnancy, (Creaven and Parke 1965). A similar decrease is observed in the hydroxylation of biphenyl (Creaven and Parke 1965) by rat liver during pregnancy. In humans the metabolism of the drugs pethidine and promazine is decreased during pregnancy (Crawford and Rudofsky 1966). This conclusion was based on a study of the pattern of urinary excretion of these drugs and their metabolites. The pregnant subjects (and also neonates) excreted a preponderance of unchanged drug over metabolized, whereas
the converse applied in the non-pregnant. The results therefore suggest that the metabolism of pethidine and promazine is inhibited during pregnancy (and the neonatal period) but do not preclude the possibility that some other cause(s) is responsible for the altered pattern of excretion. It has been suggested, for example, that during pregnancy the oestrogens interfere with the transport of certain materials (e.g. sulphobromophthalein) through the liver cell (Mueller and Kappas 1964). The processes involved in the metabolism of these two drugs are demethylation (of pethidine) and oxidation or hydroxylation (of promazine); the authors suggest that "it would be unreasonable to suppose that these are the only processes of detoxication and metabolic degradation to be influenced by changes in the environmental concentration of progestogen - oestrogen type substances". (Crawford and Rudofsky 1966).

An editorial in the Lancet (1966) on "Pregnancy and Drug Metabolism" criticises Crawford and Rudofsky for using too few subjects, and points out that the variation in excretion was too wide to allow any firm conclusions to be drawn. The pH of the urine was not controlled even though this has been shown to be very important in studies of drug excretion (Milne 1964), especially of pethidine. The editorial concludes that it is not known whether pregnant women are particularly susceptible to pethidine or phenothiazines or not.

The in vitro addition of various steroids to liver preparations has been shown to inhibit several metabolic transformation reactions. Thus it was shown that progesterone at concentrations ranging from $10^{-7}$ to $10^{-3}$M inhibited the metabolism of lysergic acid diethylamide in vitro in male rat liver microsomal preparations (Bergen et al 1962). Other steroids to show this effect included pregnanediol, oestradiol,
cortisol and testosterone. Similarly oestradiol, progesterone and testosterone have been shown to inhibit competitively the N-demethylation of ethylmorphine and the oxidation of hexobarbitone by male rat liver microsomes in vitro, (Tephly and Mannerling 1968). These steroids also inhibited the in vitro oxidation of chlorpromazine but in this case the steroids were less potent inhibitors and the inhibition was not competitive. Juchau and Fouts (1966) demonstrated that progesterone at concentrations of $10^{-5}$ and $10^{-4}$M markedly inhibited in vitro the side-chain oxidation of hexobarbitone, ring hydroxylation of zoxazolamine and p-hydroxylation of aniline in immature male rat liver microsomal preparations. To a lesser degree it also inhibited the ring hydroxylation of 3,4-benzpyrene and the N-demethylation of aminopyrine, but it did not appreciably alter the reduction of the nitro radical of p-nitrobenzoic acid, the reduction of the azo linkage of neoprontosil, the O-demethylation of codeine or the metabolism of promazine. Moreover, pretreatment of immature male rats with a high dose of progesterone 1 or 2 hours prior to killing the animals led to a decrease in in vitro oxidation of hexobarbitone and zoxazolamine but had no effect on the other pathways mentioned above. A similar inhibition was noted twenty-four and thirty-six hours after a high dose of progesterone but between two hours and twenty-four hours the level of activity returned to normal (Juchau and Fouts 1966).

It appears then that metabolic transformation reactions may also be inhibited by pregnancy, and that it is possible that this effect may be produced by high levels of steroids in the maternal blood.

Very recently there have been two reports of impaired drug metabolism in pregnancy. Halac and Sicignano (1969) have shown that conjugation of bilirubin and p-nitrophenol
with glucuronic acid in vitro by dialysed homogenates of rat liver gradually decreases below normal during pregnancy. As they were using dialysed homogenates they concluded that the decreased conjugation was not due to the presence of an inhibitor. Moreover, phenobarbitone administration during pregnancy increased the activity but not quite to the level induced by phenobarbitone in non-pregnant rats. The administration of phenobarbitone during pregnancy was not without deleterious side effects on the foetuses. Feuer and Liscio (1969) have demonstrated that the duration of action of pentobarbitone is increased in pregnant rats above that of non-pregnant rats when dosed on a weight basis. This probably indicates a slower rate of metabolism. King et al (1963) had previously reported a longer duration of action of pentobarbitone in pregnant rats. The hydroxylation of 4-methylcoumarin and the conjugation of o-aminophenol with glucuronic acid by rat liver homogenates are inhibited during pregnancy (Feuer and Liscio 1969). Pretreatment with either 4-methylcoumarin, 3-methylcholanthrene or phenobarbitone induces an increase in the pregnant animals but the level of activity is not raised to that of induced non-pregnant animals. Induction of drug-metabolizing enzymes in the livers of pregnant animals had been reported previously (Inscoe and Axelrod 1960; Pantuck et al 1968) although no inhibition in the untreated pregnant livers was noted. Kato et al (1968 b) have recently reported that the effect of phenobarbitone to increase the activities of microsomal drug-metabolizing enzymes is greater in pregnant rats than in non-pregnant.

These recent observations seem to confirm previous suspicions that drug-metabolism is inhibited during pregnancy. Some studies have indicated that the hormones of pregnancy cause this effect by inhibiting
the drug-metabolizing enzymes in the liver, while one or two studies do not find this to be so. Whether these findings in different species can be applied to humans or not is not known; there certainly do not seem to be many reports of altered quantitative pharmacological effect in pregnant humans.

Before leaving the topic of pregnancy the possible role of the placenta and the foetal liver in drug metabolism should be mentioned. It is known that the placenta plays an important role in the biosynthesis, degradation and conjugation of hormones (Dixon and Willson 1968), and since there is much evidence to suggest that steroids are endogenous substrates for drug-metabolizing enzymes (Kuntzman et al 1964; Kuntzman 1969), it would not be surprising if the placenta was capable of drug metabolism. Moreover drug metabolites are found in the foetal circulation suggesting that metabolites formed in the liver of the mother traverse the placenta, that drugs are metabolized in the placenta, or that drugs are metabolized in the foetus (Van Petten et al 1968). If the placenta and foetus can metabolize drugs this might have an effect on the overall metabolism of a drug by the mother.

It has been established for some time now that the ability to metabolize and conjugate various drugs is low or absent in the livers of foetal and new-born animals (Jondorf et al 1958; Fouts and Adamson 1959; Creaven and Parke 1965; Pantuck et al 1968) and this is associated with a lack of cytochrome P450 (Kato 1966). Until recently, however, the placenta has not been investigated for drug-metabolizing ability. Rabbit placentae were found to be able to metabolize zoxazolamine but not hexobarbitone (Dixon and Willson 1968); similarly no hydroxylation of coumarin or biphenyl was detectable in rat and rabbit placentae (Creaven and Parke 1965).
Van Petten et al (1968) demonstrated that the human placenta was capable of bringing about the oxidation of pentobarbitone, the deamination or hydroxylation of amphetamine or the metabolism of meperidine (but not by demethylation as in the rat liver), but was not capable of demethylating aminopyrine or reducing the nitro group of p-nitrobenzoic acid. Juchau (1969) also found that the human placenta did not reduce p-nitrobenzoic acid but this was not the case with rabbit and rat placentae. It had earlier been reported that the human placenta is capable of reducing azo linkages (Juchau et al. 1968). The presence of cytochrome P450 in microsomal and mitochondrial subfractions of human term placenta has been reported (Meigs and Ryan 1968) and would lend credibility to reports of placental drug-metabolizing activity.

Dixon and Willson (1968) attempted to stimulate placental drug metabolism by treating the pregnant mother rabbits with known inducers of metabolism. Chlordane but not phenobarbitone treatment induced hexobarbitone metabolism in the placentae. Cigarette smoking by pregnant women has been shown to induce the hydroxylation of 3,4-benzpyrene by human placenta and this is attributable to several polycyclic aromatic hydrocarbons present in the smoke since pretreatment of pregnant rats with these leads to increased benzpyrene hydroxylation in the placenta (Welch et al. 1968). Attempts to stimulate foetal liver metabolism by pretreatment with inducers have produced variable results, some producing stimulation (Fouts and Hart 1965; Pantuck et al. 1968) others not, (Dixon and Willson 1968; Feuer and Liscio 1969).

The metabolism of drugs by the placenta and foetal liver at the present time seems to be rather confused. They appear able to metabolize some drugs and not others,
and there are also species differences. Such metabolism that does exist may supplement the decreased ability of the maternal liver to metabolize foreign compounds and drugs. It might be argued that this would be a good thing since it protects the foetus from the pharmacological action of the drug as metabolism usually deactivates the drug. On the other hand, some metabolites are more active or more toxic so that placental metabolism would represent an increased hazard to the foetus. Moreover, metabolites are generally more polar than the parent drug, so as a result of placental metabolism these may get into the foetal tissues and build up to toxic levels since transfer of polar compounds across the placental barrier is more difficult.
C. The Effect of Oral Contraceptive Agents on Drug Metabolism

Gregory Pincus pioneered the development of oral contraception. He originally found that progesterone and progestational hormones could inhibit ovulation. Since that time, and particularly over the last five years, much progress has been made, and nowadays most oral contraceptive formulations contain a progestogen and an oestrogen (the conventional type), although recently oestrogens alone (the sequential type) and progestogen alone (the "minipill") have been used. A detailed review of oral contraception is not warranted in the present context, for this see Tyler (1967), Jackson and Schieden (1968), Diczfalussy (1968), Kalman (1969).

The conventional type of "pill" contains a progestogen and one or other of the two oestrogens, ethinyloestradiol or mestranol. The progestogens fall into two main types, those derived from 19-nortestosterone which include norethynodrel, norethisterone, lynoestrenol, ethynodiol diacetate and norgestrel (a new totally synthetic progestogen, Roland, 1966; O'Roark et al 1966), and those derived from 17α-hydroxyprogesterone which include chlormadinone acetate and megestrol acetate. The first type are closely related to the male sex hormone testosterone, while the second type are more closely related to progesterone (Fig. 1.2). The dosage regime for this type of pill is one daily for three weeks followed by one week without medication.

The sequential formulations consist of oestrogen alone and a mixture of oestrogen and progestogen. For the first fortnight of the woman's cycle the oestrogen alone is taken, then for the next week the mixture is taken, then as with the conventional type medication is stopped for a week.
Fig. 1.2. Types of Progestogen

Type I

![Testosterone](image1)

![Norethynodrel](image2)

Type II

![Progesterone](image3)

![Chlormadinone](image4)

The "minipill" or continuous progestogen type of oral contraceptive contains a small dose of progestogen (at present the two on the market both contain chlormadinone acetate) and is taken every day, without any interruption, even during menstruation.

There is now a wide range of oral contraceptives on the market nearly all of which vary slightly in their content, particularly in their overall oestrogen/progestogen balance. This balance is very important in determining side effects. Whether a "pill" is predominantly progestogenic or oestrogenic cannot be inferred from the milligram dosage since other factors play a part (Today's Drugs, 1968). A list of oral contraceptives currently on the market is shown in Table 1.2, and this also classifies each product according to its oestrogen/progestogen balance.
Table 1.
Oral Contraceptives currently available in the United Kingdom

<table>
<thead>
<tr>
<th>Name</th>
<th>Manufacturer</th>
<th>Progestogen</th>
<th>Dose (mg)</th>
<th>Oestrogen</th>
<th>Dose (µg)</th>
<th>Group</th>
</tr>
</thead>
<tbody>
<tr>
<td>Anovlar</td>
<td>Schering</td>
<td>Norethisterone</td>
<td>4</td>
<td>Ethinyl</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acetate</td>
<td></td>
<td>Oestradiol</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Conovid</td>
<td>Searle</td>
<td>Norethynodrel</td>
<td>5</td>
<td>Mestranol</td>
<td>75</td>
<td>4</td>
</tr>
<tr>
<td>Conovid-E</td>
<td>Searle</td>
<td>Norethynodrel</td>
<td>2.5</td>
<td>Mestranol</td>
<td>100</td>
<td>4</td>
</tr>
<tr>
<td>C-Quens</td>
<td>Lilly</td>
<td>Chlormadinone</td>
<td>1.5</td>
<td>Mestranol</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Demulen</td>
<td>Searle</td>
<td>Ethynodiol</td>
<td>0.5</td>
<td>Mestranol</td>
<td>100</td>
<td>3</td>
</tr>
<tr>
<td></td>
<td></td>
<td>diacetate</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Feminor</td>
<td>London Rubber</td>
<td>Norethynodrel</td>
<td>2.5</td>
<td>Mestranol</td>
<td>100</td>
<td>5</td>
</tr>
<tr>
<td>Gynovlar</td>
<td>Schering</td>
<td>Norethisterone</td>
<td>3</td>
<td>Ethinyl</td>
<td>50</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td></td>
<td>acetate</td>
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* Group classification:
1. Strong progestogen + weak oestrogen
2. Weak progestogen + weak oestrogen
3. Strong progestogen + strong oestrogen
4. Weak progestogen + strong oestrogen
5. Strongly oestrogenic (sequential type)
6. Progestogenic
The mode of action of none of the types of formulation is completely understood. There is evidence that both progestogens and oestrogens inhibit ovulation by suppressing pituitary gonadotropin secretion, as happens during normal pregnancy, and this led Tyler (1964a) to suggest that the use of oral contraceptives leads to a state of metabolic pseudopregnancy. However, it has been found that these hormones prevent conception in doses which are below the gonadotropin suppressing range, so other hypotheses have been suggested but a discussion of these is beyond the scope of this introduction, (Holmes and Mandl, 1962; Diczfalusy, 1968).

However, conception is prevented, it is apparent that the hormones circulating in the blood are responsible, so that the situation resembles that of the pregnant woman. One might, therefore, find similarities between the effect of pregnancy and oral contraception on drug metabolism.

One important point with all present forms of oral contraception is that they are taken regularly and for long periods of time by large numbers of healthy women. It is, therefore, extremely important that they produce very few side effects and are safe to use. Several relatively important effects have been attributed to the use of the pill, so before considering the effect of oral contraceptives on drug metabolism a brief mention should be made of their major side effects. The question of whether the use of oral contraceptives is associated with an increased risk of thromboembolic disease is very controversial and has not yet been resolved, although it does seem that the risk of thromboembolic disease is certainly not as great during oral contraception as during pregnancy. (Swyer 1966; World Health Organisation 1966; British Medical Research Council 1967; British Committee for the Safety of Drugs, 1968; Drill and Calhoun, 1968).
Several reports have appeared which suggest that oral contraceptives also cause liver damage (Eisalo et al 1964, 1965; Palva and Mustala 1964; Cullberg et al 1965; Larsson-Cohn 1965; Stoll et al 1965; Orellana-Alcalde and Dominguez 1966). However, many of these reports are isolated cases and other investigations have found no evidence that the pill causes liver damage (Linthorst 1964; Rice-Wray 1964; Tyler 1964 b). Swyer and Little (1965) note that all the reports suggesting hepatic impairment come from Scandinavian countries where they suggest racial, dietetic or other factors may be important. Orellana-Alcalde and Dominguez (1966) have reported from Chile, however, fifty cases, over a two year period, of cholestatic jaundice associated with the use of oral contraceptives, and this bore a close resemblance to that observed in cholestatic jaundice of pregnancy.

So far there have been few reports of any effects of oral contraception on drug metabolism. Crawford and Rudofsky (1966) at the same time as studying the effect of pregnancy on the metabolism of pethidine and promazine in humans also investigated their metabolism in subjects taking the pill. They found a similar pattern (increased urinary concentration of unmetabolized drug) of excretion to that found in pregnancy and therefore suggested that the metabolism of these drugs is rendered deficient in women taking oral contraceptives.

A very interesting case has been reported by Keeler et al (1964) of a thirty-year-old woman receiving chlorpromazine treatment for schizophrenia who was simultaneously treated for suspected endometriosis with a progestogen-oestrogen type medication similar to one used for oral contraception. Soon after the hormone treatment was commenced the response to the chlorpromazine improved, but on subsequent withdrawal of the hormone
therapy the psychotic manifestations again worsened. From this case it appears that the hormone treatment increased the effectiveness of the chlorpromazine possibly by inhibition of its metabolism.

Little appears to have been done on the effect of oral contraceptive steroids on drug-metabolizing enzyme systems. Following the suggestion of Kuntzman et al (1964) that the same hepatic microsomal enzyme systems are involved in the metabolism of drugs and endogenous steroids, and the finding that several naturally occurring steroids competitively inhibit the N-demethylation of ethylmorpine in vitro (Tephly and Mannering 1964, 1968), Juchau and Fouts (1966) investigated the effect of the synthetic progestogen, norethynodrel, on hepatic microsomal drug-metabolizing enzyme systems of immature male rats. They also studied the effect of the oral contraceptive, Enovid (the American equivalent of Conovid) on the hydroxylation of hexobarbitone by rat liver. Addition in vitro of norethynodrel produced similar results to progesterone (see section B) in that pathways involving hydroxylation or oxidation were inhibited by concentrations of $10^{-4}M$, but little effect was produced on other pathways of metabolism. The nature of the inhibition was competitive. Pretreatment of rats with norethynodrel indicated that it acted like certain other inhibitors of drug-metabolizing enzymes in that an initial inhibition was followed by stimulation (Kato, Chiesara and Vasantelli 1964) and this stimulation remained during chronic treatment. Prolonged treatment with Enovid, however, caused an inhibition of hexobarbitone metabolism in vitro. It should be noted that these effects were only brought about by very high doses of norethynodrel.

Recently it has been shown that the progestogen, medroxyprogesterone acetate, either alone or combined
with ethinyloestradiol, when administered chronically to female rats increases the \textit{in vitro} hydroxylation of aniline, O-demethylation of \textit{p}-nitroanisole and N-demethylation of aminopyrine by liver microsomal preparations (Jori \textit{et al.}, 1969). However, unlike Juchau and Pouts (1966), these authors did not find any increase produced by norethynodrel alone, nor a decrease when given in combination with mestranol.

Rumke and Noordhoek (1969) have also shown that a single dose of lynoestrenol given alone, 48 hours but not 24 or 2 hours beforehand, enhances the elimination of phenobarbitone plus phenytoin from mouse plasma and consequently diminishes their anticonvulsive effect. The hexobarbitone sleeping time is shortened and the \textit{in vitro} metabolism of hexobarbitone is accelerated by the same pretreatment. The authors suggest that these effects may be due to accelerated metabolism of these drugs after lynoestrenol treatment.

The few reports of effects of oral contraceptive agents on drug metabolism to date thus show no consistent trend. One or two reports suggest that, as in pregnancy, there is an inhibition of drug metabolism, while others show the reverse or none at all. It is very likely that the different steroids, particularly the two types of progestogens, produce different effects depending more on the way they are metabolized than on their pharmacological potency as progestogens or oestrogens.

From this introduction it can be seen that the amount of work that has been carried out on the effect of pregnancy and oral contraceptives on drug metabolism is very small. Most of the investigations described have been concerned with the effect of pregnancy on conjugation reactions. The aim, therefore, of the work described in this thesis is to investigate more fully the effect of pregnancy on drug
metabolism both at the enzymic level and in the whole animal. The possible causes of the effect of pregnancy on drug metabolism in the rat are considered, with particular reference to the natural steroids of pregnancy. The effect of oral contraception on drug metabolism has also been investigated in order to determine whether this produces a similar effect to pregnancy.
CHAPTER II

MATERIALS AND METHODS
The materials and methods described in this chapter are all concerned with the study of hepatic microsomal drug-metabolizing enzymes. Separate chapters are devoted to two particular systems – the hydroxylation of biphenyl and the glucuronidation of 4-methylumbelliferone – as these have been studied in more detail. Hexobarbitone sleeping times and the gas chromatography of phenacetin and its metabolites are dealt with in their respective chapters.

Materials

Substrates:
p-Nitrobenzoic acid, (B.D.H. Analar Grade) was used as supplied. The substrate solution used in the enzyme assay was prepared by dissolving 0.835 g. in Tris (Sigma) base solution, then adjusting the pH to 7.6 at 23°C with dilute hydrochloric acid and the volume to 500 ml, such that the concentration of Tris in the final solution was 0.05 M.

Coumarin, (B.D.H.) was recrystallized from water (carbon) and was used as a solution of 1 μmole/ml. in 1.15% w/v KCl solution, m.p. 68°C.

4-Methoxybiphenyl and 4-Ethoxybiphenyl (P.J. Creaven, gifts). Substrate solutions were prepared by dissolving 36.8 and 39.6 mg respectively in 2 g. Tween 80, and diluting the whole to 100 ml with 1.15% w/v KCl. Crystals that deposited on cooling were redissolved by warming before use.

Standards:
p-Aminobenzoic acid, B.D.H., was recrystallized from aqueous ethanol. m.p. 187°C.

7-Hydroxycoumarin (Umbelliferone) Hopkins & Williams) was recrystallized from water (carbon) m.p. 225°C.
Cofactors:
NADP (Sigma), Glucose-6-phosphoric acid, disodium salt (Koch-light), Flavinmononucleotide (Sigma), all used as supplied.

Other reagents:
Folin & Ciocalteu's reagent (B.D.H.) used as supplied.
Potassium chloride (B.D.H. Analar) was used as a 1.15% w/v solution.
Phosphate buffer, both 0.1 M and 0.2 M were prepared as described by Dawson et al (1959) from 'Analar' grade sodium dihydrogen phosphate and disodium hydrogen phosphate.
Carbon monoxide (British Oxygen Company). However, for the majority of Cytochrome P450 determinations 'coal gas' used as a source of CO.

Solvents:
All solvents were obtained from B.D.H. and were used as supplied.

Steroids: All were used as supplied.

- Progesterone
  (Pregn-4-ene-3,20-dione)
- Pregnanediol
  (5β-Pregnan-3α,20α-diol)
- Oestradiol
  (Oestra-1,3,5(10)-triene-3, 17β-diol)
- Oestrone
  3-Hydroxyoestra-1,3,5(10)-trien-17-one
- Oestriol
  (Oestra-1,3,5(10)-triene-3, 16α, 17β-triol)
- Testosterone
  (17β-hydroxyandrost-4-en-3-one)
- Norethynodrel
  (17β-hydroxy-19-nor-17α-pregn-5(10)-en-20-yn-3-one;
  Searle.)
Ethynodiol
(19-Nor-17α-pregn-4-en-20-yn-3β,17-diol): Searle.

Norgestrel
(13β-Ethyl-17-hydroxy-18,19-dinor-17α-pregn-4-en-20-yn-3-one): Schering.

Chlormadinone
(6-Chloro-17α-hydroxypregna-4,6-diene-3,20-dione): I.C.I.

Mestranol
(17α-Ethynyl-3-methoxyoestra-1,3,5(10)-trien-17β-ol): Searle

Norethisterone
(17β-Hydroxy-19-nor-17α-pregn-4-en-20-yn-3-one): Parke-Davis.

Inducing agents:
Phenobarbitone sodium (B.D.H.) Used as supplied
Methylcholanthrene (Sigma) Used as supplied.

Animals
A. Rats Two strains of rat were used, Wistar albino and Black-hooded. During the first year of this work, carried out at St. Mary's Hospital Medical School, only Wistar albino rats were used and they originated from two sources, either Oxfordshire Laboratory Animal Colonies or Allington Farm. The animals were kept in metal boxes with wire mesh floors, there being six to eight in a cage. The rats were fed on Diet 41B (Oxoid) and water ad libitum, and kept in a room on the fifth floor of the medical school along with other animals such as ferrets, rabbits, guinea-pigs and chickens.

During the second two years of this work, carried out at the University of Surrey, both Wistar albino and Black-hooded rats were used, although the latter strain was only used occasionally. The rats were bred in the University animal
house, at ground floor level under controlled heating and lighting conditions. They were all specific pathogen free. The mother rats were littered on wood chippings and the young reared to weaning (4 weeks) on the same bedding before being transferred and maintained in plastic cages, six to eight to a cage on 'Steralit'. The rats were fed on Spiller's autoclaved small animal diet and water ad libitum, and were kept in a room with no other species.

B. Rabbits

Only one strain of this species was used, namely the New Zealand white. At St. Mary's the rabbits were obtained from the same source as the rats and were maintained under similar conditions. At Surrey the rabbits were obtained from Westwood Hybrid Table Rabbits Ltd., Plymouth. They were housed in individual cages in a room with no other animals and were fed on a diet of Dixon's rabbit pellets and water ad libitum.

Preparation of Liver Fractions

The species, strain, sex, supplier, age and weight of the animals used were routinely recorded, and are given in the text where relevant.

Animals were killed by a blow on the head at approximately the same time of day, i.e. 10 a.m. ± 1 hour to avoid differences due to diurnal variation (Radzialowski and Bousquet 1968). The livers were removed rapidly and placed in ice-cold potassium chloride solution (in the case of rabbits the gall bladder was removed), chopped and weighed. The liver, or a portion of it, was then homogenised in a glass homogeniser (diameter 2.4 cm) using a loose fitting Teflon pestle (Potter and Elvehjem, 1936). The pestle was driven by a Black & Decker electric drill, and three up and three down strokes were employed. The homogenate was then diluted to give a
one in four dilution, i.e. 1 ml of KCl solution contained the equivalent of 250 mg of wet weight liver. It was then centrifuged in 50 ml plastic centrifuge tubes in an M.S.E. 'High Speed 18' refrigerated centrifuge at 0°C-4°C at 9,500 r.p.m. (10,000 x g) for ten minutes. The supernatant was carefully decanted and used in most enzyme assays as the '10,000 x g supernatant'. At all times the homogenates were kept cool in ice-water.

For the preparation of the microsomal fraction an aliquot of the 10,000 x g supernatant was centrifuged for one hour in an M.S.E. 'Superspeed 50' in a 10 x 10 ml. angle head at 40,000 r.p.m. giving an average of 105,000 x g in the centre of the tube, at a temperature between 0°C-4°C. The supernatant was carefully decanted, the pellet was rinsed with a few drops of ice-cold 1.15% KCl to remove adhering supernatant fraction, and was then resuspended in the same volume of 1.15% KCl by gentle homogenisation. This constituted the 'microsomal suspension'. Exceptionally the second centrifugation was carried out at 50,000 r.p.m. = 160,000 x g, to bring down the "light" microsomes.

**Determination of the nitroreduction of p-nitrobenzoic acid**

Nitroreductase activity of liver homogenates was measured by following the reduction of p-nitrobenzoic acid to p-aminobenzoic acid using the method developed by Fouts and Brodie (1957) and modified by Gingell (Unpublished). The outline of the method is given below as it is as yet unpublished. The incubation mixture consisted of 10,000 x g supernatant (1.0 ml), p-nitrobenzoic acid substrate solution as described under 'Materials' (2.0 ml), magnesium chloride (25 μmoles), glucose-6-phosphate (25 μmoles), flavinmononucleotide (0.125 μmoles) and NADP (0.25 μmoles) in a total volume of 5.0 ml, made up with 1.15% KCl solution.
The incubation was carried out under nitrogen in wide side arm glass tubes, stoppered with rubber bungs. Oxygen-free nitrogen was fed into the tubes by a needle inserted through the rubber stopper, and flowed out to the next tube via the side arm. The incubation tubes were cooled in iced-water, evacuated and then pre-flushed with nitrogen for ten minutes, after which time they were moved to a Mickel shaking incubator at 37°C and incubated for 30 minutes.

The reaction was terminated by immersing the tubes in ice-cold water and flushing the system with oxygen for one minute, then adding 1.0 ml of 25% w/v trichloracetic acid solution to precipitate the protein. The tubes were centrifuged at 2,000 r.p.m. for ten minutes in an M.S.E. 'Super Magnum' centrifuge at room temperature. 4.0 ml of the supernatant was taken and used in the Bratton and Marshall (1939) test as follows: 0.5 ml of 0.1% freshly prepared sodium nitrite solution was added, the tubes were shaken and allowed to stand three minutes, after which time 0.5 ml of a 0.5% ammonium sulphamate solution was added and the shaking repeated. After a further three minutes, shaking was repeated with 0.5 ml of 0.1% Bratton-Marshall (1939) reagent (N-(naphthyl)-ethylenediamine dihydrochloride). The volume was adjusted to 10 ml with water and after standing for 30 minutes to allow the colour to develop, the optical density was measured in a Unicam SP 500 at 546 nm.

All tubes were run in triplicate; a blank was run adding the substrate after incubation, while standards were run in a similar fashion adding both substrate and standard solution of p-aminobenzoic acid after incubation. Recovery of p-aminobenzoic acid was essentially complete (98± 4%). The calibration curve for the standards is shown in Fig. 2.1.
Fig. 2.1. Standard curve for the determination of p-aminobenzoic acid

![Graph showing the standard curve for the determination of p-aminobenzoic acid.]

- Optical Density
- Concentration of p-aminobenzoic acid (mMolar)

Fig. 2.2. The effect of substrate concentration on the demethylation of 4-methoxybiphenyl by rat liver preparations

![Graph showing the effect of substrate concentration on the reaction rate.]

- Reaction rate (μmoles product formed/g liver/hour)
- 4-Methoxybiphenyl concentration (μMoles per incubation)
Determination of aromatic hydroxylation

The hydroxylating activity of liver homogenates was measured using two systems, namely the hydroxylation of coumarin and of biphenyl. Both systems are found in the rabbit, but only the latter is found in the rat. (Creaven *et al* 1965a, 1965b).

A. The coumarin-7-hydroxylase activity of rabbit liver 10,000 x g preparations was determined essentially as described by Creaven *et al* (1965a). The differences were as follows: nicotinamide was not added to homogenates; NADP concentration in each incubation was raised to 0.5 μmoles; the disodium glucose-6-phosphate was omitted from the incubation; and the incubation was carried out in 15 ml glass-stoppered test tubes (unstoppered whilst incubating).

B. The biphenyl-4-hydroxylase activity of rabbit liver 10,000 x g supernatant preparations was determined as described by Creaven *et al* (1965b). However, for the determination of the activity in rat liver homogenates the method described by Creaven *et al* had to be modified quite appreciably, with consequent reappraisal of many factors. These are described in chapter three; thus only the final method adopted is described here.

Tissue fractions were prepared as described by Creaven *et al* (1965a) except that nicotinamide was not added. The incubation mixture consisted of liver 10,000 x g supernatant 0.5 ml. (≡ 125 mg. of liver), biphenyl 3.0 μmoles (in 10% Tween 80), 0.05 M Tris HCl buffer pH 8.1, 0.5 ml., magnesium chloride 10 μmoles, NADP 1.5 μmoles in a total incubation volume of 2.0 ml. made up with 1.15% KCl solution.

The incubation time was 20 minutes. The method used for the extraction and estimation of 2- and 4-hydroxybiphenyls was similar to that described by
Creaven et al (1965b), the fluorescence being measured in a Baird-Atomic Spectrophotofluorimeter with excitation and fluorescence wavelengths of 311 and 400 nm respectively.

**Determination of O-dealkylation of alkoxybiphenyls**

The dealkylating activity of rat and rabbit liver preparations was measured by following the dealkylation of 4-methoxybiphenyl and 4-ethoxybiphenyl to 4-hydroxybiphenyl (Creaven et al 1966), which was then extracted and estimated as described by Creaven et al. (1965b).

The incubation conditions used for both 4-methoxy and 4-ethoxy biphenyl were as follows: liver 10,000 x g supernatant 0.5 ml., substrate 1.0 μmoles, 0.05 M Tris HCl buffer pH 8.1, 0.5 ml., magnesium chloride 10 μmoles, NADP 0.25 μmole in a total incubation volume of 2.0 ml. made up with 1.15% KCl solution. Incubation time was 20 minutes. The effect of varying substrate concentration was determined as this was not stated before (Creaven et al 1966), and is shown in Fig. 2.2 and Fig 2.3.

The extraction and estimation of the 4-hydroxybiphenyl was as described by Creaven (1965b).

**Cytochrome P450 determination**

The following method for the estimation of cytochrome P450 is based on that described by Sladek and Mannering (1966). 6.0 ml. of 0.2 M phosphate buffer pH 7.4 and a few crystals of sodium dithionite were added to 3.0 ml. of "microsomal suspension". After mixing by inversion, the mixture was poured into each of two cuvettes, and carbon monoxide or coal gas was bubbled through one (the sample) for 30 seconds. The second cell was used as reference. The difference spectra between the two was then traced between 500 and 400 nm. using the dual-beam Pye-Unicam SP 800 spectrophotometer. The difference in absorbance between 490 nm and 450 nm was measured and the cytochrome P450 content calculated using the molar
Fig. 2. 3. The effect of substrate concentration on the 6-ethylolation of 4-ethoxybiphenyl by rat liver preparations.

Fig. 2. 4. Carbon monoxide difference spectrum of rat liver microsomes

Fig. 2. 5. Coal gas difference spectrum of rat liver microsomes
extinction coefficient of 91 cm\(^{-1}\) mM\(^{-1}\) as determined by Omura and Sato (1964a). A typical trace is shown in Fig. 2.4. It was found that there was no difference obtained between the difference spectrum produced whether carbon monoxide or coal gas was used, Fig. 2.5. Coal gas was therefore used routinely for the determination.

**Determination of the glucuronidation of 4-methylumbelliferone**

The glucuronyl transferase activity of rat and rabbit liver preparations was measured using 4-methylumbelliferone as aglycone. The method was developed from that described by Arias et al. (1958) and the development is described in Chapter four. Only the final method adopted is described here.

The primary incubation system was as follows:

- 4-methylumbelliferone 0.6 /umoles
- 0.1 M Tris HCl buffer pH 7.6
- 0.3 ml
- UDPGA 2.0 /umoles
- liver 10,000 x g supernatant, diluted 1 in 20 immediately before use,
- 0.2 ml, made up to a total volume of 1.0 ml with 1.15% KCl.

The incubation was carried out in 20 ml glass-stoppered test tubes (unstoppered for incubation) in a Mickle shaking incubator, 60 oscillations per minute, stroke 3", for 30 minutes. The reaction was terminated by placing the tubes in iced-water and adding 2.0 ml of ice-cold distilled water. 10 ml of chloroform was then added and the tubes shaken in a mechanical shaker for 5 minutes. The tubes were then centrifuged in an M.S.E. Super Magnum centrifuge at 2,000 r.p.m. for ten minutes at room temperature. The aqueous layer was then transferred to a further 10 ml of chloroform — and the shaking and centrifuging repeated. Aliquots of 0.5 ml of the aqueous layer were then incubated for 30 minutes at 37°C in test tubes in a Mickle shaking incubator with 1.0 ml of 0.1 M acetate buffer,
pH 5.0 and 500 units of \( \beta \)-glucuronidase in a total volume of 2.0 ml. Similar aliquots were carried through the same procedure without the \( \beta \)-glucuronidase. After incubation 5.0 ml of 0.5 M glycine buffer pH 10.4, was added to each tube and the fluorescence determined in an Aminco-Bowman Spectrophotofluorimeter or Baird-Atomic Spectrophotofluorimeter, excitation wavelengths being 353 nm and 368 nm respectively, and fluorescence wavelengths 442 and 450 respectively, all wavelengths being instrumental and uncorrected.

The increase in fluorescence following incubation with \( \beta \)-glucuronidase was considered to indicate the amount of 4-methyl-umbelliferone conjugated with glucuronic acid in the initial incubation.

Tubes were run in triplicate. Blanks, comprising substrate added after the initial incubation, and standards of 4-methylumbelliferone glucuronide and 4-methylumbelliferone were run through the procedure. Recoveries and calibration curves are given in Chapter four.

**Protein determination**

The protein concentration of 10,000 \( \times \) g supernatant and microsomal suspensions were determined using a modification of the method described by Lowry et al (1951). In the case of the 10,000 \( \times \) g supernatant, 0.2 ml was diluted to 10 ml with distilled water, while with the microsomal suspension 0.5 ml was diluted to 10 ml. 1.0 ml of the diluted solution was then taken for protein estimation. Four reagents were required as follows:-

A. 2.0% w/v \( \text{Na}_2\text{CO}_3 \) in 0.1N NaOH

B. 2.0% w/v NaK tartrate

C. 1% w/v \( \text{CuSO}_4 \), 5\( \text{H}_2\text{O} \)
1.0 ml each of B and C were mixed immediately prior to use, with 100 ml of A, and 10 ml of mixture was added to 1.0 ml of diluted protein solution.

After mixing and standing for 15 minutes, 0.5 ml of Folin and Ciocalteu's reagent was added and the solutions mixed immediately. After standing 40 minutes the optical densities were read at 750 nm in a Pye-Unicam SP 500. All samples were run in triplicate. A blank of distilled water was used together with standards (1 ml) of serum bovine albumin containing between 50 µg and 500 µg. The standard curve for albumen is shown in Fig. 2.6.

Statistics

All results are expressed as the mean ± standard error of the mean. Significance levels were evaluated using the Student's t test.
CHAPTER III

REAPPRAISAL OF THE METHOD FOR

THE DETERMINATION

OF BIPHENYL HYDROXYLATION IN

RAT LIVER PREPARATIONS
Introduction

Creaven et al (1965 b) have described a method for studying the enzymic hydroxylation of biphenyl by liver microsomal preparations from eleven species, using a fluorescence method for the estimation of the hydroxylation products 2- and 4-hydroxybiphenyl. The method was developed using rabbit liver preparations, and while it has been found to be satisfactory for that species, I have found that several modifications have had to be made to obtain optimal conditions for the in vitro hydroxylation of biphenyl by rat liver preparations. It was therefore necessary to reappraise the method for the determination of biphenyl-4-hydroxylase with rat liver, and these investigations are described in this chapter.

Materials

<table>
<thead>
<tr>
<th>Substance</th>
<th>m.p.</th>
<th>Supplier</th>
<th>Notes</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biphenyl</td>
<td>70°C</td>
<td>[B.D.H.] recrystallized from water</td>
<td></td>
</tr>
<tr>
<td>2-hydroxybiphenyl</td>
<td>57°C</td>
<td>(B.D.H.)</td>
<td>All purified as described by Bridges et al (1965)</td>
</tr>
<tr>
<td>4-hydroxybiphenyl</td>
<td>167°C</td>
<td>(Sigma)</td>
<td>described by Creaven et al (1965b)</td>
</tr>
<tr>
<td>Succinic acid</td>
<td></td>
<td>(B.D.H.) recrystallized from water</td>
<td></td>
</tr>
<tr>
<td>Nicotinamide</td>
<td></td>
<td>(B.D.H.)</td>
<td></td>
</tr>
<tr>
<td>NADP</td>
<td></td>
<td>(Sigma)</td>
<td>used as purchased</td>
</tr>
<tr>
<td>Disodium glucose-6-phosphate</td>
<td></td>
<td>(Sigma)</td>
<td></td>
</tr>
<tr>
<td>Tween 80, polyoxyethylene sorbitan mono-oleate</td>
<td></td>
<td>(Koch-Light) used as purchased.</td>
<td></td>
</tr>
</tbody>
</table>
Experimental

The original incubation conditions described by Creaven were as follows:

- 0.05 M Tris HCl buffer pH 8.6 1.0 ml
- 25% Liver 10,000 x g supernatant (containing 10 µmoles nicotinamide) 1.0 ml
- NADP (0.25 µmole) 0.25 ml
- Biphenyl substrate solution 0.5 ml

In a total volume of 2.7 ml.

This incubation was carried out in 25 ml beakers for 30 minutes at 37°C in a shaking incubator.

These conditions were at first modified to give a total incubation volume of 3.0 ml to allow for the additions of various other factors to the incubation. 20 ml glass-stoppered test tubes (unstoppered during incubation) were then substituted for 25 ml beakers, so that the first extraction into n-heptane could be done directly from the incubation container. Neither of these modifications produced any significant difference in activity to the original conditions.

The remaining modifications involved changing the incubation conditions to obtain the optimal conditions for rat liver preparations. The following conditions were studied: co-factor requirements, optimal period of incubation, substrate concentration, enzyme (homogenate) concentration and pH of the incubation mixture.

Throughout these experiments the termination and extraction procedure used was that used by Creaven et al (1965b).

Co-factor Requirements

A. Effect of NADP concentration

Using the original incubation conditions described by Creaven et al (1965b), and shown under the experimental section of this chapter, the variation of biphenyl hydroxylation by female rat liver with increasing NADP concent-
tration was determined (Table 3.1.A). In order effectively to increase the concentration of NADP without increasing the amount used, the incubation volume was reduced to 2.0 ml, and the effect of varying the NADP concentration on biphenyl hydroxylation again determined (Table 3.1.B).

The 2.0 ml incubation conditions used were as follows:

- 0.05 M Tris-HCl buffer pH 8.6 0.5 ml
- 25% liver 10,000 x g supernatant 0.5 ml
- MgCl₂ solution (50 μmoles/ml) 0.2 ml
- Biphenyl substrate solution (3 μmoles) 0.25 ml
- NADP (varying amounts) in 1.15% KCl 0.2 ml
- 1.15% KCl to a total volume of 2.0 ml

The results are shown in Table 3.1 and are illustrated graphically in Fig. 3.1.

Table 3.1
The effect of NADP concentration on the hydroxylation of biphenyl by rat liver preparations

<table>
<thead>
<tr>
<th>NADP added (μmoles)</th>
<th>Hydroxylase activity in μmoles product formed/g. liver/hour</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>0.0</td>
<td>0.15</td>
</tr>
<tr>
<td>0.25</td>
<td>0.40</td>
</tr>
<tr>
<td>0.5</td>
<td>0.70</td>
</tr>
<tr>
<td>0.75</td>
<td>-</td>
</tr>
<tr>
<td>1.00</td>
<td>1.42</td>
</tr>
<tr>
<td>1.5</td>
<td>2.25</td>
</tr>
<tr>
<td>3.0</td>
<td>3.53</td>
</tr>
<tr>
<td>5.0</td>
<td>3.36</td>
</tr>
<tr>
<td>10.0</td>
<td>1.62</td>
</tr>
</tbody>
</table>

A - Original 3.0 ml incubation conditions
B - 2.0 ml incubation conditions

The same liver homogenate was used in both experiments.
The results are the means of two experiments which did not show any significant differences.

The results show that the optimum level of activity of biphenyl-4-hydroxylase in rat liver 10,000 x g supernatant is reached when the concentration of NADP in the incubation is in the order of 0.75 mMolar, i.e. 1.5 μmole in a 2.0 ml incubation. A concentration of 1.5 μmoles per incubation was therefore chosen to be added routinely.

B. Effect of Glucose-6-Phosphate and Mg^{++}

G-6-P is oxidized by G-6-P dehydrogenase with the concomitant reduction of NADP to NADPH_{2} in the preparation, G-6-P may thus act as a co-factor for the hydroxylation of biphenyl; Creaven et al (1965b) found, in fact, that activity could be restored to rabbit liver microsomes by the addition of G-6-P, NADP and G-6-P dehydrogenase.

Mg^{++} is a known stimulator of many enzyme systems (Terriere and Chan 1969), so the effect of adding this alone and in combination with the G-6-P was tried.

The incubation conditions were as follows:-

<table>
<thead>
<tr>
<th>Component</th>
<th>Concentration</th>
<th>Volume</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.05 M Tris HCl buffer pH 8.6</td>
<td></td>
<td>1.0 ml</td>
</tr>
<tr>
<td>25% rat liver 10,000 x g supernatant</td>
<td></td>
<td>1.0 ml</td>
</tr>
<tr>
<td>Biphenyl substrate solution (3.0 μmoles)</td>
<td></td>
<td>0.5 ml</td>
</tr>
<tr>
<td>NADP (where indicated) 2.5 μmoles in</td>
<td>1.15% KCl</td>
<td>0.2 ml</td>
</tr>
<tr>
<td>G-6-P (0.0, 1.0 or 5.0 μmoles) in</td>
<td>1.15% KCl</td>
<td></td>
</tr>
<tr>
<td>MgCl_{2} (0.0, 5.0 or 10.0 μmoles) in</td>
<td>1.15 KCl</td>
<td>0.3 ml</td>
</tr>
</tbody>
</table>

Total 3.0 ml
**Fig. 3.1.** The effect of NADP concentration on the hydroxylation of biphenyl by rat liver preparations

![Graph showing the effect of NADP concentration on the hydroxylation of biphenyl](image)

Legend:
- A (see Table 3.1)

**Fig. 3.2.** The effect of nicotinamide at different NADP concentrations on the hydroxylation of biphenyl by rat liver preparations

![Graph showing the effect of nicotinamide on the hydroxylation of biphenyl](image)

Legend:
- No Nicotinamide
- 1 mg/ml Nicotinamide
The results of adding G-6-P and Mg\(^{++}\) both in the absence and presence of NADP are shown in Table 3.2.

**Table 3.2**

Effect of G-6-P and Mg\(^{++}\) on the hydroxylation of biphenyl by rat liver preparations

<table>
<thead>
<tr>
<th>Additions</th>
<th>Yield of 4-hydroxybiphenyl (µmoles/g liver/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Mg(^{++}) (µatoms)</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>0</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>0</td>
</tr>
<tr>
<td>10</td>
<td>0</td>
</tr>
<tr>
<td>15</td>
<td>0</td>
</tr>
<tr>
<td>5</td>
<td>1</td>
</tr>
<tr>
<td>10</td>
<td>1</td>
</tr>
</tbody>
</table>

The results are the means of three animals, and did not differ by more than ± 0.02.

The results show, both in the absence and presence of NADP, that G-6-P produces a slight increase in hydroxylating activity, and Mg\(^{++}\) produces a slightly greater increase. The effects are not additive, the increase produced by Mg\(^{++}\) in the presence of G-6-P being the same as that of Mg\(^{++}\) alone. It was decided therefore to add 10 µmoles of magnesium chloride routinely to the standard incubation, but not to add any G-6-P.
C. Effect of Nicotinamide

Nicotinamide has been added in many in vitro studies of liver microsomal mixed function oxidase reactions (Schenkman et al 1967a) to inhibit pyridine nucleotidases (Mann and Quastel, 1941) which break down NADP (Zatman et al 1953) but it does not inhibit the pyrophosphatase which destroys NADPH (Gillette, 1963, Gillette et al 1963). However, in the presence of non-limiting concentrations of NADPH, nicotinamide has been shown to inhibit several rat liver microsomal mixed function oxidases (Schenkman et al 1967a). Creaven et al (1965b) added nicotinamide routinely to liver homogenates before determining biphenyl hydroxylase activity. It was decided, therefore, to investigate the effect of varying NADP concentration in the absence and presence of nicotinamide.

The incubation conditions employed were as follows:

0.05 M Tris HCl buffer pH 8.6 0.5 ml.
25% Rat liver 10,000 x g supernatant 0.5 ml
Biphenyl substrate solution (3.0 μmoles) 0.25 ml.
NADP 1.5 μmoles in 1.15% KCl 0.25 ml.
MgCl₂ solution (50 μmoles/ml.) 0.2 ml.

1.15% KCl to Total of 2.0 ml.

Nicotinamide where used was added to the homogenate immediately after homogenisation, at a concentration of either 1 or 2 mg./ml. homogenate, as indicated.

The results are shown in Table 3.3, and are illustrated in Fig. 3.2.
Table 3.3
Effect of nicotinamide and NADP on the hydroxylation of biphenyl by rat liver preparations

<table>
<thead>
<tr>
<th>Nicotinamide added to homogenate</th>
<th>Hydroxylating activity (μmoles 4-hydroxybiphenyl/g liver/hour) in the presence of the following amounts (μmoles) of NADP</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>0.0</td>
</tr>
<tr>
<td>0</td>
<td>0.34</td>
</tr>
<tr>
<td>1 mg./ml.</td>
<td>1.58</td>
</tr>
<tr>
<td>2 mg./ml.</td>
<td>-</td>
</tr>
</tbody>
</table>

The results are the mean of three experiments, with no significant experimental differences.

The results show that nicotinamide can both enhance or inhibit the hydroxylating activity depending on the concentration of NADP used. At low concentrations of the latter, nicotinamide increases the activity, whereas at high NADP concentrations it inhibits the activity.

Because of the possible unpredictable effect of nicotinamide it was decided not to add it routinely to homogenates, but rather to use higher NADP concentrations.

Optimal Period of Incubation

The rate of production of 4-hydroxybiphenyl was determined under various conditions at incubation times of 10, 20 and 30 minutes. The results are shown in Fig. 3.3 in which A' and A" represent the original 3 ml. incubation conditions but using 1 and 2 μmoles of NADP respectively, and B' represents the revised 2 ml. conditions using 4 μmoles of NADP per incubation.
Fig. 3.3. Effect of period of incubation on the rate of hydroxylation of biphenyl by rat liver preparations.
Effect of Substrate Concentration

The following incubation conditions were employed for the investigation of this parameter:

- 0.05 M Tris HCl buffer pH 8.6
- 25% Rat liver 10,000 x g supernatant
- NADP, 4 μmoles in 1.15% KCl
- KCl, 1.15% solution
- Biphenyl solution in Tween 80 in 1.15% KCl (0.25 - 4.0 μmoles)

Total: 2.0 ml.

The incubation time was 20 minutes.

The results, shown in Table 3.4, indicate that the percentage of substrate hydroxylated was maximal at the lowest substrate concentrations (0.25 μmole/2 ml. of incubation mixture) used, but the maximum yield of 4-hydroxybiphenyl was obtained with 3.0 μmoles of substrate per incubation. This concentration was, therefore, added in the standard procedure.

Table 3.4.

The effect of substrate concentration on the rate of formation of 4-hydroxybiphenyl by rat liver 10,000 x g preparations

<table>
<thead>
<tr>
<th>Substrate Concentration (μmoles/2 ml. incubation)</th>
<th>Rate of formation of 4-hydroxybiphenyl (μmoles/g. liver/hr.)</th>
<th>% of Substrate Hydroxylated</th>
</tr>
</thead>
<tbody>
<tr>
<td>0.25</td>
<td>0.67</td>
<td>22</td>
</tr>
<tr>
<td>0.5</td>
<td>0.92</td>
<td>15</td>
</tr>
<tr>
<td>1.0</td>
<td>1.28</td>
<td>11</td>
</tr>
<tr>
<td>2.0</td>
<td>1.52</td>
<td>6</td>
</tr>
<tr>
<td>3.0</td>
<td>1.92</td>
<td>5</td>
</tr>
<tr>
<td>4.0</td>
<td>1.90</td>
<td>4</td>
</tr>
<tr>
<td>6.0</td>
<td>1.95</td>
<td>3</td>
</tr>
</tbody>
</table>

The results are the means (S.E.M. = 0.05) of three experiments.
Effect of Enzyme Concentration

The incubation conditions used for this investigation were the revised 2.0 ml. ones described under the effect of NADP using 1.5 μmoles of NADP per incubation. The volume of 25% w/v 10,000 x g rat liver supernatant added to the incubation mixture was varied and the volume made up with 1.15% w/v KCl. The results, illustrated in Fig. 3.4, show that the hydroxylating activity is linearly related to the amount of 10,000 x g liver supernatant added, up to a volume of 0.75 ml. A volume of 0.5 ml. was, therefore, added in the procedure finally adopted; this is equivalent to 125 mg. of liver.

Effect of pH

The conditions for incubation used were similar to those described in the preceding section, the pH of the Tris buffer being varied between 7.7 and 8.7 at 20° C. The results, illustrated in Fig. 3.5, show that the highest activity is obtained in the region of pH 8.0 - 8.2. 0.05 M Tris pH 8.1 was therefore used in the standard procedure.

The Recovery of 4-Hydroxybiphenyl from Enzymic Incubations

Since the incubation conditions had been altered to give an increased production of 4-hydroxybiphenyl, it was thought advisable to check the recovery of the product. For this the final 2 ml. incubation conditions were used omitting the substrate; 1.0 ml. of standard 4-hydroxybiphenyl solution (30 μg/ml. in 5% ethanol) was added to the incubation mixture either before or after incubation. 0.5 ml. of 2N HCl and 0.25 ml. of biphenyl substrate solution were added at the end of the 20-minute incubation period. The comparison of recovery, as indicated by fluorimeter reading, of standard added before and after the incubation is shown in Table 3.5. This also shows the effect, on both the standard and on the enzyme incubation, of refluxing in 3N HCl for 30 minutes after incubation.
Fig. 3.4. The effect of enzyme concentration on the hydroxylation of biphenyl by rat liver preparations.

Fig. 3.5. The effect of pH on the hydroxylation of biphenyl by rat liver preparations.
Table 3. 5
The Recovery of 4-hydroxybiphenyl from the incubation medium

<table>
<thead>
<tr>
<th>Conditions</th>
<th>Fluorimeter Reading</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Not refluxed</td>
</tr>
<tr>
<td>Standard added before incubation</td>
<td>2.13</td>
</tr>
<tr>
<td>Standard added after incubation</td>
<td>3.43</td>
</tr>
<tr>
<td>Normal enzyme incubation with biphenyl substrate</td>
<td>3.15</td>
</tr>
<tr>
<td></td>
<td>Refluxed with 3N HCl</td>
</tr>
<tr>
<td>Standard added before incubation</td>
<td>3.38</td>
</tr>
<tr>
<td>Standard added after incubation</td>
<td>3.43</td>
</tr>
<tr>
<td>Normal enzyme incubation with biphenyl substrate</td>
<td>2.94</td>
</tr>
</tbody>
</table>

The results show that the recovery of 4-hydroxybiphenyl, added before the incubation is started, is less than that added after incubation, and that acid hydrolysis restores the recovery to normal. These results suggest that the 4-hydroxybiphenyl is being conjugated, and since bacterial β-glucuronidase added to the incubation prevents the decreased recovery (Franklin 1969) it is suggested that the glucuronide is formed. The results also show that acid hydrolysis of a normal enzyme incubation does not lead to greater amounts of free 4-hydroxybiphenyl indicating that 4-hydroxybiphenyl formed during the normal enzyme incubation is not further metabolized by conjugation. Franklin (1969) has similarly shown that addition of bacterial β-glucuronidase to the enzyme incubation does not lead to an increased production of 4-hydroxybiphenyl. The recovery of 4-hydroxybiphenyl from incubations carried out under the revised conditions is no different from
that found by Creaven et al (1965b), and is therefore satisfactory.

**Summary of revised conditions for assay of liver activity**

The revised assay procedure for rat liver biphenyl-4-hydroxylase activity is, therefore, as follows:

**The incubation conditions:**

- 0.05 M Tris buffer pH 8.1 0.5 ml.
- 25% Liver 10,000 x g supernatant fraction - no nicotinamide 0.5 ml.
- MgCl₂ 10 µmoles
- NADP 1.5 µmoles in 1.15% KCl 1.0 ml.
- Biphenyl 3 µmoles
- Tween 80 10 mg.

In a total incubation volume of 2.0 ml.

Incubation period was for 20 minutes at 37°C.

The termination of reaction and extraction and determination of 4-hydroxybiphenyl was carried out as described by Creaven et al (1965b).

**Comparison between Creaven's method and that devised here.**

It has been shown here that the biphenyl-4-hydroxylase activity of rat liver preparations has different optimum requirements to that of rabbit liver (Creaven et al (1965b)).

The optimum concentration of NADP required for rat liver in the incubation medium is 0.75 mM. Thus the concentration of NADP (0.1 mM) used by Creaven et al (1965b), when studying the activity of rat liver preparations was far below optimal. These authors were, however, adding nicotinamide routinely to the homogenate, even though in fresh rabbit liver preparations the nicotinamide produced an inhibiting effect. However, in the rat I have shown that at low NADP concentrations
(i.e. the NADP is a limiting factor) nicotinamide has a stimulating effect. However, when the NADP concentrations are high, such that they are no longer limiting, the effect of nicotinamide is inhibiting. This agrees with the findings of Schenkman et al. (1967a) on the effect of nicotinamide on aminopyrine demethylation. Although nicotinamide stimulated hydroxylation of biphenyl at sub-optimal NADP concentrations, it was not possible to obtain maximal activity with a mixture of nicotinamide and NADP. Maximal activity was obtained with a minimum NADP concentration of 0.75 mM. These findings could thus account for the higher activity obtained by Creaven et al. for rat liver, using 0.25 μmoles NADP, which was 1.2 μmoles per gm. liver per hour, compared to the 0.4 μmoles per gm. liver per hour found here for 0.25 μmoles NADP and 3.5 μmoles per gm. liver per hour for 3.0 μmoles NADP.

The finding that G-6-P has very little effect on the hydroxylation of biphenyl in vitro is similar to that found by Creaven et al.

Mg²⁺ stimulates some, but not all, microsomal mixed function oxidases (Terriere and Chan, 1969) and is, therefore, added routinely in many assays. It has been shown here to increase the activity of biphenyl-4-hydroxylase to a small extent. Creaven et al. did not investigate the effect of Mg²⁺.

It is also shown here that the specific activity of rat liver preparations during the time course of the hydroxylation of biphenyl decreases when the old conditions of incubation are used, (see Fig. 3.3). Gram and Pouts (1966), using aminopyrine demethylation, have shown a similar decrease in rat liver preparations with time, although this does not occur in the liver of rabbits or mice. A similar species difference appears to exist with the stability of the enzymic hydroxylation of biphenyl. This is probably due to the more rapid breakdown of NADPH₂ in rat liver.
The optimum substrate concentration for the hydroxylation of biphenyl by rat liver preparations is $1.5 \times 10^{-3}\text{M}$ which is slightly less than for rabbit liver ($2 \times 10^{-3}\text{M}$).

The optimum pH for rat liver biphenyl-4-hydroxylase was found to be 8.1 compared with 8.6 for rabbit liver.

As a final step in the comparison of the two methods the activity of rabbit liver preparations was compared using the old and the new incubation conditions, and also using different concentrations of NADP and the old conditions of incubation. Those of the former were as follows: old conditions, 5.45, new conditions 4.06 μmoles 4-hydroxybiphenyl produced/gm.liver/hour, these results being the mean of two animals. The results of the latter are shown in Table 3.6.

**Table 3.6**

The effect of NADP concentration on the hydroxylation of biphenyl by rabbit liver preparations

<table>
<thead>
<tr>
<th>NADP added (μmoles)</th>
<th>Rate of formation of 4-hydroxybiphenyl (μmoles/gm.liver/hr)</th>
<th>Plus nicotinamide 1 mg/ml</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>No nicotinamide</td>
<td>Plus nicotinamide 1 mg/ml</td>
</tr>
<tr>
<td>0.0</td>
<td>1.01</td>
<td></td>
</tr>
<tr>
<td>0.25</td>
<td>2.91</td>
<td>5.45</td>
</tr>
<tr>
<td>0.5</td>
<td>4.08</td>
<td>5.88</td>
</tr>
<tr>
<td>1.0</td>
<td>5.88</td>
<td>5.88</td>
</tr>
<tr>
<td>2.0</td>
<td>6.09</td>
<td>5.45</td>
</tr>
<tr>
<td>4.0</td>
<td>5.88</td>
<td>5.03</td>
</tr>
<tr>
<td>8.0</td>
<td>4.61</td>
<td></td>
</tr>
</tbody>
</table>

The original 3 ml. conditions were used.
These results show that in the rabbit the conditions described by Creaven et al (1965b) give a greater enzyme activity than the optimum method for the rat described here. They also show that the conditions devised by Creaven with respect to NADP and nicotinamide, i.e. 0.25 μmoles of NADP plus nicotinamide, give almost maximum results.

Hydroxylation of biphenyl in the 2-position.

Creaven et al (1965b) found no appreciable (<0.1 μmoles/gm.liver/hour) hydroxylation of biphenyl in the 2-position by liver preparations from adult rats, although there was activity in liver preparations from young rats (0.3 μmoles/gm.liver/hour). Using the revised conditions for 4-hydroxylation and the method described by Creaven et al (1965b) for the determination of 2- and 4-hydroxybiphenyls in the same mixture, the 2-hydroxylation of biphenyl by rat liver preparations has been re-investigated. No appreciable (<0.1 μmoles 2-hydroxybiphenyl/gm.liver/hour) 2-hydroxylation was obtained in liver preparations from adult rats. In four determinations on liver preparations from young rats the extent of 2-hydroxylation ranged from 0.41 to 0.88 μmoles 2-hydroxybiphenyl formed/gm.liver/hour. The results, therefore, agree qualitatively but not quantitatively with those obtained by Creaven et al (1965b).

Induction of hydroxylation with phenobarbitone

Since the concentration of NADP was found to be a critical factor in determining the hydroxylating enzyme activity, it was decided to investigate the effect of varying NADP concentrations on the biphenyl-4-hydroxylase activity of the livers of phenobarbitone-pretreated rats. Table 3.7 shows the results from male Black-hooded rats pretreated daily for three days with a dose of 40 mg./kg. i.p. of phenobarbitone sodium.
The results show that at the level of 1.5 μmoles NADP per incubation both the control and pretreated liver preparations show maximal activity. However, the percentage induction at different NADP levels is altered considerably. It is possible that at such high rates of activity some other factor, such as substrate concentration, becomes limiting. Alternatively phenobarbitone pretreatment might increase the concentration of NADP in the liver either directly or indirectly, for example by a more rapid regeneration of NADPH due to the enzymes involved in this reaction being induced as well, (Ernster and Orrenius 1965).

Table 3. 7
The effect of NADP concentration on the biphenyl-4-hydroxylase activity of the livers of phenobarbitone pretreated male rats

<table>
<thead>
<tr>
<th>NADP added (μmoles)</th>
<th>Activity μmoles/g.liver/ hr.</th>
<th>% Induction</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Pretreated</td>
</tr>
<tr>
<td>0.25</td>
<td>0.95</td>
<td>3.62</td>
</tr>
<tr>
<td>0.75</td>
<td>3.23</td>
<td>7.24</td>
</tr>
<tr>
<td>1.50</td>
<td>4.78</td>
<td>7.83</td>
</tr>
<tr>
<td>3.00</td>
<td>4.37</td>
<td>7.32</td>
</tr>
</tbody>
</table>

These findings emphasize the importance of standardizing conditions when undertaking this kind of experiment, since one may obtain varying results with varying conditions, e.g. in this example if the NADP concentrations were not exactly defined one may obtain as much as a four-fold difference in amount of induction.
Conclusion

The results obtained in this chapter show that the optimum conditions for the determination of the hydroxylation of biphenyl by rat liver microsomal preparations differ from the optimum conditions for rabbit liver microsomal preparations as previously determined by Creaven et al (1965b). As a result a revised system has been devised for determining the hydroxylation of biphenyl by rat liver microsomal preparations. The details of this system are given in Chapter two.
CHAPTER IV

DEVELOPMENT AND INVESTIGATION OF
4-METHYLUMBELLIFERONE GLUCURONYL TRANSFERASE
DETERMINATION IN RAT AND RABBIT LIVER PREPARATIONS
Introduction

Many substrates have been used for determining the activity of glucuronyl transferase in various tissues. The most common are γ-aminophenol, p-nitrophenol and phenolphthalein (Dutton 1966). All these procedures are based on colorimetric determinations either of the product or of the decrease in substrate concentration. The estimation of glucuronyl transferase using 4-methylumbelliferone (4-MU) as substrate is, however, based on fluorimetry, a far more sensitive procedure than colorimetry, and for this reason was used as the method of choice. Since this method is more suitable for tissue which is high in glucuronyl transferase activity per unit of protein, it is particularly suited to use on liver preparations. This method has been used by several workers using different tissues, (Arias 1962, - human liver; Taketa 1962, - mouse liver; Bollet et al, 1959 - rat stomach mucosa) but none appears to have investigated in detail the characteristics of the enzyme using 4-MU as substrate in rat liver preparations.

The method is similar to that devised by Arias (1962) for human liver and is based on the fact that 4-MU is highly fluorescent while its glucuronide is non-fluorescent. After initially incubating 4-MU with uridine diphosphate glucuronic acid (UDPGA) and liver homogenate the mixture is washed with chloroform to denature protein and to extract unconjugated 4-MU without removing the glucuronide. 4-MU is liberated from its conjugate by further incubation with β-glucuronidase (Mead et al 1955) and the subsequent increase in fluorescence is taken as a measure of original glucuronyl transferase activity. The method described by Arias (1962) was found unsatisfactory for rat liver, the chief reason being that a greater amount of
UDPGA is required. It has been reported (Dutton 1966) that rat liver has a high requirement for UDPGA in in vitro determinations of glucuronyl transferase activity because of the rapid breakdown of the coenzyme by pyrophosphatase activity. However, since rats were to be used as the experimental animal in the study of other enzymes it was necessary to use rats for the measurement of glucuronyl transferase. Therefore the characteristics of 4-MU glucuronyl transferase in rats were determined, and the findings are described in this chapter.

Materials

4-Methylumbelliferone (Koch-Light) was re-crystallised as described by Mead et al (1955). The substrate solution for the final standard procedure was prepared by dissolving 35.2 mg of the recrystallised substrate in 100 ml. of 1.15% W/v KCl solution.

4-Methylumbelliferone glucuronide was prepared as described by Woollen and Walker (1965) from acetobromomethylglucuronate and 4-methylumbelliferone. The acetobromomethyl glucuronate was prepared as described by Bollenback et al (1955).

Uridine diphosphate glucuronic acid (UDPGA), uridine diphosphate glucose (UDPG), NAD, NADP (all from Sigma) were used as supplied. Glucuronic acid, ATP, ADP, AMP (all from B.D.H.) were used as supplied. B-glucuronidase (Sigma) prepared from bovine liver had a pH optimum of 5.

Tris buffer 0.1 M pH 7.6 ) prepared as described by Dawson et al (1959)
Acetate buffer 0.1 m pH 4.6 ) prepared as described by Dawson et al (1959)
Glycine buffer 0.5 M pH 10.4 )

Methods and Results

Adult female Wistar albino rats weighing 150-200 g. were used in all experiments unless otherwise stated. Each animal was killed by cervical dislocation after an initial stunning. The liver was immediately removed
and placed in ice-cold 1.15% \( \frac{W}{v} \) KCl solution, then homogenised in a Potter-Elvehjem homogeniser with a Teflon pestle. The homogenate, diluted to give a 1 in 4 dilution, was centrifuged in an MSE High Speed 18 centrifuge at 10,000 x g for 10 minutes. The supernatant from this was used as a source of the glucuronyl transferase.

Where rabbit liver homogenates were used, New Zealand White does were killed by cervical dislocation and the liver homogenised and centrifuged in a similar manner to the rat livers.

Before evaluating the conditions of incubation it was necessary to plot a standard curve for the fluorescence of 4-MU and also to check the recovery of products using authentic 4-MU glucuronide.

**Fluorescence of 4-MU**

A standard curve for the fluorescence of 4-MU was produced by measuring the fluorescence of varying amounts (0.001 - 0.01 \( \mu \)moles) of 4-MU dissolved in 2.0 ml of the acetate buffer, diluted with 5.0 ml of the glycine buffer, in a 1 cm\(^2\) quartz cuvette in either an Aminco-Bowman or a Baird Atomic spectrophotofluorimeter. Concentration of 4-MU plotted against fluorescence intensity gave a straight line (Fig. 4.1). The excitation wavelengths were 368 and 353 nm and fluorescence was measured at 450 and 482 nm (instrumental readings) in the Baird and Aminco fluorimeters respectively.

**Recovery of 4-MU Glucuronide**

The amount of \( \beta \)-glucuronidase required to hydrolyze an amount of 4-MU glucuronide in excess of that produced in the first incubation was determined. A solution of 0.5 ml containing 0.01 \( \mu \)moles of 4-MU-glucuronide was incubated with 1.5 ml of the acetate buffer containing
varying amounts of β-glucuronidase (50 - 500 units). After 30 minutes incubation at 37°C, 5.0 ml of the glycine buffer was added and the fluorescence determined. Tubes containing 100, 200 and 500 units of β-glucuronidase activity gave similar readings. Therefore 200 units of β-glucuronidase activity was chosen as the quantity to use in future experiments.

The experiment was then repeated keeping the amount of β-glucuronidase constant and varying the amount of 4-MU glucuronide (0.001 - 0.01 μmoles). The results indicated that the fluorescence of 4-MU liberated by the hydrolysis increased in direct proportion to the amount of 4-MU glucuronide added (Fig. 4.2).

The overall recovery of 4-MU glucuronide was determined as follows. 4-MU (0.01 and 0.05 μmoles) was added to 3.0 ml of liver homogenate and co-factors which had been previously incubated at 37°C for 30 minutes (see later). This mixture was washed twice by shaking with 10 ml of chloroform and 0.5 ml of the remaining aqueous phase was incubated at 37°C for 30 minutes with 1.5 ml of the acetate buffer containing 200 units of β-glucuronidase. The fluorescence of the 4-MU liberated showed that at both concentrations the recovery of 4-MU glucuronide was 83%.

The possibility of 4-MU glucuronide formed during the initial incubation being broken down by β-glucuronidase present in the liver was investigated. 4-MU glucuronide was added before the initial incubation, and the extraction carried out as described above. Recovery of 4-MU added prior to incubation was 82% showing that little or no β-glucuronidase was present (or at least was not active under the prescribed conditions.)
**Fig. 4.1.** Standard curve for the fluorescence of 4-methylumbelliferone

![Graph showing fluorescence vs. 4-Methylumbelliferone added (µmoles x 10^3)]

**Fig. 4.2.** Standard curve for the hydrolysis of 4-methylumbelliferone glucuronide by β-glucuronidase

![Graph showing Fluorimeter reading vs. 4-Methylumbelliferone glucuronide added (µmoles x 10^3)]
Determination of 4-methylumbelliferone glucuronide

As a result of the foregoing experiments the following standard procedure was adopted for determining the amount of 4-MU glucuronide formed in the primary incubation. The incubation was stopped by the addition of 2.0 ml of ice-cold distilled water and 10 ml of chloroform. The tubes were shaken vigorously for 10 minutes after which time they were centrifuged at 2000 r.p.m. in an MSE Super Magnum for 15 minutes. The procedure was repeated with a second 10 ml portion of chloroform; the chloroform extracts were discarded. The aqueous layer (0.5 ml aliquots) was incubated with 1.5 ml of the acetate buffer containing 200 units of \( \beta \)-glucuronidase for 30 minutes at 37\(^\circ\)C in a Mickle shaking incubator. Similar aliquots were carried through the same procedure but with the omission of the \( \beta \)-glucuronidase, and these served as blanks. 50 ml of the glycine buffer was added and the fluorescence determined in either an Amino-Bowman or Baird Atomic Spectrophotofluorimeter. The increase in fluorescence following incubation with \( \beta \)-glucuronidase was taken to represent the amount of 4-MU conjugated with glucuronic acid in the initial incubation.

Primary Incubation Conditions

A. Rat The incubation conditions first used were similar to those described by Bollet et al. (1959) and are given below:

\[
\begin{align*}
0.1 \text{ M Tris buffer pH 7.6} & \quad 0.5 \text{ ml} \\
4\text{-MU} & \quad 0.2 \mu\text{moles} \\
\text{UDPGA} & \quad 0.2 \mu\text{moles} \quad \text{in 1.15\% KCl} \\
\text{Magnesium chloride} & \quad 50 \mu\text{moles} \\
\text{Liver 10,000 x g supernatent diluted 1 in 10} & \quad 0.5 \text{ ml} \\
\text{Total volume} & \quad 2.2 \text{ ml}
\end{align*}
\]
The incubation was carried out in 20 ml glass-stoppered test tubes (unstoppered during incubation) at 37°C for 30 minutes in a Mickle shaking incubator. These incubation conditions did not give much glucuronyl transferase activity, and so it was decided to investigate them in more detail.

**Effect of UDPGA**

The effect obtained by varying UDPGA concentration was first studied using the conditions described above. A linear response in activity was obtained even when the amount of UDPGA added to the incubation mixture was as high as 5 μmoles. The incubation conditions were therefore modified by decreasing the total volume from 2.2 ml to 1.0 ml, thereby increasing the effective concentration of UDPGA; the new conditions were:

- 0.1 M Tris buffer pH 7.6: 0.3 ml
- Magnesium chloride, none
- 4-MU: 0.6 μmole
- UDPGA: different amounts in 1.15% KCl: 0.5 ml
- Liver 10,000 x g supernatant diluted 1 in 10: 0.2 ml
- Total volume: 1.0 ml

Using these conditions the optimum concentration of UDPGA was found to be 5 μmoles per incubation (Fig. 4.3). This concentration of UDPGA is very high, and since it is very expensive methods were sought to reduce the amount required (see later). These results were obtained at St. Mary's Hospital Medical School using female Wistar albino rats obtained from Oxfordshire Laboratory Animal Colonies. However, on moving to the University of Surrey where the Wistar albino rats were bred, the concentration of UDPGA required for optimal 4-MU glucuronyl transferase activity was found to be 2.0 μmoles per incubation. This concentration of UDPGA was considered to be
economically viable, in view of the fact that introducing other cofactors (see later) might introduce other variables, and was therefore adopted in the standard procedure for the assay.

Using female Black-hooded rats the optimum concentration of UDPGA, using the 1.0 ml incubation conditions described above, was found to be in the order of 0.5 μmoles per incubation, which is considerably less than required for the Wistar albino rats.

**Effect of substrate concentration**

The conditions of the incubation for investigating this parameter, using Wistar rats at Surrey, were:

- 0.1 M Tris buffer pH 7.6 0.5 ml
- UDPGA 1.0 μmole
- 4-MU different amounts in 1.15% KCl 1.2 ml
- Liver 10,000 x g supernatant diluted 1 in 10 0.5 ml
- Total volume 2.2 ml

The effect of varying the substrate concentration from 0.01 μmoles to 2.0 μmoles per incubation is shown in Fig. 4.4.

At the time, the determination was carried out using sub-optimal levels of UDPGA. The determination was therefore repeated using two concentrations of 4-MU, namely 0.3 and 0.6 μmoles per 1.0 ml incubation mixture as described above, to which varying amounts of UDPGA were added. The levels of enzymic activity were the same at the two substrate concentrations. A substrate concentration of 0.3 μmoles per incubation was therefore chosen for use in the standard procedure finally adopted. The apparent Michaelis-Menten constant for rat liver microsomal glucuronyl transferase with respect to 4-MU was calculated to be 5 x 10^{-5} M.
Fig. 4.3. Effect of UDPGA concentration on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations.

Fig. 4.4. Effect of substrate concentration on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations.
Effect of Magnesium

The effect of Mg\textsuperscript{++} on 4-MU glucuronidation by rat liver was determined using the following incubation conditions:-

- 0.1 M Tris buffer pH 7.6 0.5 ml
- UDPGA 1.0 \mu mole
- 4-MU 1.0 \mu mole
- Magnesium chloride, different amounts
- Liver 10,000 x g supernatant diluted 1 in 10 0.5 ml

Total volume 2.2 ml

Fig. 4.5 shows the effect of Mg\textsuperscript{++} to be inhibitory at higher concentrations (100-250 \mu moles per incubation), and to have no effect at lower concentrations (10-50 \mu moles per incubation). No Mg\textsuperscript{++} was added, therefore, in the standard procedure.

Effect of protein concentration

For this determination the modified conditions described under the effect of UDPGA were used. Amounts of 10,000 x g supernatant (0.25 - 2.0 ml) were diluted to 10.0 ml with 1.15\% KCl solution and 0.2 ml of the resulting dilution used in the determination as the source of enzyme. Fig. 4.6 shows the variation of activity, expressed as \mu moles of 4-MU conjugated per tube per hour, with varying homogenate dilutions. The relationship is linear up to a concentration of homogenate equivalent to about 3.0 mg of liver (wet weight) per incubation. Since rat liver 10,000 x g supernatant contains about 120 mg of protein per g. wet weight, activity is linear with respect to protein concentration up to 0.5 mg protein per ml. of incubation mixture.
Fig. 4.5. Effect of Mg$^{++}$ concentration on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations.

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Fig. 4.6. Effect of enzyme concentration on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations.
In the standard assay procedure finally adopted, therefore, an amount of homogenate equivalent to 2.5 mg of liver was used, i.e. 0.2 ml of the 10,000 x g supernatant diluted twentyfold with 1.15% KCl solution.

**Effect of length of incubation**

The rate of production of 4-MU glucuronide with length of incubation was determined using the incubation conditions described under protein concentration for primary incubation periods of 15, 30, 45 and 60 minutes. Fig. 4.7 shows the rate of production of 4-MU glucuronide with time. The relationship is seen to be linear up to about 45 minutes.

A period of 30 minutes was chosen for the primary incubation time in the standard procedure.

**Effect of pH**

The conditions of incubation were similar to those used in the last section, except that the pH of the Tris buffer was varied between 6.6 and 8.6. The results are shown in graphical form in Fig. 4.8. The highest activity was obtained in the region of pH 7.4 - 7.6. 0.1 M Tris pH 7.6 was therefore used in the standard procedure.

**Effect of other factors**

As mentioned above, with regard to UDPGA concentration, the amount required at St. Mary's for rat liver (5.0 μmoles) was very high. In an attempt to reduce the amount of UDPGA required, in order to reduce the cost, various conditions were altered. Firstly the incubation volume was reduced, and then the effect of adding various compounds to have a sparing effect on UDPGA was investigated. The choice of these compounds was based on a consideration of the fact that the oxidation of UDPG to UDPGA requires NAD (Strominger et al 1957). Thus the effect of adding UDPG, NAD
Fig. 4.7 Effect of length of incubation on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations.

Fig. 4.8 Effect of pH on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations.
and glucuronic acid to the incubation medium was investigated. The effect of several other nucleotides was also investigated.

**UDPG**

UDPG (1.0 μmole) added to the incubation in place of UDPGA resulted in negligible glucuronyl transferase activity. The effect of adding various concentrations of UDPG together with 1.0 μmole of UDPGA is shown in Fig. 4.9. The addition of 0.1 μmole of UDPG per incubation together with 1.0 μmole of UDPGA almost doubled the activity; no further increase was obtained when greater amounts of UDPG were added, and in fact with very high concentrations, an inhibition was obtained. When 2.0 μmoles UDPGA were added with 0.1 μmole of UDPG the activity increased from 29.6 to 44.6 μmoles/g liver/hr., an increase of 50%. With the optimal concentration of UDPGA, i.e. 5 μmoles per incubation, no increase could be produced by the addition of UDPG.

**NAD**

NAD (1.0 μmole) added to the incubation in place of UDPGA resulted in no glucuronyl transferase activity. The effect of adding various concentrations of NAD together with 1.0 μmole UDPGA is shown as Fig. 4.10. The addition of 0.05 μmoles of NAD more than doubled the activity; with higher concentrations of NAD only a slightly greater activity was obtained; no inhibition was produced even when 5.0 μmoles of NAD were added. When the amount of UDPGA added was increased to 2.0 μmoles, 0.2 μmole of NAD increased the activity from 29.6 to 49.4 μmoles/g liver/hr., an increase of 67%. With 5.0 μmoles of UDPGA no increase could be produced in the activity by the addition of NAD. These observations were made with rats at St. Mary's; at Surrey no activation was produced by NAD in the presence of sub-optimal levels of UDPGA.
Fig. 4.9 Effect of UDPG on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations in the presence of UDPGA.

![Graph showing the effect of UDPG on the activity of glucuronyl transferase.]

Fig. 4.10 Effect of NAD on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations in the presence of UDPGA.

![Graph showing the effect of NAD on the activity of glucuronyl transferase.]

Glucuronic Acid

As with the UDPG and NAD, glucuronic acid alone produced no glucuronyl transferase activity. By adding various concentrations together with 1.0 μmole of UDPGA, however, the activity could be increased above that in the presence of UDPGA alone; the results are shown in Fig. 4.11. The addition of 5.0 and 7.5 μmoles of glucuronic acid is shown to produce an increase in the order of 50% in the glucuronyl transferase activity, but 10 μmoles completely inhibited the glucuronyl transferase.

Combinations of UDPG, NAD and glucuronic acid

Various stimulatory concentrations of each of these substances were added in different combinations, both in the presence and absence of added UDPGA. The results are shown in Table 4.1.

Table 4.1

<table>
<thead>
<tr>
<th>Compounds added*</th>
<th>Level of activity / (μmoles substrate conjugated/gm.liver/hour)</th>
</tr>
</thead>
<tbody>
<tr>
<td>UDPGA</td>
<td>UDPG</td>
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<td>-</td>
<td>-</td>
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<tr>
<td>-</td>
<td>+</td>
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<td>+</td>
<td>+</td>
</tr>
</tbody>
</table>

* Amounts added per incubation were UDPGA 1 μmole, UDPG 0.5 μmole, NAD 1 μmole and glucuronic acid 5 μmoles.

/ The results are the mean of two experiments, there being no significant difference between them.
Fig. 4.11. Effect of glucuronic acid on the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations in the presence of UDPGA.
In the absence of UDPGA the only combination to increase the glucuronyl transferase activity was that of UDPG plus NAD. Combinations of UDPG, NAD and glucuronic acid in the presence of UDPGA did not produce additive effects, and in some cases less stimulation was produced by multiple combinations than singly.

Other compounds

Pogell and Leloir (1961) have shown that ATP and UDP-N-acetylglucosamine activate glucuronyl transferases particularly in the rat, possibly by inhibiting the pyrophosphatase activity which contributes to the breakdown of UDPGA. At Surrey several other nucleotides were, therefore, investigated for their effect on the rat liver glucuronyl transferase activity; those used were NADP, AMP, ADP, and ATP. All were used at a concentration of 1.0 µmole per incubation together with 1.0 µmole of UDPGA. None of them produced any stimulatory effect.

B. Rabbit

The optimum conditions of incubation for the determination of 4-MU glucuronyl transferase in rabbit liver preparations were determined using methods similar to those described for the rat. The effect of varying the following parameters was determined: UDPGA, substrate Mg++ and protein concentrations, pH of the media and length of the initial incubation.

In respect of the substrate and protein concentrations, pH of the medium and length of incubations the optimum conditions were found to be similar to the rat. Optimum UDPGA and Mg++ concentrations, however, differed markedly from the rat. The effect of varying Mg++ concentration is shown in Fig. 4.12. There is an initial stimulation followed by gradual fall off to almost complete inhibition. The effect of varying UDPGA
Fig. 4.12 Effect of Mg\(^{++}\) on the activity of 4-methylumbelliferone glucuronyl transferase in rabbit liver preparations.

Fig. 4.13 Effect of UDPGA on the activity of 4-methylumbelliferone glucuronyl transferase in rabbit liver preparations.
concentration is shown in Fig. 4.13. The optimal concentration is in the region of 0.25 μmoles per incubation.

Discussion

The 4-MU glucuronidation by rat liver preparations was found to be low and inconsistent when determined using a method similar to that described by Bollet et al. (1959) for stomach mucosa. The activity could be improved by increasing the concentration of UDPGA, but even by increasing it to 1.5 mM the activity was still not maximal. This is in agreement with the observations of Dutton (1966) that rat liver possesses high pyrophosphatase activity which is responsible for breakdown of UDPGA to glucuronic acid-1-phosphate. Incubation volume was therefore decreased to 1.0 ml to increase effectively the concentration of UDPGA. The optimum concentration of UDPGA was then found to be in the region of 5 mM. With change of laboratories and source of rats, however, the optimum concentration of UDPGA required changed to somewhat less than half of this concentration. By this time the modified method had been developed and further investigation into the cause of this difference was not undertaken. So in view of the high cost of UDPGA, this was considered fortunate. The most likely explanation is that the rate of breakdown of the UDPGA by pyrophosphatase is lower in the Surrey rats. In rabbit liver the pyrophosphatase activity is low (Pogell and Leloir 1961) and this would account for the much smaller amount of UDPGA required for optimum 4-MU glucuronyltransferase activity in the rabbit.

Various other characteristics of the system were investigated although the optimum concentration of UDPGA was not always employed. Thus, for the determinations of pH optimum and effect of Mg++, below optimal
levels were used, but for the effect of homogenate concentration and length of incubation the optimum level was used.

Mg\(^{++}\) did not stimulate the glucuronyl transferase activity in rat liver preparations, although Bollet et al. (1959) and Taketa (1962) found that it did so in stomach mucosa and mouse liver respectively. I also found that Mg\(^{++}\) in low concentration had a stimulatory effect in rabbit liver. The rat liver is perhaps richer in endogenous Mg\(^{++}\) than these other tissues.

The glucuronyl transferase activity was only directly proportional to very small amounts of liver protein. This is almost certainly due to the quenching effect of protein on the fluorescence of the 4-methylumbelliferone released in the second incubation, since insufficient shaking with the chloroform, and therefore insufficient protein precipitation, leads to very low and inconsistent fluorimeter readings. The method is therefore only suitable for small amounts of tissue relatively rich in glucuronyl transferase activity.

The activity was found to be proportional to length of incubation up to three quarters of an hour. This seems rather a long time in view of the fact that the UDPGA is supposedly broken down by pyrophosphatase activity and that there is only a small amount of tissue present. The optimum UDPGA concentration, however, was determined on the basis of a thirty minute incubation so that if the UDPGA is being broken down it will not fall below the optimum level until after this period. The results also show that the enzyme is quite stable under these conditions.

Attempts were made to reduce the amount of UDPGA required in order to reduce the cost. Strominger et al. (1957) showed that guinea-pig liver soluble fractions dehydrogenated UDPG to UDPGA via an NAD-linked enzyme.
In the experiments described here neither UDPG nor NAD added alone stimulated glucuronyl transferase activity; added in combination though they did increase the activity slightly. When added separately with below optimum levels of UDPGA, however, they caused a large increase in activity. It is unlikely, therefore, that they are acting as precursors of UDPGA and thus stimulating activity. Pogell and Leloir (1961) have shown similar effects with addition of UDP-\(\text{N}\)-acetyl-glucosamine or ATP to rat liver microsomes on \(p\)-nitrophenol glucuronyl transferase but were unable to show any effect with UDPG. These authors found that part of the activation of glucuronyl transferase was caused by inhibition of the breakdown of UDPGA to glucuronic acid-1-phosphate, glucuronic acid and uridine by phosphatases, but that there was an additional increase which was unexplained. This unexplained increase manifested itself as an activation of the rat liver glucuronyl transferase in the presence of the optimum concentration of UDPGA. Moreover, only certain nucleotides showed this phenomenon. In the experiments described in this chapter no activation of glucuronyl transferase was produced by UDPG or NAD in the presence of optimum UDPGA concentration. Now it is unlikely that the NAD and UDPG produce their effects by competing with UDPGA for the pyrophosphatase since they produce their effects at very low concentrations and do not produce greater stimulation with increasing concentration. It is also unlikely that the activation is similar to that observed by Pogell and Leloir (1961), since the effects are not additive and no stimulation is observed in the presence of excess UDPGA. It is interesting to note that the effect of NAD in stimulating the glucuronidation was not observed with the rats at Surrey, and the optimum level of UDPGA required was less. The effect of various other nucleotides (NADP, ATP, ADP, AMP)
were also studied at Surrey, and did not stimulate the glucuronidation either. It was thus not possible to follow up the investigation of this stimulation.

The stimulation produced by addition of glucuronic acid can probably be explained on the basis of inhibition of phosphatase activity by product inhibition. Thus, with gradual increase in concentration of glucuronic acid the stimulation produced increases as the breakdown of UDPGA by pyrophosphatase decreases. The abrupt fall in activity to zero is probably attributable to the alteration in pH produced by the high concentration of the acid.

Pogell and Leloir (1961) have also shown that addition of EDTA to rat liver microsomes stimulated the activity of p-nitrophenol glucuronyl transferase. The EDTA inhibits the pyrophosphatase responsible for the breakdown of UDPGA. Addition of EDTA, therefore, seemed a possible method of increasing the effective concentration of UDPGA. However, Miettinen and Leskinen (1963) have shown that EDTA also inhibits β-glucuronidase, so its use in the present method was not possible as this involved a second incubation with β-glucuronidase. By virtue of the same fact it was impractical to add any other β-glucuronidase inhibitors, such as saccharic acid to the primary incubation, although the results show that there was little β-glucuronidase activity present in the hepatic tissue, so that addition of inhibition would have produced little apparent increase in the activity of glucuronyl transferase.

The final method adopted for determining the activity of 4-methylumbelliferone glucuronyl transferase in rat liver preparations is described in Chapter two.
CHAPTER V.

EFFECTS OF PREGNANCY ON HEPATIC DRUG-METABOLIZING ENZYMES IN RATS AND RABBITS
Introduction

Previous studies carried out on the effect of pregnancy on drug-metabolizing enzymes have been described in detail in Chapter one. A brief resume is given here. Cessi (1952) found that the glucuronyl transferase activity of guinea-pig liver preparations from pregnant animals was 50% that of non-pregnant. There is then a large gap in the literature until 1965 when Creaven and Parke (1965) found that in liver preparations from pregnant rabbits the 7-hydroxylation of coumarin, the 4-hydroxylation of biphenyl and the conjugation of o-aminophenol with glucuronic acid were inhibited to the following respective extents; 50%, 83% and 35%, while in pregnant rat liver preparations the hydroxylation of biphenyl was decreased by 60%. Pulkinnen (1966) has since shown that sulphate conjugation of p-nitrophenol by rat liver is decreased in pregnant animals.

Since this work was commenced Halac and Sicignano (1969) have shown an inhibitory effect of pregnancy on glucuronide conjugation of bilirubin and p-nitrophenol by rat liver preparations. They find that during the course of pregnancy the conjugating capacity of the maternal liver towards both substrates gradually declines until delivery, after which it returns to normal within two days.

Feuer and Liscio (1969) have also shown that the effect of pregnancy on rat liver drug-metabolizing enzymes is an inhibitory one. The reactions they studied were the 3-hydroxylation of 4-methylcoumarin, and the conjugation of o-aminophenol with glucuronic acid.

Thus it appears from the literature that the effect of pregnancy on drug-metabolizing enzymes is an inhibitory one.
The work described in this chapter is concerned with evaluating the effect of pregnancy in both rats and rabbits on the activity of hepatic drug-metabolizing enzymes responsible for the following reactions:

- 2- and 4-hydroxylation of biphenyl,
- 7-hydroxylation of coumarin,
- reduction of \( p \)-nitrobenzoic acid to \( p \)-aminobenzoic acid
- and the conjugation of 4-methylumbelliferone with glucuronic acid.

As cytochrome P450 has been shown to play an important role in drug metabolism the levels in livers from pregnant and non-pregnant rats and rabbits have been determined.

In order to obtain some insight into the mechanism of decrease in the activities of drug-metabolizing enzymes in pregnant animals, the effects of phenobarbitone and methylcholanthrene treatment on these enzyme activities were investigated.

Since certain drug metabolites are found in the foetal circulation it is possible that the placenta and/or the foetal liver are sites of drug metabolism (Van Petten et al 1968). If this is so, then the overall capacity of the pregnant mother could be influenced. Therefore, placental and foetal liver preparations were investigated for drug-metabolizing activity using similar substrates and methods as for adult liver preparations.

**Materials and Methods**

The rats used were similar to those described in Chapter two, and weighed between 200 g. and 300 g.

Several methods were used for mating the rats and determining the length of pregnancy. The appearance of copulation plugs was tried but gave
unsatisfactory results as these sometimes fell out or were undetectable. Vaginal smears were also taken in another series every morning, and where sperm were present this indicated day one of pregnancy in that animal. However, this proved a rather laborious, time-consuming task and was abandoned. The method finally adopted was as follows: two male rats were placed in a large cage with four mature female rats during the late afternoon (several boxes were set up at a time); the animals were left together for 36 hours, i.e. until early morning two days later, when the males were removed. This was taken as day one of pregnancy of those animals that were later found to be pregnant. After ten days the females were palpated on the abdomen, and in those that were pregnant the foetuses could be detected. All the animals were placed, at this stage, in boxes on their own. Those that were not pregnant were used as controls (it was sometimes necessary to supplement the number of controls with similar animals that had not been mated). Using this method it was possible to obtain about 60% of the rats pregnant and be able to tell within a day the actual time of mating.

Rabbits were mated using two different techniques. The first, used at St. Mary's, consisted of placing a female rabbit in a buck's cage and watching until mating was achieved. This occasionally took a long time but nevertheless gave a rate of about 80% pregnant; the exact time of mating was thereby known. At Surrey the pregnant rabbits were obtained from Westwood Hybrid Table Rabbits Ltd., Plymouth, and were despatched one week before the expected delivery date. 100% success was obtained by this method, but I had to rely on the word of the breeders as to when mating had occurred,
and also one had to realise that the animals may have been stressed by the travelling while pregnant.

Animals were killed and tissue homogenates prepared as described under methods in Chapter two. The methods used for determining the hydroxylation of biphenyl and coumarin, the reduction of p-nitrobenzoic acid, the conjugation of 4-methylumbelliferone, and cytochrome P450 and protein concentrations in liver preparations are also described in Chapter two.

For studying these parameters in placenta and foetal livers of rats similar methods were used. Placentae or foetal livers from one litter were pooled. Only tissues from almost full-term rats were used, as in early pregnancy these tissues are very small. For preparing microsomal suspensions from the placenta and foetal livers, resuspension of the microsomal pellets was made in less than the original volume in order to give a more concentrated suspension.

Results

1) Rats
   a) Effect on body and liver weights

The first feature to be noted in pregnant rats was the large increase in liver weight that took place, (Table 5.1). This has been noted in the past (e.g. Campbell and Kosterlitz 1949) but does not seem to have been considered in relation to drug-metabolizing enzymes. Enzyme activity results are usually expressed in terms of μmoles product/gm.liver/hour, which does not take into account any alteration in liver weight. This method of expressing results is satisfactory if no alteration in size of the liver occurs. However, if an alteration does occur in liver size, then this must be taken into account and the total activity of the liver expressed as well.
Table 5.1 shows the effect of pregnancy in the rat on the body and liver weights at two different stages of pregnancy. Table 5.1 also shows the increase that takes place in the maternal liver during pregnancy. It appears that the liver reaches its maximum size (or very nearly so) by the 15th day of pregnancy and possibly even earlier. The ratio of liver to body weight is greater in the 15-day pregnant animals than the controls for that group; this would indicate that thereafter the increase in body weight outpaces the increase in liver size.

In the following work, therefore, enzyme results are expressed in terms of μmoles product/gm.liver/hour, i.e. as a function of unit liver weight, and this is referred to as "specific activity", as well as in terms

<table>
<thead>
<tr>
<th>Group</th>
<th>No. of rats</th>
<th>Weight Body (g)</th>
<th>Liver (g)</th>
<th>Ratio of Liver/Body x 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-pregnant (19-20 day</td>
<td>10</td>
<td>237 ± 6</td>
<td>7.9 ± 0.6</td>
<td>3.3</td>
</tr>
<tr>
<td>controls)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant (19-20 days)</td>
<td>10</td>
<td>325 ± 10</td>
<td>10.9 ± 0.4</td>
<td>3.4</td>
</tr>
<tr>
<td>Non-pregnant (15-16 day</td>
<td>6</td>
<td>260 ± 13</td>
<td>7.5 ± 0.8</td>
<td>2.9</td>
</tr>
<tr>
<td>controls)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Pregnant (15-16 days)</td>
<td>7</td>
<td>314 ± 12</td>
<td>10.3 ± 0.9</td>
<td>3.3</td>
</tr>
</tbody>
</table>
of μmoles product/liver/hour, i.e. as a function of total liver weight, and this is referred to as "total activity" since this reflects the animal's total capacity for drug metabolism.

b) **Effect on the hydroxylation of biphenyl.**

Before the assay procedure for the determination of the hydroxylation of biphenyl was revised for rat liver preparations, several experiments were carried out using the original method described by Creaven et al. (1965b). The activity of biphenyl-4-hydroxylase from livers of pregnant and non-pregnant rats is shown in Table 5.2.

**Table 5.2**

<table>
<thead>
<tr>
<th>Non-pregnant</th>
<th>Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver weight (g)</td>
<td>Activity</td>
</tr>
<tr>
<td></td>
<td>μmoles product/ g.liver/ hr.</td>
</tr>
<tr>
<td>7.0</td>
<td>0.44</td>
</tr>
<tr>
<td>9.0</td>
<td>0.46</td>
</tr>
<tr>
<td>8.0</td>
<td>0.24</td>
</tr>
<tr>
<td>7.5</td>
<td>0.36</td>
</tr>
<tr>
<td>8.0</td>
<td>0.77</td>
</tr>
<tr>
<td>9.5</td>
<td>0.32</td>
</tr>
<tr>
<td>8.0</td>
<td>0.54</td>
</tr>
<tr>
<td>8.0±0.3</td>
<td>0.48±0.07</td>
</tr>
</tbody>
</table>

* Omitted from mean values.
The table shows the individual results obtained from seven 20-day pregnant and seven non-pregnant control rats; quite a wide range of results is obtained with both groups and they overlap markedly. The average results for the non-pregnant animals agree very closely with those obtained by Creaven and Parke (0.47 μmoles/gm. liver/hour) (1965). The results for the pregnant rats, however, are considerably different. Creaven and Parke obtained an average value of 0.16 μmoles/gm. liver/hour, which amounted to a 60% inhibition; in the series of experiments described above the mean value for the pregnant animals was 0.36 ± 0.06 μmoles/gm. liver/hr., an inhibition of only 25%, which is not significant. These experiments were carried out (as was discovered afterwards) at below optimal levels of NADP, and therefore it was thought that varying levels of this cofactor in the liver may have accounted for the wide range of values obtained. If this was so, then perhaps the inhibitory effect of pregnancy on hydroxylation was due to a decrease in cofactor levels. The effect of varying the NADP concentration on the biphenyl-4-hydroxylase activity in pregnant rats was therefore determined using the revised conditions of assay. The effect is shown in Fig. 5.1. The illustration, which is the average of three determinations, shows that at all concentrations of NADP, the effect of pregnancy is an inhibitory one, the degree of inhibition being slightly higher at higher concentrations of NADP. The optimal concentration of NADP is similar for both pregnant and non-pregnant animals, so that it can be concluded that the NADP concentration is not a limiting factor of the hydroxylation in the pregnant rat.

The effect of pregnancy in the rat on the hydroxylation of biphenyl is shown in more detail in Tables 5.3 and 5.4. Table 5.3 shows the effect of 19-20 days
Fig. 5. The effect of NADP concentration on the hydroxylation of biphenyl by liver preparations from pregnant rats.
pregnancy, the results being expressed as "specific activity", "total activity" and also in terms of microsomal protein concentration.

Table 5.3
Effect of 19-20 days pregnancy on the hydroxylation of biphenyl by rat liver microsomal preparations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>Percentage Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>228 ± 7</td>
<td>332 ± 7</td>
<td>+ 45%</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>6.9 ± 0.7</td>
<td>10.4 ± 0.4</td>
<td>+ 51%</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (µmoles/gm.liver/hour)</td>
<td>3.34 ± 0.30</td>
<td>2.29 ± 0.30</td>
<td>- 31%</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (µmoles/liver/hour)</td>
<td>23.1 ± 3.7</td>
<td>23.8 ± 3.3</td>
<td>+ 3</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (µmoles/mg.protein/hour)</td>
<td>0.11 ± 0.01</td>
<td>0.07 ± 0.02</td>
<td>- 33%</td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (µmoles/gm.liver/hour)</td>
<td>0.14</td>
<td>0.13</td>
<td>- 7</td>
</tr>
</tbody>
</table>

The results were obtained from 12 animals in each group. Significant differences represented by *= p = 0.01, \( \neq p = 0.001 \).

The results show that there is a significant decrease (31%) in the specific activity of the liver from 19-20 day pregnant rats towards the hydroxylation of biphenyl in the 4-position. There is only very little 2-hydroxylation of biphenyl in both the pregnant and non-pregnant animals. The total capacity of the livers from pregnant animals towards 4-hydroxylation of biphenyl
is shown to be increased slightly above that of the non-pregnant, since the decrease in enzyme activity is accompanied by an increase in liver weight. When the activity is expressed in terms of microsomal protein concentration there is a slightly greater inhibition than of the specific activity since the microsomal protein content of the pregnant liver is slightly greater than that of the non-pregnant (see later).

Later work (described in Chapter seven) on the effect of pregnancy on the duration of action of hexobarbitone showed that the latter was increased considerably in full-term (or nearly full-term) rats, but at fifteen days the action was only prolonged by a slight amount. This suggested that the metabolism of hexobarbitone was not inhibited to such a great extent in 15-16 day pregnant rats as in 19-20 day pregnant rats. It was therefore of interest to evaluate the effect of 15-16 days pregnancy on the drug-metabolizing enzymes described above. By determining the activity of these enzymes at a different stage of pregnancy, it was also hoped to obtain a greater insight into the mechanism of the inhibition. The effect of 15-16 days pregnancy on liver microsomal biphenyl hydroxylation in the rat was determined and the results are shown in Table 5.4.

When the results are expressed as a function of unit liver weight a decrease of 13% in the liver hydroxylase activity of the 15-16 day pregnant rats is obtained, but this is not statistically significant. When, however, the results are expressed as a function of total liver weight the liver hydroxylase activity of the pregnant rats is 17% greater than the non-pregnants. This is expected since the slight decrease in specific activity is accompanied by a considerable increase in liver weight. As there is no alteration in microsomal
Table 5.4

Effect of 15-16 days pregnancy on the hydroxylation of biphenyl by rat liver microsomal preparations.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-Pregnant</th>
<th>Pregnant</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (g)</td>
<td>260 ± 13</td>
<td>312 ± 14</td>
<td>+ 20%</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>7.5 ± 0.8</td>
<td>10.0 ± 1.0</td>
<td>+ 33%</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (μmoles/gm/liver/hour)</td>
<td>4.12±0.36</td>
<td>3.60±0.34</td>
<td>- 13%</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (μmoles/liver/hour)</td>
<td>30.9±5.6</td>
<td>36.0±6.7</td>
<td>+ 17%</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (μmoles/mg/protein/hour)</td>
<td>0.142±0.017</td>
<td>0.018±0.014</td>
<td>- 17%</td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (μmoles/gm/liver/hour)</td>
<td>0.14</td>
<td>0.12</td>
<td>- 14%</td>
</tr>
</tbody>
</table>

The results were obtained from six animals in each group. Significant differences represented by

* p = 0.02, / p = 0.05

protein content at 15-16 days pregnancy, the activity expressed in terms of protein concentration or specific activity show a similar decrease. As with the full-term pregnant animals there is very little 2-hydroxylase activity present.

c) Effect on the protein content of the liver.

The protein content of the 10,000 x g supernatant and of the microsomal suspensions of livers from pregnant and non-pregnant rats were measured. The results are
shown in Table 5.5. The results show there is no difference in the concentration of 10,000 x g supernatant protein, but there is a substantial increase of 57% in total liver content of 10,000 x g supernatant protein in the 19-20 day pregnant rats. There is a slight, but statistically insignificant, increase in the concentration of microsomal protein. The total microsomal liver protein content is increased by 55% in 15-16 day pregnant animals and by 68% in 19-20 day pregnant animals. The results therefore show that the composition of the 'pregnant' liver with regard to protein is proportionally similar to the non-pregnant.

**Table 5.5**

<table>
<thead>
<tr>
<th>Protein</th>
<th>Non-pregnant</th>
<th>15-16 Day Pregnant</th>
<th>19-20 day Pregnant</th>
</tr>
</thead>
<tbody>
<tr>
<td>10,000 x g Supernatant</td>
<td>110.5±1.7 (6)</td>
<td>-</td>
<td>110.4±1.5 (6)</td>
</tr>
<tr>
<td>(mg/gm.liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>10,000 x g Supernatant</td>
<td>696±9.5 (6)</td>
<td>-</td>
<td>1093±15.1 (6)</td>
</tr>
<tr>
<td>(mg/liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal</td>
<td>28.9±1.1 (14)</td>
<td>30.5±1.3 (7)</td>
<td>31.8±1.2 (12)</td>
</tr>
<tr>
<td>(mg/gm.liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal</td>
<td>202.3±13.1 (14)</td>
<td>314.2±22.0 (7)</td>
<td>340.3±18.6 (12)</td>
</tr>
<tr>
<td>(mg/liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

Figures in parentheses represent the number of observations. Statistical difference from controls is indicated by ≠ p = 0.001, ∦ p = 0.01.
d) Effect on reduction of p-nitrobenzoic acid

The pattern of reduction of p-nitrobenzoic acid in pregnant and non-pregnant rat livers is shown in Tables 5.6 and 5.7. In the almost full-term rats the specific activity of the liver, although slightly increased on average, is not significantly different in the pregnant animals from the non-pregnant. When the increase in liver weight during pregnancy is taken into consideration and the total liver capacity expressed, a 60% increase of the pregnant above the non-pregnant is obtained. The activity expressed in terms of protein concentration shows a slight but insignificant increase in activity in the pregnant animals.

The activity in the liver preparations from 15-16 day pregnant rats follows a similar pattern to the 19-20 day pregnant animals.

Table 5.6

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>240 ± 5</td>
<td>345 ± 15</td>
<td>+ 44%</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>8.6 ± 0.5</td>
<td>11.7 ± 0.6</td>
<td>+ 36%</td>
</tr>
<tr>
<td>Specific activity (μmoles/gm. liver/hr)</td>
<td>1.97 ± 0.08(8)</td>
<td>2.32 ± 0.24(8)</td>
<td>+ 18</td>
</tr>
<tr>
<td>Specific activity (μmoles/mg. protein/hr.)</td>
<td>0.072 ± 0.004</td>
<td>0.084 ± 0.013</td>
<td>+ 17</td>
</tr>
<tr>
<td>Total activity (μmoles/liver/hr.)</td>
<td>16.9 ± 1.0</td>
<td>27.1 ± 3.9</td>
<td>+ 60%</td>
</tr>
</tbody>
</table>

The number of animals used in each group was six. Significantly different * p = 0.001; / p = 0.05.
Table 5.7

Effect of 15-16 days pregnancy on the reduction of p-nitrobenzoic acid by rat liver microsomal preparations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>260 ± 13</td>
<td>314 ± 12</td>
<td>+ 21*</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>7.5 ± 0.8</td>
<td>10.3 ± 0.9</td>
<td>+ 37*</td>
</tr>
<tr>
<td>Specific activity (μmoles/gm.liver/hr)</td>
<td>1.68 ± 0.28</td>
<td>2.32 ± 0.24</td>
<td>+ 38</td>
</tr>
<tr>
<td>Specific activity (μmoles/mg/protein/hr.)</td>
<td>0.058 ± 0.009</td>
<td>0.075 ± 0.006</td>
<td>+ 31</td>
</tr>
<tr>
<td>Total activity (μmoles/liver/hr)</td>
<td>12.9 ± 2.5</td>
<td>23.7 ± 2.8</td>
<td>+ 84*</td>
</tr>
</tbody>
</table>

The number of animals used in each group was six.
\* Significantly different p = 0.05

e) **Effect on Cytochrome P450**

The cytochrome P450 content of rat liver microsomes, expressed in terms of μmoles, calculated using a molar extinction coefficient of 91 mM⁻¹ cm⁻¹ (Omura and Sato, 1964a) in non-pregnant and 19-20 day pregnant rat livers, is shown in Table 5.8.
Table 5.8

Effect of 19-20 days pregnancy on the cytochrome P450 content of rat liver microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>Percentage Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>229 ± 6.0</td>
<td>326 ± 16</td>
<td>+ 42 $^*$</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>7.6 ± 0.4</td>
<td>10.7 ± 0.5</td>
<td>+ 40 $^*$</td>
</tr>
<tr>
<td>P 450 content (μmol/gm. liver)</td>
<td>21.2 ± 1.3</td>
<td>15.7 ± 1.0</td>
<td>- 26 $^*$</td>
</tr>
<tr>
<td>P 450 content (μmol/mg.protein)</td>
<td>0.73 ± 0.03</td>
<td>0.52 ± 0.02</td>
<td>- 29 $^*$</td>
</tr>
<tr>
<td>P 450 content (μmol/liver)</td>
<td>161.1 ± 8.8</td>
<td>168.0 ± 6.9</td>
<td>+ 4</td>
</tr>
</tbody>
</table>

The number of animals used in each group was ten. Significantly different $^*$ $p = 0.05$, $^/$ $p = 0.001$

The results show that the cytochrome P450 content seems to follow the pattern of the hydroxylation of biphenyl. The specific activity is reduced by 26% and this is significant. There is no significant difference in the total content of cytochrome P450. The cytochrome P450 and microsomal protein are measured on the same fraction so that any slight variation in specific cytochrome P450 content due to the resuspension process is eliminated. The cytochrome P450 content when expressed in terms of protein concentration is decreased by 29% in the liver microsomes of 19-20 day pregnant rats and the difference is highly significant.

The cytochrome P450 content of the liver microsomes of 15-16 day pregnant rats is shown in Table 5.9.
Table 5.9

Effect of 15-16 days pregnancy on the cytochrome P450 content of rat liver microsomes

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>Percentage Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>260 ± 13</td>
<td>314 ± 12</td>
<td>+ 21%</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>7.5 ± 0.8</td>
<td>10.3 ± 0.9</td>
<td>+ 37%</td>
</tr>
<tr>
<td>P450 content (µmole/gm liver)</td>
<td>20.5 ± 2.6</td>
<td>16.9 ± 1.2</td>
<td>- 17%</td>
</tr>
<tr>
<td>P450 content (µmole/mg protein)</td>
<td>0.70 ± 0.06</td>
<td>0.55 ± 0.02</td>
<td>- 21%</td>
</tr>
<tr>
<td>P450 content (µmole/liver)</td>
<td>146.1 ± 16.1</td>
<td>168.6 ± 9.3</td>
<td>+ 15%</td>
</tr>
</tbody>
</table>

The number of animals used in each group was six.

* Significantly different p = 0.05

The results show that in the liver microsomes from 15-16 day pregnant rats the pattern of cytochrome P450 again follows that of the hydroxylation of biphenyl, the specific content and that expressed in terms of protein concentration being reduced by some 30%, but these decreases are not statistically significant, while the total liver content of microsomal cytochrome P450 is increased by 15%, though this is not significantly different either.

Thus, the cytochrome P450 content of the liver of 15-16 day pregnant animals, expressed per g. of liver, is slightly, although not significantly, lower than non-pregnants, but greater than that of 19-20 day pregnant animals.
f) **Effect on conjugation of 4-methylumbelliferone with glucuronic acid.**

Table 5.10 shows the effect of 19-20 days pregnancy in rats on the hepatic 4-methylumbelliferone glucuronyl transferase activity.

**Table 5.10**

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>240 ± 5</td>
<td>345 ± 15</td>
<td>+ 44</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>8.6 ± 0.5</td>
<td>11.7 ± 0.6</td>
<td>+ 36 *</td>
</tr>
<tr>
<td>Specific activity (μmoles/gm/liver/hr)</td>
<td>105.9 ± 5.8</td>
<td>77.6 ± 8.3</td>
<td>- 27 /</td>
</tr>
<tr>
<td>Specific activity (μmoles/mg. protein/hr.)</td>
<td>3.90 ± 0.45</td>
<td>2.88 ± 0.46</td>
<td>- 26 /</td>
</tr>
<tr>
<td>Total activity (μmoles/liver/hr)</td>
<td>907 ± 68</td>
<td>906 ± 109</td>
<td>0</td>
</tr>
</tbody>
</table>

The number of animals used in each group was six.
Significantly different *p = 0.001, /p = 0.05.

The conjugation of 4-MU with glucuronic acid also shows a similar pattern in 19-20 day pregnant rats to the biphenyl hydroxylation, namely the specific activity is reduced, the total liver activity is unaltered, while expressed in terms of protein concentration the activity is decreased.
g) **Effect of phenobarbitone pretreatment during pregnancy**

Three factors concerned with drug metabolism have been shown to be reduced in the livers of pregnant rats, namely the 4-hydroxylation of biphenyl, the conjugation of 4-MU with glucuronic acid and cytochrome P450. These factors, together with the other two measured - the reduction of p-nitrobenzoic acid and microsomal protein, which are not decreased, are all inducible in normal control rats by pretreatment with phenobarbitone. It was therefore of interest to see whether these factors could be induced in pregnant rats.

The evaluation of this effect was aggravated by the fact that conflicting results were obtained in two sets of experiments carried out. Both sets of results are shown in Table 5.11 (a and b). In Table a, the results are the mean of three rats in each group and the phenobarbitone pretreated were given a single daily injection of 50 mg./kg. of phenobarbitone sodium in normal saline i.p. three days before killing. Animals were killed 24 hours after the last dose. Table b. shows the results obtained from two animals in each group but the phenobarbitone pretreated rats were given phenobarbitone sodium as an 0.1% solution in the drinking water (Marshall & McLean 1969) for the last five days prior to killing. The discrepancy does not arise in the effect of phenobarbitone, but rather in the effect of pregnancy alone. Table 5.11b, shows that 19-20 days pregnancy in these rats did not produce a decrease in the 4-hydroxylation of biphenyl or cytochrome P450 as it had done previously.

The effect had always been one of inhibition although the degree of inhibition varied from 0% - 50%.
Table 5. 11a
Effect of phenobarbitone pretreatment of pregnant and non-pregnant rats on hepatic drug-metabolizing enzymes, cytochrome P450 and microsomal protein.

<table>
<thead>
<tr>
<th>Group A</th>
<th>Non-pregnant</th>
<th>N.P. Pre-treated</th>
<th>Pregnant</th>
<th>Pregnant Pre-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Body weight (grams)</td>
<td>227 ± 6</td>
<td>243 ± 8</td>
<td>321 ± 7</td>
<td>336 ± 8</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>6.0 ± 0.4</td>
<td>8.9 ± 0.7</td>
<td>9.2 ± 0.5</td>
<td>11.2 ± 0.8</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (μmoles/gm. liver/hour)</td>
<td>3.23 ± 0.24</td>
<td>4.81 ± 0.81</td>
<td>2.55 ± 0.31</td>
<td>5.30 ± 0.6</td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (μmoles/gm. liver/hour)</td>
<td>0.15</td>
<td>0.21</td>
<td>0.16</td>
<td>0.23</td>
</tr>
<tr>
<td>P450 (μmoles/gm.liver)</td>
<td>24.5 ± 1.4</td>
<td>33.0 ± 2.5</td>
<td>18.6 ± 1.2</td>
<td>22.0 ± 1.3</td>
</tr>
<tr>
<td>Microsomal Protein (mg/gm.liver)</td>
<td>30.8 ± 1.2</td>
<td>38.5 ± 1.6</td>
<td>32.4 ± 1.1</td>
<td>39.5 ± 1.5</td>
</tr>
</tbody>
</table>
Table 5, lb
Effect of phenobarbitone pretreatment of pregnant and non-pregnant rats on hepatic drug-metabolizing enzymes, cytochrome P450 and microsomal protein

<table>
<thead>
<tr>
<th>Group B</th>
<th>Non-pregnant</th>
<th>Non-pretreated</th>
<th>Pregnant</th>
<th>Pregnant pre-treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>208,220 (214)</td>
<td>191,196 (194)</td>
<td>280,292 (286)</td>
<td>263,296 (280)</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>6.6,6.7 (6.7)</td>
<td>7.1,6.7 (6.9)</td>
<td>10.0,12.4 (11.2)</td>
<td>10.9,10.8 (10.9)</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (μmoles/gm. liver/hour)</td>
<td>3.67,3.85 (3.76)</td>
<td>7.80,7.05 (7.43)</td>
<td>3.95,4.23 (4.09)</td>
<td>7.71,6.86 (7.29)</td>
</tr>
<tr>
<td>Reductase (μmoles/gm. liver/hour)</td>
<td>1.34,1.12 (1.23)</td>
<td>1.89,1.48 (1.69)</td>
<td>1.42,1.62 (1.52)</td>
<td>2.32,2.11 (2.22)</td>
</tr>
<tr>
<td>P.450 (μmoles/gm. liver)</td>
<td>13.0,13.4 (13.2)</td>
<td>40.1,44.3 (42.2)</td>
<td>12.9,13.5 (13.2)</td>
<td>23.2,25.6 (24.4)</td>
</tr>
</tbody>
</table>

Figures in brackets represent the mean of the two determinations.

No immediate cause was apparent although it was noted that the animals used in this set of experiments were younger than any used previously. Nevertheless, both sets of experiments show similar effects of phenobarbitone. The 4-hydroxylation of biphenyl is elevated to the same level by phenobarbitone pretreatment in pregnant and non-pregnant; the percentage increase, at least in the first set, is therefore greater in the pregnant than the non-pregnant - 108% and 49% respectively. The 2-hydroxylation of biphenyl is also induced slightly in both non-pregnant and pregnant animals. The microsomal protein content which normally is increased by phenobarbitone pretreatment, (Conney, 1967), is increased in both the
Cytochrome P450 is raised in both the pregnant and non-pregnant animals by phenobarbitone pretreatment, but the level in the non-pregnant is not attained by the pregnant in either group. The reduction of p-nitrobenzoic acid is increased by phenobarbitone pretreatment in both non-pregnant and pregnant rats.

h) **Effect of methylcholanthrene pretreatment during pregnancy**

Two pregnant and two non-pregnant animals were pretreated with 20 mg/kg of methylcholanthrene daily for three days prior to killing. The effects on liver biphenyl hydroxylation, cytochrome P450 and microsomal protein are shown in Table 5.12.

**Table 5.12**

Effect of methylcholanthrene pretreatment of pregnant and non-pregnant rats on hepatic hydroxylation of biphenyl, cytochrome P450 and microsomal protein.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant controls</th>
<th>Non-pregnant Pretreated</th>
<th>Pregnant Pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Biphenyl-4-hydroxylase (μmoles product/gm.liver/hr)</td>
<td>3.20, 3.60 (3.40)</td>
<td>6.36, 5.96 (6.16)</td>
<td>6.69, 5.15 (5.92)</td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (μmoles product/gm.liver/hr)</td>
<td>0.14, 0.18 (0.16)</td>
<td>2.13, 2.55 (2.34)</td>
<td>2.80, 2.28 (2.54)</td>
</tr>
<tr>
<td>Cytochrome P450 (μmoles/gm.liver)</td>
<td>18.4, 18.6 (18.5)</td>
<td>24.2, 24.7 (24.5)</td>
<td>23.8, 23.6 (23.7)</td>
</tr>
<tr>
<td>Microsomal Protein (mg/gm.liver)</td>
<td>29.9, 30.4 (30.2)</td>
<td>29.1, 30.5 (29.8)</td>
<td>32.1, 29.9 (31.0)</td>
</tr>
</tbody>
</table>

The number of animals used in each group was two.
Although the determinations were carried out in only two animals the results show definite characteristics. Both 2- and 4- hydroxylation of biphenyl and cytochrome P450 are induced in pregnant and non-pregnant rats; microsomal protein remains unchanged by pretreatment.

i) Effect of second pregnancy

To see whether similar effects of pregnancy on drug-metabolizing enzymes were produced in a subsequent pregnancy a group of six rats was allowed to litter and the young were weaned after 4 weeks. Three of the six were again mated and the other three served as controls. 20 days after mating the six animals were killed and the various parameters measured. The results, expressed in terms of unit liver weight, are shown in Table 5.13.

Table 5.13
Effect of second pregnancy on liver microsomal enzymes, cytochrome P450 and microsomal protein in the rat

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>290 ± 9</td>
<td>377 ± 12</td>
<td>+ 30</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>10.6 ± 0.3</td>
<td>12.6 ± 0.5</td>
<td>+ 19</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (μmoles product/gm.liver/hr)</td>
<td>4.20 ± 0.28</td>
<td>3.51 ± 0.31</td>
<td>- 16</td>
</tr>
<tr>
<td>Biphenyl-2-hydroxylase (μmoles product/gm.liver/hr)</td>
<td>0.22</td>
<td>0.17</td>
<td>- 23</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid reductase (μmoles product/gm.liver/hr)</td>
<td>3.22 ± 0.25</td>
<td>3.07 ± 0.12</td>
<td>- 5</td>
</tr>
<tr>
<td>Cytochrome P450 (μmole/gm.liver)</td>
<td>16.7 ± 1.2</td>
<td>14.2 ± 1.1</td>
<td>- 15</td>
</tr>
<tr>
<td>Microsomal Protein (mg/gm.liver)</td>
<td>27.3 ± 1.0</td>
<td>29.7 ± 0.9</td>
<td>+ 9</td>
</tr>
</tbody>
</table>
The rats were, as a result of the first pregnancy, larger and so were the livers. The livers of the pregnant animals did not increase in size to such a great extent as with the first pregnancy. The enzyme activities of the liver followed a similar pattern to those during the first pregnancies, although the effects were not so marked. Thus the hydroxylation of biphenyl and cytochrome P450 were decreased while the nitroreductase and microsomal protein remained virtually unaltered.

2) Rabbits

The drug-metabolizing activity of the livers of pregnant rabbits has not been so extensively investigated. The results that have been obtained in full-term pregnant rabbits are shown in Table 5.14.

The only significant difference between the non-pregnant and pregnant animals is in the hydroxylation of coumarin; in the pregnant rabbit liver this is decreased by 68%. The hydroxylation of biphenyl, p-nitrobenzoic acid reductase, 4-MU glucuronidation microsomal protein and cytochrome P450 show no significant difference between pregnant and non-pregnant rabbit livers when expressed as a function of liver weight. As there is no increase in liver weight in the pregnant rabbit, there is obviously no difference in total liver content of these parameters, except of coumarin-hydroxylating capacity, which is decreased.

Inhibition of the hydroxylation of coumarin was also found in liver preparations from two 14-day and two 21-day pregnant rabbits to the extent of 46% and 55% respectively. The hydroxylation of biphenyl was not inhibited at either of these stages of pregnancy.
Table 5. Effect of pregnancy on hepatic drug-metabolizing enzymes, cytochrome P450 and microsomal protein in the rabbit.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant</th>
<th>Pregnant</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>4.0 ± 0.2(8)</td>
<td>4.5 ± 0.2(5)</td>
<td>+ 13</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>115 ± 11(10)</td>
<td>111 ± 10 (5)</td>
<td>- 3</td>
</tr>
<tr>
<td>Microsomal protein (mg/gm.liver)</td>
<td>22.1 ± 0.9(4)</td>
<td>21.4 ± 1.5(4)</td>
<td>- 3</td>
</tr>
<tr>
<td>Cytochrome P450 (μmoles/gm.liver)</td>
<td>37.3 ± 5.5(4)</td>
<td>36.2 ± 1.6(4)</td>
<td>- 3</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (μmoles/gm.liver/hr)</td>
<td>4.03 ± 0.3(10)</td>
<td>4.59 ± 0.21(5)</td>
<td>+ 14</td>
</tr>
<tr>
<td>Coumarin-7-hydroxylase (μmoles/gm.liver/hr)</td>
<td>1.12 ± 0.15(8)</td>
<td>0.36 ± 0.03(4)</td>
<td>- 68*</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid reductase (μmoles/gm.liver/hr)</td>
<td>1.09 ± 0.24(4)</td>
<td>1.29 ± 0.21(3)</td>
<td>+ 18</td>
</tr>
<tr>
<td>4-MU glucuronyl transferase (μmoles/gm.liver/hr)</td>
<td>161 ± 10 (3)</td>
<td>135 ± 15 (3)</td>
<td>- 16</td>
</tr>
</tbody>
</table>

Figures in parentheses show the number of animals used.

*Significantly different from controls, p = 0.001

The effect of phenobarbitone pretreatment of pregnant rabbits was investigated by dosing two female rabbits with 50 mg/kg of phenobarbitone sodium i.p. for the three days, prior to killing on the penultimate day of gestation. The results, shown in Table 5.15, show that all the enzymes measured were induced in the pregnant and non-pregnant rabbit livers, although not all to the same extent.
Table 5.15

Effect of phenobarbitone pretreatment of pregnant and non-pregnant rabbits on hepatic drug-metabolizing enzymes, cytochrome P450 and microsomal protein

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Non-pregnant pretreated (mean of 3 animals)</th>
<th>Pregnant (mean of 2 animals)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (kg)</td>
<td>3.2 ± 0.2</td>
<td>4.3, 4.5</td>
</tr>
<tr>
<td>Liver weight (g)</td>
<td>105 ± 9</td>
<td>103, 100</td>
</tr>
<tr>
<td>Microsomal protein (mg/gm.liver)</td>
<td>25.2 ± 1.4</td>
<td>25.7, 26.0</td>
</tr>
<tr>
<td>Cytochrome P450 (µmole/s/gm.liver)</td>
<td>85.4 ± 4.1</td>
<td>58.1, 77.0</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylase (µmole/gm.liver/hour)</td>
<td>5.88 ± 0.09</td>
<td>6.55, 8.55</td>
</tr>
<tr>
<td>Coumarin-7-hydroxylase (µmole/gm.liver/hour)</td>
<td>1.52 ± 0.2</td>
<td>0.93, 0.97</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid reductase (µmole/gm.liver/hour)</td>
<td>1.92 ± 0.26</td>
<td>2.60, 2.28</td>
</tr>
<tr>
<td>4 MU glucuronyl transferase (µmole/gm.liver/hour)</td>
<td>260 ± 21</td>
<td>149, 163</td>
</tr>
</tbody>
</table>

3) Role of the placenta

Using conditions similar to those used for investigating drug-metabolizing enzyme systems in liver preparations, no detectable activity was found in placental homogenates (25% w/v) from 20-day pregnant rats in the following systems: - 2- and 4-hydroxylation of biphenyl (4 observations) and reduction of p-nitrobenzoic acid (3 observations), but conjugation of 4-MU with glucuronic acid was detected at a low rate, 0.7 µmoles/gm. placenta/hour (4 observations) which in terms of total
placental activity is 0.4% of the total activity of the maternal liver. The protein content of the placental microsomal fraction (4.5 mg/gm. wet weight placenta) was, however, much lower than that of the liver microsomal fraction (25-30 mg/gm wet weight liver). More concentrated placental homogenates (up to 60% w/v) were then used in the enzyme determinations, but again no hydroxylation or nitro-reduction could be detected.

No cytochrome P450 (5 observations) could be detected in microsomes resuspended in the original volume of KCl solution (this gave a protein concentration of about 0.4 mg per ml in the cytochrome P450 determination). Placental microsomes were, therefore, resuspended in smaller volumes of KCl solution to give increased concentration of protein. The highest protein concentration used was 6.5 mg per ml in the cytochrome P450 determination, but even then no cytochrome P450 could be detected.

Similar determinations were carried out on placentae from pregnant rats that had been pretreated with phenobarbitone (2 animals) or methylcholanthrene (2 animals) as previously described. Again no hydroxylation of biphenyl or cytochrome P450 could be detected, and the conjugation of 4-MU with glucuronic acid was not induced above the control level.

4) Role of the foetal liver

Foetal liver preparations from control, phenobarbitone and methylcholanthrene pretreated 20-day pregnant rats were investigated for drug-metabolizing activity and cytochrome P450 content. The results are shown in Table 5. 16.
Table 5.16

Drug-metabolizing enzyme activity and cytochrome P450 content of foetal livers from normal, phenobarbitone and methylcholanthrene treated pregnant rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Phenobarbitone treated</th>
<th>Methylcholanthrene treated</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylation of biphenyl (μmoles/gm. foetal liver/hour)</td>
<td>0.1(2)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Reduction of p-nitrobenzoic acid (μmoles/gm. foetal liver/hour)</td>
<td>0.1(3)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Conjugation of 4-MU with glucuronic acid (μmoles/gm. foetal liver/hour)</td>
<td>9.1(3)</td>
<td>6.4(2)</td>
<td>8.4(1)</td>
</tr>
<tr>
<td>Cytochrome P450 (μmumoles/mg. microsomal protein)</td>
<td>0.02 (3)</td>
<td>0.08(2)</td>
<td>0.08(1)</td>
</tr>
</tbody>
</table>

The results show that there is no appreciable hydroxylation of biphenyl, reduction of p-nitrobenzoic acid or cytochrome P450 in the foetal livers of control rats. There is, however, a small amount of conjugation of 4-MU with glucuronic acid but expressed as a percentage of the total capacity of the mother this is only 1.0%. Pretreatment of the mother rats with phenobarbitone or methylcholanthrene does not affect the conjugation of 4-MU with glucuronic acid in foetal liver, but it appears to induce the formation of cytochrome P450, to a slight extent.
However, the total amount of cytochrome P450 represents only 0.1% of the total amount in the maternal liver.

**Discussion**

The study of the effect of pregnancy in the rat on the hydroxylation of biphenyl showed a similar qualitative effect to that described by Creaven and Parke (1965), namely one of inhibition. In quantitative terms, though, the effect was different; I found only 25% inhibition of hydroxylation using a similar method to Creaven, and even then the results for individual animals varied over a wide range. These widely varying results can probably be attributed to the fact that the concentration of the cofactor, NADP, used was insufficient for optimum activity so that the amount present in the liver homogenate varied from rat to rat. Even so, using the revised conditions for determining biphenyl hydroxylation, which employ an optimum amount of NADP, a similar inhibition of 31% was obtained in the pregnant rats. This effect is only manifested in 19-20 day pregnant rats; in 15-16 day pregnant animals a slight decrease in activity was obtained but it was not significant. The results are similar to those found by Feuer and Liscio (1969) that in 14-20 day pregnant rats the 3-hydroxylation of 4-methylcoumarin activity is inhibited by 50%.

A similar decrease in 4-methylumbelliferone glucuronyl transferase activity was observed in the 19-20 day pregnant animals. This also agrees with the finding of Feuer and Liscio (1969) that o-aminophenol glucuronyl transferase is inhibited during pregnancy in the rat, and with the findings of Halac and Sicignano (1969) that the glucuronyl transferases responsible for conjugating bilirubin and p-nitrophenol in rat liver are inhibited during pregnancy. The amount of inhibition reported by both of these authors was in the order of 50% which is greater than that found here.
The decrease in the activity of these two enzymes in pregnancy was accompanied by a decrease in the concentration of cytochrome P450. Various workers have attempted to correlate hydroxylase and other enzyme activities thought to involve cytochrome P450 with the actual content of the cytochrome (Orrenius et al, 1965; Orrenius and Ernster, 1964; Remmer et al 1968). Although this is sometimes possible, it frequently is not (Kratz and Staudinger, 1967; Degkwitz et al, 1968) and other factors have been implicated. For example the rate of reduction of cytochrome P450, and the activity of the enzyme NADPH-cytochrome c reductase have been suggested as limiting factors (Davies et al, 1969) rather than the content of P450. I have not measured the activity of these enzymes in pregnant animals, but it seems possible that in this instance it is the concentration of cytochrome P450 that could be the limiting factor. It would be of interest to measure the rate of cytochrome P450 reduction and the activity of NADPH-cytochrome c reductase in pregnant rats in relation to the 3-hydroxylation of 4-methylcoumarin since this is decreased twice as much as the P450 content (Feuer and Liscio, 1969).

It has been suggested that the reduction of p-nitrobenzoic acid to p-aminobenzoic acid is mediated by cytochrome P450 (Gillette et al 1968). Their evidence for this is based on two facts, a) carbon monoxide blocks nitroreduction; b) the rate of nitro-reduction is proportional to the amount of cytochrome P450 in liver microsomes from animals previously treated with either phenobarbitone or carbon tetra-chloride. It has been shown here, however, that pregnancy in the rat causes a decrease in the concentration of cytochrome P450 in the liver but does not alter, or indeed slightly increases, nitroreduction. One possible explanation, of course, is that P450 is not,
in fact, involved in nitroreduction. It had been suggested that nitroreduction could take place at an earlier stage in the electron transport system of liver microsomes (Gillette et al 1968): if the cytochrome P450 content was decreased, this could possibly channel the system towards increased activity in this respect. An explanation that fits in with the ideas of Gillette is that during pregnancy some compound is present that can combine with cytochrome P450, and by so doing can cause an apparent decrease in the cytochrome P450 content and block hydroxylation reactions, but not interfere with nitroreduction. Sasame and Gillette (1969) have in fact shown that substances that cause type I spectral changes in liver microsomes either enhance or do not alter nitroreduction, although they combine with cytochrome P450. When type I substances are added to microsomal preparations in vitro they do not alter the CO-difference spectrum of reduced microsomes; however, when rats are pretreated with a type I compound for short periods there is an apparent decrease in cytochrome P450 content possibly due to the complex between P450 and substrate not having the same spectral properties (H. Rahman) i.e. the complex between substrate and P450 produces an apparent decrease in P450. The possibility exists therefore that during pregnancy in the rat a substance(s) combines with cytochrome P450 to alter the CO-difference spectrum and inhibit hydroxylation, but not nitroreduction. This point will be considered later when the causes of these effects of pregnancy are considered.

All the above inhibitory effects are based on expressing the results as a function of unit liver weight, i.e. the specific activity or concentration. It has been shown previously (Paschkis and Cantarow 1958) and it has been shown here, that in the rat the liver
increases in size during the course of pregnancy. Although it has been reported that the ratio of liver weight to body weight is increased in pregnant rats (Bokelmann and Schering, 1932) I only found this to be so in the rats used here in the 15-16 day pregnant animals; in the almost full-term the ratio was the same. This might suggest that the liver weight has reached its maximum by the 15th day of pregnancy, or even before, and thereafter the body weight increases without a corresponding increase in liver weight.

Now, when the results are expressed as a function of total liver weight there is no longer a decrease in hydroxylation of biphenyl, glucuronidation of 4-MU or content of cytochrome P450. Although there is no difference in these parameters, there is an increase in nitroreduction and microsomal protein. Thus the total content of these two parameters in 19-20 day pregnant animals is significantly different from that of non-pregnant. In the 15-16 day pregnant animals the slight inhibitions of biphenyl-4-hydroxylase and cytochrome P450 content are converted to increases when expressed as total liver amounts, but these differences are not significant.

Thus the total liver content of biphenyl-4-hydroxylase, 4-MU glucuronyl transferase and cytochrome P450 in pregnant rats is no different from that of non-pregnant, and therefore the capacity of the pregnant rat to metabolize drugs and other foreign compounds should be as great as that of non-pregnant. Indeed, in some instances, e.g. when the major route of metabolism involves nitroreduction, the capacity of the pregnant rat is increased, since the total liver content of p-nitrobenzoic acid reductase and microsomal protein is greater than the non-pregnant. In the livers of 15-16 day pregnant rats all five parameters are increased indicating that
15-16 day pregnant rats should have a greater capacity to metabolize drugs than the non-pregnant.

Having shown that the total capacity of these pregnant rats to metabolize drugs is no less than non-pregnant, it must be pointed out that, in the 19-20 day pregnant rats at least, the ratio of the liver weight to body weight is the same as in non-pregnant. Thus the activity of these enzymes in respect to body weight will be decreased. This may be of importance when the foreign compounds in the diet are considered; obviously the mother is going to eat a greater amount of food and will therefore have larger amounts of "anutrients" to metabolize. Thus, an effectively decreased ability to deal with this situation would seem to be a bad thing.

The results have also been expressed in terms of the microsomal protein content of the liver. The liver microsomal protein content itself was slightly increased in both 15-16 day and 19-20 day pregnant rats, but the increase was not significant. This slight increase, however, had the effect of enhancing the percentage inhibition of those parameters that were inhibited, when the results were expressed in terms of protein content. The fact that the 10,000 x g supernatant and microsomal protein concentrations in the livers of pregnant rats did not differ significantly from non-pregnant, would suggest that the composition of the "pregnant" liver as far as protein is concerned is similar to the non-pregnant. One would have expected, therefore, that the specific activity of the drug-metabolizing enzymes would not be changed in pregnancy, since it is associated with the microsomal protein. Therefore, in spite of the fact that the total capacity of the pregnant rat to metabolize drugs is similar to the
non-pregnant, something happens to the liver during pregnancy so that the specific activities of certain enzymes, together with cytochrome P450 content, are decreased.

What then are the possible causes of these effects? In view of the fact that it is thought that the same microsomal enzymes are involved in the metabolism of steroids and drugs (Kuntzman 1969) a reasonable explanation might be that there is competition between the high levels of sex hormones circulating in the maternal blood during pregnancy and the drugs and foreign compounds that act as substrates for the enzyme measured. Several groups of workers have in fact shown that steroid hormones, including progesterone and various oestrogens, are capable of inhibiting drug-metabolizing enzymes both competitively and non-competitively in vitro (Juchau and Fouts, 1966; Tephly and Mannering 1968). If one can explain the decrease in activity of the enzymes by competition from these steroids, how can this explain the decrease in cytochrome P450 concentration observed in the liver of the pregnant rat? A possible explanation is the formation of a steroid-cytochrome P450 complex, as mentioned previously, which decreases or does not possess a CO-difference spectrum.

The presence of high steroid levels in the maternal blood may produce profound effects on the functioning of the liver cell, by, for example, interfering with protein synthesis and control thereof. It is known that oestrogens can affect protein synthesis in the uterus (Greenman and Kenny, 1964; Gorski and Morgan, 1967) so it would be possible that protein synthesis in the liver could be affected. Campbell and Kosterlitz (1949) have shown that during the latter stages of
pregnancy in the rat there is an increased content of RNA phosphate with no increase in DNA phosphate in the maternal liver, which is indicative of increased protein synthesis. Moreover, the increase still takes place if the foetuses are removed but the placentae left intact, which would indicate that the effect is brought about by the hormones produced by the placenta (Campbell and Kosterlitz, 1949). The question then arises as to why an increase in protein synthesis should be accompanied by a decrease in cytochrome P450 content. This could be a question of priorities, there being more important proteins required during pregnancy than cytochrome P450, the result being that the new liver tissue that is formed during pregnancy, although as rich, or even moreso, in microsomal protein as non-pregnant, is not as rich in cytochrome P450.

This idea can be explained by the hypothesis suggested by Parke (1968a) (see Chapter one) that stimulation of drug-metabolizing enzymes brought about by inducers, such as phenobarbitone, is due to induction of one or more genetic systems by de-repression of an operator (gene(s). Whatever is responsible for the effects of pregnancy on protein synthesis, whether it is a steroid or not, it would need to stimulate production of a repressor substance or act as a repressor itself if the site of control is nuclear. In this way synthesis of proteins involved in drug metabolism, i.e. mixed function oxidases, or other components of the microsomal hydroxylation system could be repressed enabling the hepatic cell to synthesise other more important proteins.

Phenobarbitone pretreatment of non-pregnant rats is known to increase cytochrome P450 as well as various drug-metabolizing enzyme activities. It has been shown here that phenobarbitone treatment of pregnant rats
increases cytochrome P450 and the enzymes that are inhibited by pregnancy. The enzymes are increased to the same level as in the non-pregnant, but the cytochrome P450 is not induced to such a great extent. The results are in agreement with those of Feuer and Liscio (1969) and Halac and Sicignano (1969) all of whom have shown that various drug-metabolizing enzymes can be induced during pregnancy in the rat by pretreatment with phenobarbitone. Feuer and Liscio (1969) say that their results suggest that various factors required for the synthesis of drug-metabolizing enzymes are present in the liver of pregnant rats but are inhibited. Another possible explanation is that the phenobarbitone upsets the balance of protein synthesis in the maternal liver and thereby induces cytochrome P450 at the expense of other possibly more important proteins. Halac and Sicignano (1969) demonstrated that the administration of phenobarbitone during pregnancy was not without consequences to the wellbeing of the litter. Resorption occurred and the viability of the foetuses was adversely affected. This could fit in with the interpretation suggested above, thus, phenobarbitone would derepress the operator gene, thereby allowing the synthesis of cytochrome P450 and mixed-function oxidases to take place, again in preference to proteins needed for foetal growth.

Are all these changes, though, brought about by the increased steroid levels during pregnancy? This question is discussed more fully in the next chapter where the effects of addition in vitro of progestogens and oestrogens to enzyme incubation mixtures and the effect of pretreatment of female rats with these steroids on liver microsomal drug-metabolizing enzyme systems are investigated.

What other possible explanations are there?
The effects observed could be due to a deficiency of some component that is required for cytochrome P450 synthesis. It is well known that during gestation the pregnant animal has a greatly increased requirement for iron, which is an important part of haemoproteins and consequently of cytochrome P450. Thus, during pregnancy a deficiency of iron in the diet could lead to decreased production of cytochrome P450 and hence decreased drug-metabolizing enzyme activity. The fact that phenobarbitone induces cytochrome P450 and drug-metabolizing enzyme activity during pregnancy does not rule out this possibility because, if the explanation suggested above is true, the pretreatment would channel the available iron to cytochrome P450 formation at the expense of other proteins. Thus in normal pregnancy an insufficiency of iron in the diet might lead to synthesis of other proteins at the expense of cytochrome P450 and other iron proteins. Such an explanation might account for the fact that certain individual rats failed to show a decrease in cytochrome P450 or biphenyl hydroxylation, although the overall picture showed that a significant decrease was obtained. Thus those rats that showed no inhibition may have had sufficient iron in the diet. However, all rats were fed on the same diet ad libitum. At present no other explanation of these discrepancies is apparent. It could be possible that there is a seasonal variation in the effect of pregnancy on drug-metabolizing enzymes.

It has been established that under many conditions characterized by rapid growth of liver cells, microsomal drug-metabolizing enzyme activities are lower than normal (Fouts, 1963b). For example, microsomal preparations of foetal or newborn livers, show decreased drug-metabolizing activity (Fouts and Adamson, 1959;Jondorf et al 1958; Fouts and Hart,1965) as do livers which are
rapidly growing after partial hepatectomy (Fouts et al. 1961). Tumour bearing rats have also been shown to possess less drug-metabolizing activity in the liver (Adamson and Fouts 1961) and a reduced cytochrome P450 content (Kato et al. 1968a). The presence of growth promoting factors has been reported in all of these groups of rats and in pregnant rats, (Kato et al. 1968a). It is possible then that a similar mechanism is functioning in all these conditions, a mechanism in which growth promoting factors (hormones?) are stimulating one form of growth at the expense of the other, the 'other' being the cytochrome P450 and drug-metabolizing enzymes. Pretreatment with an inducer of drug-metabolism then channels the effect of the growth hormone to stimulating cytochrome P450 and drug-metabolizing enzymes rat the expense of the other growth.

In this respect it is interesting to note that it is over the last seven days of pregnancy in the rat that the greatest increase in body weight takes place (see Table 7.2, Chapter seven) due to the rapid growth of the foetuses, and it is over the same period that the RNA content of the liver increases (Campbell and Kosterlitz 1949), and that the inhibitory effect of pregnancy on drug-metabolizing enzymes occurs, since there is no significant decrease in cytochrome P450 content or enzyme activity in 15-16 day pregnant rats.

It would seem that the effect of a second pregnancy on drug-metabolizing enzymes in the rat liver is similar to the first, but less marked. The fact that the liver does not increase in size to the same extent as in the first pregnancy may account for this, since the total capacity will remain the same.

The results obtained with pregnant rabbits were different from those with the pregnant rats. The only
parameters that differed in the non-pregnant and pregnant rabbits were the body weight and hepatic coumarin hydroxylation. The body weight was increased, while the coumarin hydroxylation was decreased quite considerably. Hydroxylation of biphenyl, glucuronidation of 4-MU and cytochrome P450 content, all of which were altered in the rat liver by pregnancy, were not altered in the rabbit liver by pregnancy. Neither were the reduction of p-nitrobenzoic acid nor the microsomal protein content, two parameters that were not altered in the rat either. Moreover, the liver weight was not increased as it was in the rat. The result for hydroxylation of biphenyl does not agree with the findings of Creaven and Parke (1965) while that on coumarin hydroxylation is in excellent agreement. However, they used a different strain of rabbit – the Chinchilla. The fact that the coumarin hydroxylation is inhibited at earlier stages of pregnancy as well, might suggest that this enzyme is inhibited by direct competition from steroids in the blood. However, Mikhail et al. (1961) reported that progesterone levels in the pregnant rabbit are maximal at mid-pregnancy in which case one might expect more inhibition at 15 days. Phenobarbitone pretreatment of pregnant rabbits during the last three days of pregnancy, as in the rat, induces the hepatic drug-metabolizing enzymes measured and cytochrome P450.

It would thus appear that there is a species difference between the rat and the rabbit in the effect of pregnancy on drug-metabolizing enzymes and cytochrome P450. The differences could possibly be accounted for by different dietary requirement or content of iron, but are more probably due to fundamental differences that are responsible for other species differences in drug metabolism but which are as yet unsolved.
The results obtained with placental and foetal liver tissue show that neither of these tissues is able to hydroxylate biphenyl or reduce p-nitrobenzoic acid to any measurable extent. Cytochrome P450 is not detectable either in these tissues. However, both of these tissues are capable of conjugating 4-MU with glucuronic acid. These results, therefore, do not agree with those of Juchau (1969) who showed that rat placentae are able to reduce p-nitrobenzoic acid to p-aminobenzoic acid. Dixon and Willson (1968) showed that rabbit placentae and foetal livers were unable to metabolize hexobarbitone, but were able to metabolize zoxazolamine. Thus it would seem that in this respect hexobarbitone and biphenyl belong to the same class.

It seems, therefore, that the placenta and foetal livers are unlikely to influence much the overall metabolism of drugs administered to the pregnant mother.

Treatment of pregnant rats for the last three days of pregnancy with inducers of drug-metabolizing enzymes - phenobarbitone or methylcholanthrene - had no effect on the hydroxylation of biphenyl, reduction of p-nitrobenzoic acid, conjugation of 4-MU with glucuronic acid or cytochrome P450 by the placentae. The effect of phenobarbitone agrees with the observations of Dixon and Willson (1968) that phenobarbitone failed to stimulate hexobarbitone or zoxazolamine metabolism in the placenta. There are several possible explanations for the lack of induction by phenobarbitone; the phenobarbitone may not penetrate the placenta, but this is unlikely; or, there may be no potential drug-metabolizing tissue present, so it cannot be induced.

Similar pretreatment with enzyme inducers does not affect the conjugation of 4-MU with glucuronic acid by foetal liver preparations, but it does appear to induce the formation of cytochrome P450, although by only a very small amount.
The results described in this chapter have shown some effects of pregnancy in rats and rabbits on drug-metabolizing enzymes and cytochrome P450 in maternal and foetal livers and placentae. Suggestions have been made as to the possible causes of some of these effects. Some experiments designed to investigate the role of the hormones produced during pregnancy in this context are described in the following chapter. Other experiments that could be designed to investigate the causes of these effects in pregnancy could include studying the effect of growth hormone and iron (both deficiency and supplements during pregnancy) on drug-metabolizing enzymes in non-pregnant rats.
CHAPTER VI.

EFFECTS OF PROGESTOGENS, OESTROGENS AND ORAL CONTRACEPTIVE STEROIDS ON HEPATIC DRUG-METABOLIZING ENZYMES
Introduction

The question has been posed as to what is the cause of the inhibitory effect of pregnancy on certain drug-metabolizing enzymes in rat liver. Hsia et al (1963) have suggested that the high blood level of progestogens in the human during pregnancy might inhibit glucuronide formation, so it may be possible that the same explanation could account for the inhibitory effects of pregnancy on this and other metabolic transformations of drugs in the rat. If these inhibitory effects are caused by the hormones of pregnancy are they due to competition of the steroids with the drugs for the drug-metabolizing enzymes or to non-competitive inhibition, or to some other mechanism of action? What also are the effects of the semi-synthetic steroids which are used as oral contraceptive agents?

Several workers have investigated the effects of various steroidal hormones on the activity of drug-metabolizing enzymes in vitro and found inhibitory effects. Thus several steroids, including progesterone and oestradiol, were found to be competitive inhibitors of the metabolism of ethylmorphine, hexobarbitone and zoxazolamine in vitro (Tephly and Mannering, 1964 and 1968, Juchau and Pouts, 1966). Progesterone, but not oestradiol, was found to inhibit guinea-pig liver glucuronyl transferases responsible for conjugating g-aminophenol, p-nitrophenol and 4-methylumbelliferone. (Hsia et al 1963). Pretreatment of animals with steroids has also been investigated to some extent. Juchau and Pouts (1966) reported that one hour after a large dose of either progesterone or norethynodrel the in vitro metabolism of hexobarbitone and zoxazolamine in rat liver was inhibited. However, twenty-four hours afterwards, the metabolism of both compounds was increased by the pretreatment with norethynodrel but was unaltered
by the progesterone treatment. Thus norethynodrel was found to be an inducer of certain drug-metabolizing enzymes.

The work described in this chapter, therefore, is directed towards determining the effects of the natural steroids of pregnancy on drug-metabolizing enzymes to ascertain whether these hormones play any part in the overall inhibitory effects of pregnancy on these enzymes in the rat liver. The effects on these enzymes of synthetic steroids used as oral contraceptives have also been studied to see whether they have an effect similar to the endogenous hormones or whether they may act as inducers of drug metabolism as previously suggested (Juchau and Fouts, 1966).

Remmer et al., (1966) have investigated the effect of in vitro addition of various compounds on the absorption spectrum of cytochrome P450. They have shown that the addition of any substrate that is hydroxylated will change the absorption spectrum of the oxidised P450, giving rise to one of two different types of spectra indicating that two different types of binding to cytochrome P450 occur. The effects of progesterone and oestradiol on the absolute absorption spectrum of cytochrome P450 have now been studied to see whether they give rise to type I, type II or neither type of spectrum. The effects of these compounds on the CO-difference spectrum of reduced microsomes have also been determined to see whether the steroids can combine with cytochrome P450 and thereby interfere with the normal estimation procedure of this enzyme.

Materials and Methods

The experiments described in this chapter concern the effect of the following steroids – progesterone, pregnanediol, oestradiol, norethynodrel, ethynodiol, norethisterone, chlormadinone and mestranol on the
activity of the following drug-metabolizing enzyme systems of rat and rabbit liver preparations:

- 4-hydroxylation of biphenyl,
- 7-hydroxylation of coumarin,
- dealkylation of 4-methoxybiphenyl and of 4-ethoxybiphenyl,
- reduction of p-nitrobenzoic acid,
- cytochrome P450,
- conjugation of 4-MU with glucuronic acid.

The structures of the steroids used and the methods for the determination of the enzyme activities are given in Chapter

The experiments performed were of two types:

a) those in which the steroids were added directly to the enzyme system in vitro. This type of experiment was designed to show any direct inhibitory effect of the steroids.

b) those in which rats were pretreated for varying lengths of time with the steroids and then the activity of the hepatic microsomal enzymes determined in vitro. Pretreatment for short periods of time, e.g. one hour, is likely to reveal any inhibitory effect which may not be shown by simple in vitro addition of the steroid, for example, effects brought about by a metabolite of the steroid. Pretreatment for twenty-four hours or three days will reveal any inducing properties that the steroids may possess (Conney, 1967). Finally, pretreatment for longer periods of time may show effects that are produced by chronic exposure to steroids such as are found during natural pregnancy and the pseudo-pregnancy state produced by oral contraceptive agents.

**In vitro additions of steroids**

In studying the effect of direct addition of a steroid to enzyme preparations in vitro, the steroid was dissolved in a suitable volume of acetone such that the effects of final concentrations of $10^{-5}$, $10^{-4}$, and $10^{-3}$ M could be observed. The volume of acetone added was
never greater than 0.1 ml., and was often considerably less, depending on the solubility of the steroid. A similar volume of acetone was added to control, standard and blank enzyme incubations. Acetone (0.1 ml) produced a slight decrease in activity of the hydroxylating enzymes, but this decrease was less than 10% of the activity in the absence of acetone; smaller volumes did not alter the activity of any of the enzymes. Pregnanediol is insoluble in acetone, and was therefore added in dimethyl sulphoxide (DMSO). This solvent alone did not alter the enzyme activity.

The nature of the inhibitory effect of progesterone on the demethylation of 4-methoxybiphenyl was examined using the Lineweaver-Burk (1934) plot. To obtain the data for this, three inhibitor concentrations and six substrate concentrations were used. The substrate solutions were all prepared from the original (12 μmoles/ml) solution by diluting with 2% w/v Tween 80 in 1.15% w/v potassium chloride solution such that the volume of substrate solution added to each incubation was 0.5 ml. This was done to eliminate any possible extraneous effect of the Tween 80, such as its effect on the solubility of the steroids.

Effects of the in vitro addition of steroids on cytochrome P450 were measured in a similar manner to that described by Remmer et al (1968). Female Wistar albino rats weighing approximately 150 gm. were given phenobarbitone sodium orally (0.1% solution in the drinking water) for seven days prior to killing. Untreated rats were used as controls. The cytochrome b_5 and cytochrome P450 were determined in both the phenobarbitone pretreated and the control animals. The cytochrome b_5 was determined in a similar manner to the cytochrome P450 as described in Chapter two, except that the difference spectrum between oxidised and reduced
microsomes (reduced with 0.1 ml. of a 2% NADPH₂ solution to 3 ml. of microsomes) was determined and the difference between the absorbance at 424 and 409 nm. was used as a measure of the cytochrome b⁵ content. The increase in cytochrome b⁵ caused by phenobarbitone induction is not as great as the increase in cytochrome P₄₅₀. Thus by diluting the induced microsomal suspension to give the same concentration of cytochrome b⁵ as in the control, the resulting difference spectrum between control and diluted induced microsomes is the absolute spectrum of cytochrome P₄₅₀. Various steroids were then added to the induced microsomal suspension, dissolved in 0.01 ml. acetone, and any change in the absorption pattern measured.

A simpler method was also used for studying the effect of steroids on the absorption spectrum of oxidised cytochrome P₄₅₀, in which microsomes from phenobarbitone induced rats alone were used. A sample (3 ml) of microsomal suspension diluted 1:2 with 0.2M phosphate buffer, was placed in each of the two cuvettes. To one cuvette (the sample) the steroid dissolved in acetone (0.1 ml.), was added to give a final concentration of 10⁻⁴ or 10⁻⁵M; to the other cuvette (the reference) acetone (0.1 ml.) alone was added, and the difference spectrum between the sample and reference cuvettes was determined between 450 and 350 nm.

The effect of several steroids on the CO-difference spectrum of reduced cytochrome P₄₅₀ was also investigated. For this the steroid, dissolved in acetone (0.1 ml.), was added to the sample cuvette before the dithionite. Acetone alone was added to the reference cuvette. The difference spectrum was then obtained in the usual manner with CO and compared with that obtained without the steroid.
Pretreatment with steroids

All steroids were administered in ethyl oleate, except for pregnanediol which was given in dimethyl-sulphoxide. Control animals received solvent alone at a dose level of 2 ml/kg. In acute studies, i.e. where only one single dose was given, the rats received a single intraperitoneal injection either one or twenty-four hours prior to killing. In subacute studies, i.e. three days pretreatment, the rats were given a daily intraperitoneal injection at the same time each day, and were killed twenty-four hours after the final dose. The rats that were chronically treated with steroids received a daily dose by either intraperitoneal or intramuscular injection at approximately the same time each day and were finally killed twenty-four hours after the last injection. The volume of vehicle injected for intramuscular injections was in the order of 0.1 - 0.2 ml.

For studying the effect of prolonged pretreatment of female rats with low doses of progestogen it was obviously impractical to give the compounds by injection so the oral route was used. The steroids were dissolved in arachis oil which was then evenly dispersed in a ration of powdered rat chow. For the control animals arachis oil alone was used. A suitable ration for one rat for one day was found to be 20 g. of diet so that 0.1 mg. of steroid was mixed with each 20 gm. of food. The animals were kept in groups of six and each group was given 120 gm. of food daily at approximately 10.00 a.m. Thus the daily dose per animal was about 0.1 mg. of steroid (0.5 mg/kg).
Results

   a. In the rat
      i) The hydroxylation of biphenyl.

      The effects of the addition in vitro of several progestogens and oestrogens on the hydroxylation of biphenyl by rat liver preparations were determined using both the original method of Creaven et al (1965b) and the modified method described in Chapter three. The major difference was the lower concentration of NADP employed in the former. The effects of the addition of progesterone, norethynodrel, norethisterone, ethynodiol diacetate, pregnanediol, oestradiol, oestrone and testosterone to liver preparations (10,000 x g supernatant) of mature female rats on the hydroxylation of biphenyl are shown in Table 6.1.

      The results show that all types of steroids examined - progestogens, oestrogens and androgens - are capable of producing inhibition of the hydroxylation of biphenyl by rat liver homogenates in vitro, but only at relatively high concentrations. Both progesterone and oestradiol produced about the same degree of inhibition at a concentration of $10^{-3}M$, as did testosterone, which indicates that this action is fairly generalised. The synthetic progestogens derived from 19-nortestosterone: norethynodrel, ethynodiol diacetate and norethisterone acetate, all produced a slightly greater inhibitory effect. The concentrations used in these experiments are very close to the limit of solubility of these steroids, and although this is enhanced by the presence of Tween 80, it could be that many of the differences in inhibitory action of the different steroids may be attributed to differences in solubility.
Although the concentration of steroid required to give a significant inhibition of the hydroxylation reaction is high, it is very similar to the concentration of substrate (biphenyl) used. In the original method the biphenyl concentration was $2 \times 10^{-3} \text{M}$, whilst in the modified method it was $1.5 \times 10^{-3} \text{M}$. This difference probably accounts for the slightly greater inhibition obtained using the modified method.

### Table 6.1

Effect of in vitro addition of progestogens and oestrogens on the hydroxylation of biphenyl by rat liver preparations

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Concentration (M)</th>
<th>Percentage inhibition using</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Original Method</td>
</tr>
<tr>
<td>Progesterone</td>
<td>$10^{-5}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>$37 \pm 3$</td>
</tr>
<tr>
<td>Pregnanediol</td>
<td>$10^{-4}$</td>
<td>3</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>$10^{-5}$</td>
<td>1</td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>5</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>$68 \pm 3$</td>
</tr>
<tr>
<td>Norethisterone</td>
<td>$10^{-4}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>$32 \pm 2$</td>
</tr>
<tr>
<td>Ethynodiol diacetate</td>
<td>$10^{-4}$</td>
<td>0</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>$27 \pm 3$</td>
</tr>
<tr>
<td>Clomadinone acetate</td>
<td>$10^{-4}$</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>-</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>$10^{-4}$</td>
<td>2</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>$35 \pm 2$</td>
</tr>
<tr>
<td>Oestrone</td>
<td>$10^{-3}$</td>
<td>$20 \pm 4$</td>
</tr>
<tr>
<td>Mestranol</td>
<td>$10^{-3}$</td>
<td>-</td>
</tr>
<tr>
<td>Testosterone</td>
<td>$10^{-3}$</td>
<td>-</td>
</tr>
</tbody>
</table>

The results are the mean of at least three separate determinations.
ii) The dealkylation of 4-methoxybiphenyl and of 4-ethoxybiphenyl.

The effect of several steroids in vitro on dealkylation by rat liver preparations was also investigated. The systems studied were the demethylation of 4-methoxybiphenyl and the de-ethylation of 4-ethoxybiphenyl (Creaven et al 1966). The incubation conditions used were similar to those described by Creaven et al (1966) for studying dealkylation by rabbit liver preparations, and are summarised in Chapter two. It is not known whether these conditions are optimal, particularly with respect to NADP, for rat liver preparations as they have not been re-investigated. However, since the steroids produced similar levels of inhibition of biphenyl hydroxylation with both the original and modified incubation systems, it is likely that the NADP concentration employed in the dealkylation experiments would prove adequate for the determination of enzyme inhibition.

The effects of progesterone, norethynodrel, norethisterone acetate, ethynodiol diacetate, oestradiol and oestrone on the dealkylation of 4-methoxybiphenyl and 4-ethoxybiphenyl by liver 10,000 x g supernatant preparations from mature female rats are shown in Table 6. 2.

All of the steroids used showed some degree of inhibition at the concentrations used — $10^{-4}$ and $10^{-3}M$. The inhibition of dealkylation was greater than the inhibition of aromatic hydroxylation for a similar concentration of steroid, but this could be accounted for by the fact that the substrate concentration used in the dealkylations is $0.35 \times 10^{-3}M$ which is about one-sixth of the substrate concentration used in the aromatic hydroxylation. The effect produced by the synthetic progestogens norethynodrel, norethisterone
and ethynodiol was again somewhat greater than that produced by progesterone. The oestrogens produced a less marked effect.

Table 6. 2
Effect of in vitro addition of steroids on the dealkylation of 4-methoxybiphenyl and of 4-ethoxybiphenyl by rat liver preparations.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Concentration (M)</th>
<th>Percentage of inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>4-Methoxybiphenyl</td>
</tr>
<tr>
<td></td>
<td></td>
<td>demethylation</td>
</tr>
<tr>
<td>Progesterone</td>
<td>$10^{-4}$</td>
<td>33</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>66</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>$10^{-4}$</td>
<td>26</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>74</td>
</tr>
<tr>
<td>Norethisterone acetate</td>
<td>$10^{-4}$</td>
<td>23</td>
</tr>
<tr>
<td></td>
<td>$10^{-3}$</td>
<td>56</td>
</tr>
<tr>
<td>Ethynodiol diacetate</td>
<td>$10^{-3}$</td>
<td>55</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>$10^{-3}$</td>
<td>43</td>
</tr>
<tr>
<td>Oestrone</td>
<td>$10^{-3}$</td>
<td>12</td>
</tr>
</tbody>
</table>

The results are the mean of three determinations: no appreciable difference between individual results was obtained.

As progesterone produced a slightly greater inhibition of demethylation than of aromatic hydroxylation, it was decided to use the former system to investigate the nature of the inhibition of the hepatic mixed-functional oxygenases by progesterone. The rate of demethylation
of 4-methoxybiphenyl by rat liver 10,000 x g supernatant was measured at several substrate concentrations without inhibitor and in the presence of three concentrations of progesterone. The data obtained from this determination which are the mean of at least three determinations, are shown in Table 6.3: the values of $K_m$ and $V_{max}$ were obtained graphically from a Lineweaver Burk (1934) plot of $\frac{1}{V}$ against $\frac{1}{S}$, which is shown in Fig. 6.1.

**Table 6.3**

Demethylation of 4-methoxybiphenyl at various substrate concentrations in the presence and absence of progesterone

<table>
<thead>
<tr>
<th>Substrate concentration (µmoles/incubation)</th>
<th>Velocity (µmoles 4-hydroxybiphenyl produced/gm.liver/hr.)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Progesterone concentration None</td>
</tr>
<tr>
<td>1.0</td>
<td>0.43</td>
</tr>
<tr>
<td>0.5</td>
<td>0.40</td>
</tr>
<tr>
<td>0.25</td>
<td>0.30</td>
</tr>
<tr>
<td>0.17</td>
<td>0.22</td>
</tr>
<tr>
<td>0.125</td>
<td>0.19</td>
</tr>
<tr>
<td>0.1</td>
<td>0.16</td>
</tr>
</tbody>
</table>

$K_m = 59.5x10^{-6}$

$V_{max} = 0.44$

The near consistency of $V_{max}$ and the characteristic configuration of the plotted data indicate that the mode of inhibition of the demethylation of 4-methoxybiphenyl by progesterone is competitive in nature.
Fig. 6. 1. Inhibition of the microsomal demethylation of 4-methoxybiphenyl by progesterone in mature female rats.

$v = \mu$moles 4-hydroxybiphenyl produced per gram liver per hour
$s = \text{substrate concentration}$

- $\Delta \Delta$ $10^{-3}\text{M}$ Progesterone;
- $\Delta$ $0.5 \times 10^{-3}\text{M}$ Progesterone,
- $\circ \circ$ $10^{-4}\text{M}$ Progesterone;
- $\circ \circ \circ$ No Progesterone
iii) The reduction of p-nitrobenzoic acid

The effect of progesterone, oestradiol, ethynodiol diacetate and mestranol on the reduction of p-nitrobenzoic acid by liver preparations from mature female rats is shown in Table 6.4.

Table 6.4

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Concentration (M)</th>
<th>Percentage Inhibition</th>
</tr>
</thead>
<tbody>
<tr>
<td>Progesterone</td>
<td>$10^{-3}$</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>15</td>
</tr>
<tr>
<td>Ethynodiol diacetate</td>
<td>$10^{-3}$</td>
<td>10</td>
</tr>
<tr>
<td></td>
<td>$10^{-4}$</td>
<td>0</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>$10^{-3}$</td>
<td>19</td>
</tr>
<tr>
<td>Mestranol</td>
<td>$10^{-3}$</td>
<td>19</td>
</tr>
</tbody>
</table>

The results are the mean of three determinations: no appreciable difference between individual results obtained.

The results show that with this enzyme system also the effect of adding various steroids to the enzyme incubation is inhibitory, although the effects produced are less marked than those observed with aromatic hydroxylation and the dealkylations. The substrate concentration used was $2 \times 10^{-3} \text{M}$, which is the same as the concentration of biphenyl used in the original incubation conditions for the determination of the 4-hydroxylation of biphenyl. Thus the percentage
inhibitions of the aromatic hydroxylation and nitro-reduction reactions by progesterone at a concentration of $10^{-3}$M and for similar substrate concentrations were 37% and 30% respectively. A minor, but possibly important factor, which might play a role here, is the presence of Tween 80 in the hydroxylation incubations but not in the reduction incubations. The presence of Tween 80 may help to maintain the steroids in solution.

iv) Cytochrome P450
Neither progesterone, at final concentration of $10^{-4}$ and $10^{-3}$M, nor oestradiol, at a final concentration of $10^{-4}$M, produced any effect on the CO-difference spectrum; nor did hexobarbitone ($10^{-4}$M).

In the mature female rats used in these experiments phenobarbitone pretreatment induced the cytochrome b$_5$ to almost the same extent as the cytochrome P450, 120% and 140% respectively (three determinations). The absolute spectrum of cytochrome P450 obtained was, therefore, only a very small peak. Nevertheless, hexobarbitone and aniline added at final concentrations of $10^{-4}$M caused detectable shifts in the spectrum typical of type I and type II substances respectively. Neither progesterone ($10^3$ or $10^{-4}$M) nor oestradiol ($10^{-4}$M) caused detectable shifts of the peak.

Using the other method whereby microsomes from induced animals alone were used and difference spectra measured, again very small peaks were obtained. Hexobarbitone ($10^{-4}$M) produced a typical type I difference spectrum but neither progesterone ($10^{-3}$M) nor oestradiol ($10^{-4}$M) produced any detectable difference spectrum.

It must be emphasized that with all these experiments involving the absolute spectrum of cytochrome P450
only very small peaks were obtained, and so detecting differences was very difficult.

b. In the rabbit

The effect of adding progesterone, norethynodrel and norethisterone acetate at final concentration of $10^{-3}$M to incubations of rabbit liver 10,000 x g supernatant on the 7-hydroxylation of coumarin, 4-hydroxylation of biphenyl, demethylation of 4-methoxybiphenyl and de-ethylation of 4-ethoxybiphenyl are shown in Table 6.5.

<table>
<thead>
<tr>
<th>Steroid</th>
<th>Percentage inhibition of the following activities:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>7-hydroxylation of coumarin</td>
</tr>
<tr>
<td>Progesterone</td>
<td>47</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>60</td>
</tr>
<tr>
<td>Norethisterone</td>
<td>-</td>
</tr>
</tbody>
</table>

The results are the mean of three determinations.

The results show that, as with the rat, the effect of these progestogens is inhibitory, although again only in high concentrations. The inhibition produced is also less than that which is produced by similar concentration of the steroids on the rat enzymes. The difference in effect on the two aromatic oxygenases is probably accounted for by the difference in substrate
concentrations used. For biphenyl this is $2 \times 10^{-3}$ M and for coumarin $0.33 \times 10^{-3}$ M. This explanation is supported by the fact that the two dealkylation reactions, where the substrate concentration is also $0.33 \times 10^{-3}$ M, show a similar degree of inhibition to the 7-hydroxylation of coumarin.

2. Pretreatment studies with steroidal hormones

i. Progesterone

The effect of intraperitoneal pretreatment of mature female rats with progesterone on the hydroxylation of biphenyl was investigated by determination of the enzyme activity in vitro at various time intervals after injection. The results are shown in Table 6.6. The dose of progesterone, 25 mg/kg in solution in ethyl oleate, was given intraperitoneally 1 and 24 hours prior to sacrifice. Those animals pretreated for three days received three daily doses of 25 mg/kg, and were killed 24 hours after the final injection.

<table>
<thead>
<tr>
<th>Period of pretreatment</th>
<th>Enzyme activity (umoles product/gm.liver/hr)</th>
<th>Percentage change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>Pretreated</td>
<td></td>
</tr>
<tr>
<td>None</td>
<td>$3.68^{\pm}0.22 (13)$</td>
<td>-</td>
</tr>
<tr>
<td>1 hour</td>
<td>$3.59^{\pm}0.24 (9)$</td>
<td>$3.66^{\pm}0.15(8)$</td>
</tr>
<tr>
<td>24 hours</td>
<td>$3.59^{\pm}0.24 (9)$</td>
<td>$3.15^{\pm}0.10(6)$</td>
</tr>
<tr>
<td>3 days</td>
<td>$4.27^{\pm}0.21 (6)$</td>
<td>$4.12^{\pm}0.22(6)$</td>
</tr>
</tbody>
</table>

Numbers in parentheses represent the number of animals used for that determination.
The results show that none of the pretreatments produced any significant change in the hydroxylation of biphenyl by liver preparations.

These results are in contrast to those of Table 6.7, which shows the effect of three days progesterone pretreatment on biphenyl hydroxylation and cytochrome P450 content of liver preparations of immature male and female rats. It should be noted, however, that the experiment has only been carried out on two rats in each group, so the results can only be considered as an indication that induction occurs.

Table 6.7
Effect of pretreatment of immature male and female rats with progesterone on the hydroxylation of biphenyl and cytochrome P450 content of liver preparations

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Sex</th>
<th>Controls</th>
<th>Pretreated</th>
<th>Percentage difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight gain (grams)</td>
<td>M</td>
<td>16,15 (16)</td>
<td>20,21 (21)</td>
<td>+ 31</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>14,17 (16)</td>
<td>16, 15 (16)</td>
<td>0</td>
</tr>
<tr>
<td>4-Hydroxylation of biphenyl (μmoles product/gm. liver/hour)</td>
<td>M</td>
<td>4.82,5.09 (4.96)</td>
<td>5.66,5.58 (5.62)</td>
<td>+ 14</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>4.91,5.15 (5.03)</td>
<td>5.46,5.52 (5.49)</td>
<td>+ 9</td>
</tr>
<tr>
<td>2-Hydroxylation of biphenyl (μmoles product/gm. liver/hour)</td>
<td>M</td>
<td>0.87,0.94 (0.91)</td>
<td>1.01,1.09 (1.05)</td>
<td>+ 15</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>1.24,1.06 (1.15)</td>
<td>1.47,1.58 (1.53)</td>
<td>+ 33</td>
</tr>
<tr>
<td>Cytochrome P450 concentration (μmoles/mg. protein)</td>
<td>M</td>
<td>0.93,0.94 (0.94)</td>
<td>1.13,1.13 (1.13)</td>
<td>+ 20</td>
</tr>
<tr>
<td></td>
<td>F</td>
<td>0.92,0.90 (0.91)</td>
<td>1.05,1.08 (1.07)</td>
<td>+ 18</td>
</tr>
</tbody>
</table>

The figures in parentheses are the means of the two individual results.
The results show that pretreatment of immature rats of both sexes with progesterone causes an upward trend in hydroxylating enzymes and cytochrome P450.

The effects of one hour and three days pretreatment, of mature female rats with progesterone, on other liver parameters, are shown in Table 6.8. The doses used were similar to those used for Table 6.6. The results are the means of three animals in each case.

Table 6.8
Effect of pretreatment of mature female rats with progesterone on various liver parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>1 hour pretreated</th>
<th>3 days pretreated</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Pre-treated</td>
</tr>
<tr>
<td>Body weight (grams)</td>
<td>189±5</td>
<td>194±2</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>6.1±0.4</td>
<td>6.0±0.4</td>
</tr>
<tr>
<td>Cytochrome P450 (μmoles/mg/protein)</td>
<td>0.66±0.02</td>
<td>0.65±0.02</td>
</tr>
<tr>
<td>p-Nitrobenzoic acid reduction (μmoles/gm liver/hr)</td>
<td>1.79±0.15</td>
<td>1.81±0.16</td>
</tr>
<tr>
<td>Microsomal protein (mg/gm liver)</td>
<td>27.5±0.4</td>
<td>26.7±0.7</td>
</tr>
<tr>
<td>4-MU glucuronidation (μmoles/gm liver/hr)</td>
<td>87.0±13</td>
<td>83.5±3</td>
</tr>
</tbody>
</table>

Again it was found that pretreatment with progesterone either for one hour or three days produced no significant effect on the parameters measured.
The effect of pretreating mature female rats with progesterone at a dose of 1 mg. per animal daily for 18 days is shown in Table 6.9. The hormone was administered by intramuscular injection to prolong its effect.

**Table 6.9**

*Effect of chronic pretreatment of female rats with progesterone on various liver parameters*

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Pretreated</th>
<th>Percentage change</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>215 ± 5</td>
<td>205 ± 6</td>
<td>-5</td>
</tr>
<tr>
<td>(Before)</td>
<td>232 ± 4</td>
<td>235 ± 8</td>
<td>+1</td>
</tr>
<tr>
<td>(After)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>8.8 ± 0.4</td>
<td>6.9 ± 0.4</td>
<td>-22</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylation ((\mu)moles/product/gm.liver/hour)</td>
<td>2.78 ± 0.19</td>
<td>3.06 ± 0.09</td>
<td>+10</td>
</tr>
<tr>
<td>(p)-nitrobenzoic acid reduction ((\mu)moles product/gm.liver/hour)</td>
<td>2.75 ± 0.27</td>
<td>2.72 ± 0.18</td>
<td>-1</td>
</tr>
<tr>
<td>4 MU glucuronidation ((\mu)moles conjugated/gm.liver/hour)</td>
<td>85 ± 4</td>
<td>77 ± 2</td>
<td>-9</td>
</tr>
<tr>
<td>Microsomal protein (mg/gm.liver)</td>
<td>23.2 ± 0.8</td>
<td>26.5 ± 0.7</td>
<td>+14</td>
</tr>
<tr>
<td>Cytochrome P450 ((\mu)moles/mg.protein)</td>
<td>0.58 ± 0.03</td>
<td>0.59 ± 0.01</td>
<td>+2</td>
</tr>
</tbody>
</table>

The results are the mean of three animals.

The liver weight is decreased in the treated animals; none of the other parameters is significantly changed although the microsomal protein content and hydroxylation of biphenyl are slightly increased.
ii. Pregnanediol

The compound used was 4 pregnane-3α-20α-diol, at a dose of 4 mg/kg, and the effect on various liver parameters of giving this dose (dissolved in dimethyl sulphoxide) daily for three days intraperitoneally to mature female rats is shown in Table 6.10.

Table 6.10
Effect of three days pretreatment of mature female rats with pregnanediol on various liver parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Pretreated</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Hydroxylation of biphenyl</td>
<td>3.88 ± 0.33</td>
<td>4.34 ± 0.18</td>
<td>+ 12</td>
</tr>
<tr>
<td>(μmoles product/gm.liver/hour)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>N-glucuronidation</td>
<td>85 ± 8</td>
<td>102 ± 7</td>
<td>+ 20</td>
</tr>
<tr>
<td>(μmoles conjugated/gm.liver/hour)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction of p-Nitrobenzoic acid</td>
<td>1.29 ± 0.6</td>
<td>1.23 ± 0.09</td>
<td>- 5</td>
</tr>
<tr>
<td>(μmoles product/gm.liver/hour)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>25.5 ± 0.9</td>
<td>24.1 ± 0.8</td>
<td>- 5</td>
</tr>
<tr>
<td>(mg/gm.liver)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>0.72 ± 0.02</td>
<td>0.73 ± 0.02</td>
<td>+ 1</td>
</tr>
<tr>
<td>(μmoles/mg. protein)</td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The results are the means of determinations on six animals.

The slight increases in biphenyl hydroxylation and 4-MU glucuronidation are not statistically significant and it would, therefore, appear that pretreatment of mature female rats with pregnanediol for three days does not have any marked effect on the liver, its content of cytochrome P450 and microsomal protein and certain of its drug-metabolizing enzymes.
iii. Oestradiol

Oestradiol, chosen as a representative female hormone, was given, dissolved in ethyl oleate, to female rats by intraperitoneal injection at a dose of 5 mg/kg. Animals were pretreated for one hour, and daily for three days, before being killed and the following liver parameters measured: 4-hydroxylation of biphenyl; reduction of p-nitrobenzoic acid; 4-MU glucuronidation; cytochrome P450 and microsomal protein. The results are shown in Table 6.11.

**Table 6.11**

Effect of pretreatment of mature female rats with oestradiol on various liver parameters.

<table>
<thead>
<tr>
<th>Parameter</th>
<th>One Hour's pretreatment</th>
<th>Three days' pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Pretreated</td>
</tr>
<tr>
<td>Body weight (grams)</td>
<td>189 ± 5</td>
<td>190 ± 2</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>6.1 ± 0.3</td>
<td>6.5 ± 0.4</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylation (µmoles product/gm.liver/hour)</td>
<td>4.45±0.40</td>
<td>4.56±0.22</td>
</tr>
<tr>
<td>p-nitrobenzoic acid reduction (µmoles product/gm.liver/hour)</td>
<td>1.79±0.15</td>
<td>1.88±0.20</td>
</tr>
<tr>
<td>4-MU glucuronidation (µmoles conjugated gm.liver/hour)</td>
<td>87±3</td>
<td>93±3</td>
</tr>
<tr>
<td>Cytochrome P450 (µmoles/mg.protein)</td>
<td>0.66±0.02</td>
<td>0.60±0.04</td>
</tr>
<tr>
<td>Microsomal protein (mg./gm.liver)</td>
<td>26.8±0.7</td>
<td>26.2±0.4</td>
</tr>
</tbody>
</table>

The results are the mean of: ≠ three rats ≠ five (except 4 MUglucuronidation) rats, in each group.
Pretreatment for one hour only with oestradiol does not have any effect on any of the parameters measured. Three days pretreatment does, however, produce some slight effects, namely that the body weight, the microsomal protein content, the hydroxylation of biphenyl and 4-MU glucuronidation are decreased by small amounts, but none of these is significant. The nitroreduction and cytochrome P450 content are unaffected.

Several mature female rats were then pretreated for 18 days with a dose of 0.1 mg (intramuscularly) of oestradiol per animal daily and the same parameters measured. The results are shown in Table 6.12.

Table 6.12
Effect of chronic pretreatment of female rats with oestradiol on various liver parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Pretreated</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Before</td>
<td>215 ± 5</td>
<td>201 ± 5</td>
<td>- 7</td>
</tr>
<tr>
<td>After</td>
<td>232 ± 4</td>
<td>224 ± 6</td>
<td>- 3</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>8.8 ± 0.4</td>
<td>8.8 ± 0.5</td>
<td>0</td>
</tr>
<tr>
<td>Biphenyl-4-hydroxylation (μmoles product/gm.liver/hour)</td>
<td>2.78 ± 0.19</td>
<td>3.16 ± 0.07</td>
<td>+14</td>
</tr>
<tr>
<td>p-nitrobenzoic acid reduction (μmoles product/gm.liver/hour)</td>
<td>2.75 ± 0.27</td>
<td>2.56 ± 0.49</td>
<td>- 7</td>
</tr>
<tr>
<td>4 MU glucuronidation (μmoles conjugated/gm.liver/hr)</td>
<td>85 ± 4</td>
<td>88 ± 4</td>
<td>+ 4</td>
</tr>
<tr>
<td>Microsomal protein (mg./gm.liver)</td>
<td>23.2 ± 0.8</td>
<td>21.8 ± 0.8</td>
<td>- 6</td>
</tr>
<tr>
<td>Cytochrome P450 (μmoles/mg.protein)</td>
<td>0.58 ± 0.03</td>
<td>0.62 ± 0.02</td>
<td>+ 7</td>
</tr>
</tbody>
</table>

The results are the mean of three animals in each group.
Apart from a slight increase in the hydroxylation of biphenyl there are no differences between the liver parameters of the treated and untreated rats.

iv. Progesterone plus oestradiol

A combination of progesterone (25 mg/kg) and oestradiol (5 mg/kg) was administered to mature female rats by intraperitoneal injection in ethyl oleate daily for three days. The usual liver parameters were measured and the results are shown in Table 6.13. The effects of chronic (18 days) pretreatment of mature female rats with a daily intramuscular injection of progesterone (1 mg) plus oestradiol (0.1 mg) on the same parameters are also shown in Table 6.13.

The results show that there are no significant differences between the pretreated and control rats in either group.
Table 6.13
Effect of pretreatment of mature female rats with a combination of progesterone and oestradiol on various liver parameters

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Three days pretreatment</th>
<th>Eighteen days pretreatment</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls</td>
<td>Pretreated</td>
</tr>
<tr>
<td>Change in body weight (gms)</td>
<td>+2 ± 0.1</td>
<td>-5 ± 0.5</td>
</tr>
<tr>
<td>Liver weight (gms)</td>
<td>6.7 ± 0.7</td>
<td>7.2 ± 0.7</td>
</tr>
<tr>
<td>4-Hydroxylation of Biphenyl (umoles product/gm.liver/hour)</td>
<td>4.09 ± 0.41</td>
<td>4.42 ± 0.36</td>
</tr>
<tr>
<td>Reduction of p-nitrobenzoic acid (umoles product/gm.liver/hour)</td>
<td>1.38 ± 0.11</td>
<td>1.55 ± 0.20</td>
</tr>
<tr>
<td>4-MU glucuronol transferase (umoles conjugated/gm.liver/hour)</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cytochrome P450 (umoles/mg.protein)</td>
<td>0.60 ± 0.03</td>
<td>0.57 ± 0.04</td>
</tr>
<tr>
<td>Microsomal Protein (mg/gm.liver)</td>
<td>33.0 ± 0.2</td>
<td>31.0 ± 0.8</td>
</tr>
</tbody>
</table>

* The number of rats used in this group was three.

/ The number of rats used in this group was six.
3. Pretreatment studies with contraceptive steroids

i. Acute treatment

The effect of various periods of norethynodrel pretreatment on the hydroxylation of biphenyl by rat liver has been studied. The effects were investigated using both the original assay procedure for biphenyl which employed a low concentration of NADP, and the revised conditions which use a much higher concentration of NADP. In those investigations using the old assay procedure the rats were given a single dose of 4 mg. of norethynodrel dissolved in DMSO (0.1 ml) by intraperitoneal injection at various time intervals prior to killing; those animals that were pretreated for three days received a similar daily dose and were killed twenty-four hours after the final injection. Control animals received DMSO alone. In the investigations using the modified assay procedure the use of DMSO was abandoned, and instead the norethynodrel was administered in ethyl oleate using a similar dose pattern; controls for these received ethyl oleate alone. Table 6.14 shows the results for both assay procedures.

Using the original conditions of incubation the effect of pretreatment with norethynodrel was found to be as follows: after one hour the 4-hydroxylation of biphenyl was inhibited to a small extent, but this was not significant; after four and twelve hours the activity had returned to normal, but after twenty-four hours the activity was increased significantly by 66%. Twenty-four hours after the final dose of three daily doses, however, the activity was not enhanced.

Using the modified incubation conditions, a different picture was obtained. Again, after one hour, the activity was inhibited, but it was also inhibited after twenty-four hours to an even greater extent. After three days treatment the activity was not changed.
Table 6. 14

Effect of pretreatment of female rats with norethynodrel on the hydroxylation of biphenyl by liver microsomal preparations

<table>
<thead>
<tr>
<th>Pretreatment period</th>
<th>Activity using old method (umoles product/g liver/hr.)</th>
<th>% Difference</th>
<th>Activity using new method (umoles product/g liver/hr.)</th>
<th>% Difference</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Controls (DMSO)</td>
<td>Pretreated (in DMSO)</td>
<td></td>
<td>Controls (Ethyl oleate)</td>
</tr>
<tr>
<td>None</td>
<td>0.62 ± 0.06 (12)</td>
<td>-</td>
<td>-</td>
<td>3.68 ± 0.22 (13)</td>
</tr>
<tr>
<td>1 hour</td>
<td>0.70 ± 0.06 (8)</td>
<td>0.56 ± 0.09 (8)</td>
<td>-20</td>
<td>4.03 ± 0.19 (18)</td>
</tr>
<tr>
<td>4 hours</td>
<td>0.65 ± 0.09 (3)</td>
<td>0.63 ± 0.03 (3)</td>
<td>-3</td>
<td>-</td>
</tr>
<tr>
<td>12 hours</td>
<td>0.69 ± 0.07 (3)</td>
<td>0.73 ± 0.05 (3)</td>
<td>+6</td>
<td>-</td>
</tr>
<tr>
<td>24 hours</td>
<td>0.56 ± 0.06 (7)</td>
<td>0.93 ± 0.09 (7)</td>
<td>+66*</td>
<td>4.11 ± 0.23 (12)</td>
</tr>
<tr>
<td>3 days</td>
<td>0.51 ± 0.09 (3)</td>
<td>0.46 ± 0.10 (3)</td>
<td>-10</td>
<td>2.69 ± 0.28 (6)</td>
</tr>
</tbody>
</table>

Figures in parentheses represent the number of determinations.
Significant difference * p = 0.05  ≠ p = 0.01
Thus there is a marked difference in the effect observed after twenty-four hours pretreatment with norethynodrel using the two methods; the only other difference, apart from the method that is, was the fact that one group received the norethynodrel in DMSO, the other in ethyl oleate.

ii. Subacute treatment

Several semi-synthetic progestogens and oestrogens were examined for inducing ability (or any other effect that might arise) in mature female rats by giving the animals three consecutive daily doses of the steroid dissolved in ethyl oleate by intraperitoneal injection. They were killed twenty-four hours after the final dose and the usual parameters measured. The steroids used, together with the dose, were as follows: norethynodrel (20 mg/kg), chlormadinone acetate (20 mg/kg), norgestrel (10 mg/kg) and mestranol (2 mg/kg). A combination of norethynodrel (20 mg/kg) and mestranol (2 mg/kg) was also used. The known inducer, phenobarbitone sodium, was also used at a dose of 40 mg/kg. The results obtained are shown in Table 6.15. The results are expressed as a mean percentage of the controls, and the number of animals used in each group is given in parenthesis.

The results in Table 6.15 show that pretreatment of female rats with high doses of progestogen alone does not have any significant effect on liver weight, microsomal protein, cytochrome P450, 4-hydroxylation of biphenyl, reduction of p-nitrobenzoic acid or 4-MU glucuronidation. All of these parameters, except microsomal protein, are increased by similar pretreatment with a low dose of phenobarbitone. The semi-synthetic oestrogen, mestranol, increases liver weight, but does not affect any of the other parameters, and since it increases the liver size the actual amount of the other parameters must be increased. Norethynodrel and mestranol combined treatment does not produced any alteration in these liver parameters.
Table 6.15
Effect of pretreatment of mature female rats with contraceptive steroids on certain liver parameters
(Results are expressed as a percentage of the controls)

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Norethynodrel (6)</th>
<th>Chlorimadinone acetate (6)</th>
<th>Norgestrel (3)</th>
<th>Mestranol (6)</th>
<th>Norethynodrel &amp; Mestranol (6)</th>
<th>Pheno-barbitone (3)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body weight (grams)</td>
<td>96</td>
<td>100</td>
<td>92</td>
<td>91</td>
<td>97</td>
<td>100</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>101</td>
<td>107</td>
<td>102</td>
<td>127*</td>
<td>105</td>
<td>122*</td>
</tr>
<tr>
<td>Microsomal Protein</td>
<td>111</td>
<td>105</td>
<td>96</td>
<td>104</td>
<td>102</td>
<td>109</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>100</td>
<td>115</td>
<td>101</td>
<td>98</td>
<td>98</td>
<td>157*</td>
</tr>
<tr>
<td>4-Hydroxylation of biphenyl</td>
<td>104</td>
<td>113</td>
<td>113</td>
<td>102</td>
<td>102</td>
<td>150*</td>
</tr>
<tr>
<td>Reduction of p-nitrobenzoic acid</td>
<td>100</td>
<td>130</td>
<td>109</td>
<td>110</td>
<td>98</td>
<td>154*</td>
</tr>
<tr>
<td>4-MU glucuronidation</td>
<td>99</td>
<td>110</td>
<td>116</td>
<td>105</td>
<td>100</td>
<td>135*</td>
</tr>
</tbody>
</table>

*Significantly different.  \( p = 0.05 \). Figures in parentheses represent the number of animals in each group. Mean values did not vary by more than ± 2%.
iii. Chronic treatment

Mature female rats were treated intraperitoneally daily for eighteen days with ethynodiol diacetate (5 mg/kg), mestranol (0.5 mg/kg) and a mixture of both. The steroids were given in ethyl oleate; controls were given ethyl oleate alone. Ethynodiol diacetate was used in this study instead of norethynodrel since the supply of the latter became exhausted. Twenty-four hours after the last injection the animals were killed and the usual liver parameters measured. The results are shown in Table 6.16. The values are the mean of six animals in each group. The results show that pretreatment of female rats for eighteen days with the progestogen, ethynodiol, alone does not significantly alter any of the parameters measured. Pretreatment with the oestrogen, mestranol does not allow such a rapid increase in body weight as the controls, but increases significantly the liver weight; none of the other factors is altered. Pretreatment with a mixture of the two steroids also prevents such a rapid increase in body weight, which is presumably due to the mestranol; the liver, however, is not increased in weight, but the hydroxylation of biphenyl is increased significantly by 32% and the 4-MU glucuronidation is slightly, but not significantly, increased.

Table 6.17 shows the effect of 120 days treatment of rats with norethynodrel, chlormadinone acetate and ethynodiol diacetate on body weight, liver weight, microsomal protein, cytochrome P450, biphenyl hydroxylation, p-nitrobenzoic acid reduction and glucuronidation of 4-methylumbelliferone. Prolonged oral pretreatment with the two closely related progestogens, norethynodrel and ethynodiol diacetate, did not allow the rats to gain as much weight as the controls during the course of the experiment. Chlormadinone acetate treatment did not produce this effect. The liver weight is reduced by
<table>
<thead>
<tr>
<th>Parameter</th>
<th>Controls</th>
<th>Ethynodiol diacetate</th>
<th>Mestranol</th>
<th>Ethynodiol diacetate plus Mestranol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Increase in body weight (grams)</td>
<td>32 ± 2</td>
<td>24 ± 1</td>
<td>10* ± 1</td>
<td>7* ± 2</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>6.9 ± 0.3</td>
<td>7.2 ± 0.2</td>
<td>8.5* ± 0.2</td>
<td>6.7 ± 0.4</td>
</tr>
<tr>
<td>4-Hydroxylation of biphenyl</td>
<td>3.37 ± 0.11</td>
<td>3.66 ± 0.18</td>
<td>3.23 ± 0.22</td>
<td>4.47* ± 0.19</td>
</tr>
<tr>
<td>(umoles product/gm.liver/hour)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Reduction of p-nitrobenzoic acid</td>
<td>2.80 ± 0.28</td>
<td>2.54 ± 0.15</td>
<td>2.92 ± 0.12</td>
<td>2.80 ± 0.18</td>
</tr>
<tr>
<td>(umoles product/gm.liver/hour)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>4-MU</td>
<td>53 ± 8</td>
<td>60 ± 11</td>
<td>55 ± 8</td>
<td>64 ± 5</td>
</tr>
<tr>
<td>(umoles conjugated/gm.liver/hour)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>0.79 ± 0.02</td>
<td>0.79 ± 0.01</td>
<td>0.83 ± 0.02</td>
<td>0.79 ± 0.03</td>
</tr>
<tr>
<td>(umoles/mg.protein)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>25.2 ± 0.4</td>
<td>23.8 ± 1.1</td>
<td>24.2 ± 0.9</td>
<td>25.3 ± 0.5</td>
</tr>
<tr>
<td>(mg./gm.liver)</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Significantly different from controls  \( p = 0.01 \)
Table 6.17
Effect of 120 days oral pretreatment of female rats with norethynodrel, chlormadinone acetate or ethynodiol diacetate on certain hepatic drug-metabolizing enzymes, cytochrome P450, and microsomal protein

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>Chlormadinone acetate</th>
<th>Norethynodrel</th>
<th>Ethynodiol diacetate</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gain in body weight (grams)</td>
<td>76 ± 4</td>
<td>73 ± 8</td>
<td>/</td>
<td>52 ± 9</td>
</tr>
<tr>
<td>Liver weight (grams)</td>
<td>8.4 ± 0.4</td>
<td>7.4* ± 0.3</td>
<td>8.3 ± 0.3</td>
<td>8.3 ± 0.6</td>
</tr>
<tr>
<td>4-Hydroxylation of biphenyl (μmoles product/g. liver/hour)</td>
<td>4.82 ± 0.25</td>
<td>3.96* ± 0.32</td>
<td>4.63 ± 0.3</td>
<td>3.74 ± 0.36</td>
</tr>
<tr>
<td>Reduction of nitrobenzoic acid (μmoles product/g. liver/hour)</td>
<td>2.69 ± 0.07</td>
<td>2.83 ± 0.39</td>
<td>2.66 ± 0.18</td>
<td>2.61 ± 0.21</td>
</tr>
<tr>
<td>4-MUGlucuronidation (μmoles conjugated/g. liver/hour)</td>
<td>60.7 ± 8.2</td>
<td>71.3 ± 11.2</td>
<td>64.8 ± 7.1</td>
<td>-</td>
</tr>
<tr>
<td>Cytochrome P450 (μmoles/gm. liver)</td>
<td>0.74 ± 0.02</td>
<td>0.70 ± 0.01</td>
<td>0.73 ± 0.02</td>
<td>0.73 ± 0.03</td>
</tr>
<tr>
<td>Microsomal protein (mg./gm. liver)</td>
<td>29.0 ± 1.3</td>
<td>31.5 ± 0.9</td>
<td>27.8 ± 0.68</td>
<td>26.9 ± 1.2</td>
</tr>
</tbody>
</table>

Significant difference from controls *p = 0.1, / p = 0.002.
The results are the means of six animals in each group.
chlormadinone acetate pretreatment (but this is only slightly significant \( p = 0.1 \)); the other compounds do not affect the liver weight. The 4-hydroxylation of biphenyl is decreased in both the chlormadinone acetate and the ethynodiol diacetate pretreated animals, but this decrease is only slightly significant \( p = 0.1 \) in both cases. The reduction of \( p \)-nitrobenzoic acid, 4-MU glucuronidation, cytochrome P450 and microsomal protein are all slightly, but insignificantly, higher in the chlormadinone treated animals, and are not affected by norethynodrel or ethynodiol diacetate treatment.

**Discussion**

**In vitro studies**

The results obtained in the first section of this chapter show that at high concentrations both progestogens and oestrogens are capable of inhibiting hepatic microsomal drug-metabolizing systems in vitro. In the case of hydroxylation of biphenyl the synthetic progestogen derived from progesterone, chlormadinone, produced only about half the inhibition that progesterone itself produced, whilst those synthetic progestogens derived from testosterone, norethynodrel, norethisterone and ethynodiol, produced a much greater inhibition than the progesterone. With the dealkylation reactions, however, progesterone and the synthetic progestogens derived from testosterone produced similar degrees of inhibition. In this respect these results agree with those of Juchau and Fouts (1966) who found that progesterone and norethynodrel produced similar degrees of inhibition of the side-chain oxidation of hexobarbitone, but that norethynodrel produced a greater inhibition of the ring hydroxylation of zoxazolamine than progesterone. Here the similarity ends, for Juchau and Fouts (1966) obtained
70 - 80% and 30 - 40% inhibition of oxidation reactions using concentrations of steroids of $10^{-4}$M and $10^{-5}$M respectively, although the concentration of substrates was in the order of $10^{-3}$M. Similar inhibitory effects were observed by Tephly and Mannering (1968) when studying the effect of in vitro addition of several steroids, including progesterone and oestradiol on the oxidation of ethylmorphine and hexobarbitone by male rat liver microsomal preparations. Thus, the concentrations of steroids that were found to result in inhibition in the present experiments are much higher than were found by previous workers. It is interesting to note, however, that for the same concentration of steroid the inhibition of the dealkylations, where the substrate concentration is $3 \times 10^{-4}$M, is greater than the inhibition of the aromatic hydroxylation where the substrate concentration is $1.5 \times 10^{-3}$M.

The nature of the inhibition of the demethylation of 5-methoxybiphenyl by progesterone was found to be competitive. Juchau and Fouts (1966) and Tephly and Mannering (1968) also found the nature of the inhibitions mentioned above to be competitive.

Progestogens and oestrogens, both natural and synthetic, also inhibit the reduction of p-nitrobenzoic acid to p-aminobenzoic acid, but again only at high concentrations of steroids. The inhibition produced is much less than that of the aromatic hydroxylation or dealkylation, and Juchau and Fouts (1966) were unable to demonstrate any inhibitory effect of either progesterone or norethynodrel at $10^{-4}$M and $10^{-5}$M on the reduction of p-nitrobenzoic acid. Thus these steroids appear to inhibit oxidation reactions to a much greater extent than they do reduction reactions.

Inhibition of aromatic hydroxylation and dealkylation by progestogens was also demonstrated with rabbit liver
preparations as well as with the rat enzymes. However, the results were quantitatively different, inhibition being greater with the rat enzymes, particularly in the case of biphenyl hydroxylation. There was also a striking difference with the rabbit preparations in the extent of inhibition of the two different aromatic hydroxylations, the inhibition of the hydroxylation of coumarin being some four times that of the hydroxylation of biphenyl.

The above results provide further evidence that the liver microsomal enzymes that catalyze certain oxidations of drugs and foreign compounds also catalyze oxidations of steroid hormones (Kuntzman et al 1964). Now Remmer et al (1968) have stated that any substrate that can be hydroxylated will change the absorption spectrum of oxidised cytochrome P450, giving rise to one of two types of spectrum, called type I and type II. One might expect, therefore, that progesterone and oestradiol would combine with cytochrome P450 to give either type I or type II spectra. The results obtained here did not demonstrate either type of change, although typical type I and type II difference spectra were obtained with hexobarbitone and aniline respectively. However, all the differences obtained were very small, and there was a lot of interference from light scattering so that firm conclusions could not be reached. It was, however, shown quite definitely that addition of either progesterone or oestradiol produced no decrease in the CO-difference spectrum of reduced microsomes. This observation can be interpreted in several ways. Firstly, if it is assumed that a complex is formed between the steroid and the cytochrome P450, either in the oxidised or the reduced form, then this complex does not interfere with the formation of the CO-ligand complex. A second possibility is that a
complex is not formed between the cytochrome P450 and steroid, or that formation of a complex requires the intact endoplasmic reticulum. Whichever explanation is correct, the fact remains that the mere presence of progesterone or oestradiol is not sufficient to cause an apparent decrease in the cytochrome P450 CO-difference spectrum.

It is pertinent to question if these results in vitro shed any light on the cause of the effect of pregnancy on drug-metabolizing enzymes. It would certainly seem that if the inhibitory effect of pregnancy is due to substrate competition from endogenous steroids then the amount present in the liver would have to be very high indeed. For example, in order to produce a steroid concentration of $10^{-4}$M in the determination of biphenyl hydroxylation, it would require the equivalent of 0.5 mg of progesterone to be present in each gram of liver. Furthermore, although inhibition of the reduction of p-nitrobenzoic acid was not observed with pregnant animals, the inhibition seen with addition of steroids in vitro is not unreasonable since it has been shown that the reduction of nitro groups may also be mediated through cytochrome P450 (Gillette et al. 1968). However, Juchau and Fouts (1966) did not find any inhibition of nitroreduction in vitro by progesterone or norethynodrel at concentrations of $10^{-4}$ and $10^{-5}$M.

Nevertheless it is of interest that in rabbit liver preparations progesterone produces much less inhibition of biphenyl hydroxylation than in rat liver preparations, whereas the inhibition of coumarin hydroxylation is quite large, a picture which is reminiscent of the effect of pregnancy on aromatic hydroxylations in the rabbit.

The fact that neither progesterone nor oestradiol affected the CO-difference spectrum of reduced cytochrome P450 does not rule out the possibility that they would
not affect it if present initially in the intact cell, but it adds weight to the idea that the simple presence of steroids does not decrease the effective concentration of cytochrome P450, as found in pregnancy in the rat.

**Pretreatment studies: a. Naturally occurring steroids**

The results obtained in the second part of this chapter show that pretreatment of mature female rats with progesterone and oestradiol, both alone and in combination, and with pregnanediol for various lengths of time are without significant effect on the hepatic microsomal content of cytochrome P450 and protein and on the hydroxylation of biphenyl, the reduction of p-nitrobenzoic acid, and the glucuronidation of 4-methylumbelliferone by rat liver microsomes. These results in part agree with those obtained by Juchau and Pouts (1966) who found that progesterone pretreatment of immature male rats caused inhibition of the hydroxylations of hexobarbitone and zoxazolamine in liver microsomes both one and twenty-four hours after administration, although activity returned to normal in between; the pretreatment did not, however, affect the hydroxylation of 3,4-benzpyrene or aniline, or pathways involving N-demethylation, O-demethylation or sulphoxidation. It should be pointed out that these authors were using a large dose of progesterone (50 mg/kg.) and they were also using immature male rats. These differences may account for the differences between their results and those described here.

It was interesting to note that three days pretreatment of young male and female rats with a high dose of progesterone caused an upward trend in hydroxylating activity, particularly of biphenyl in the ortho position, and in cytochrome P450 content, (cf. Juchau and Pouts 1966). At this stage of development the drug-metabolizing activity of the young animal is undergoing rapid changes
and it is perhaps not surprising that a different effect is produced. Indeed, Kato and Takanaka (1968) have shown that the inducing effect of phenobarbitone is much more marked in young rats than in old.

Do these pretreatment studies shed any further light on the role of steroid hormones in pregnancy with respect to drug-metabolizing enzymes? If they do then the answer must be that the steroids, at least progesterone and oestradiol, and probably their metabolites, are not responsible for the effect. However, what relation do these pretreatments with steroids bear to pregnancy? During pregnancy the steroids are being produced continuously so that presumably the liver is having to metabolize them all the time. Thus there could conceivably be an inhibitory concentration of steroid present all the time. Now, none of the pretreatment studies carried out here produce this effect exactly. However, one would expect that after a brief pretreatment time with a high dose of steroid there would be sufficient circulating in the blood to produce a reasonable level in the liver. Thus, one hour's pretreatment might have been expected to produce this effect. Such a pretreatment period with either progesterone or oestradiol did not alter any of the parameters measured.

It was shown in Chapter five, however, that it was only towards the end of pregnancy that inhibition of certain drug-metabolizing enzymes occurred, which might suggest that prolonged "exposure" to steroids was required to produce the effect, whether by direct competition or by some other means. Eighteen days' intramuscular pretreatment of rats was therefore carried out, but this did not alter the level of drug-metabolizing enzymes or cytochrome P450. Neither did any of the treatments cause an increase in liver size, which is associated with pregnancy, although progesterone did lead to an increased body weight.
It should, of course, be recognized that only one dose regimen has been used in these studies and that different effects may be produced by other doses. Nevertheless, one might have expected some indications of inhibition if, in fact, these steroids produce such an effect.

It would appear that neither progesterone nor oestradiol pretreatment affect hepatic microsomal drug metabolism in the same manner as was observed with addition of these steroids in vitro. These findings are not contradictory since in the latter instance extremely high levels of steroid were required to produce inhibition and comparable levels were unlikely to be produced either in the pretreatment experiments or pregnancy.

The evidence provided here does not support the idea that either natural progestogens or oestrogens are responsible for the inhibitory effect of pregnancy on drug-metabolizing enzyme systems in the rat.

Pretreatment studies: b. Oral contraceptive steroids

The results obtained in the third section of this chapter indicate that synthetic steroids used as oral contraceptives do not produce any generalized inhibitory or inductive effects on hepatic microsomal drug-metabolizing enzymes in the mature female rat.

The effect of pretreatment of female rats with a large dose of norethynodrel produced contrasting effects in several experiments on the hydroxylation of biphenyl. When the hydroxylating activity was determined using the original assay conditions (Creaven et al 1965b) and the norethynodrel was administered in dimethyl sulfoxide an effect similar to that produced by certain other drug-metabolizing enzyme inhibitors (such as SKF 525-A) where there is an initial inhibition followed by an enhancement
of the enzyme activity, was obtained. This finding is similar to that obtained by Juchau and Fouts (1966). However, when given in ethyl oleate, and with the hydroxylating enzyme activity determined using the revised method, norethynodrel produced inhibition of the hydroxylation both one and twenty-four hours after administration. There seem to be two possible explanations for these results. One is that the solvent played a part in the effect, the dimethyl sulphoxide perhaps giving a more rapid absorption of the steroid so that it was metabolized and removed more quickly and in so doing produced induction, whereas the ethyl oleate gave rise to a slow release of the steroid so that it was still around in sufficient quantity twenty-four hours later to produce an inhibition. The second possible explanation is that norethynodrel increased the cofactor levels in the liver, so that once its initial inhibitory effect was over, it caused stimulation under the assay conditions in which there was a limiting concentration of cofactor (NADP), but inhibition where the cofactor was optimum (high levels of NADP were found to inhibit the hydroxylation of biphenyl).

In spite of these results, three days' pretreatment with norethynodrel did not affect the hydroxylation of biphenyl. Indeed, three days' pretreatment of mature female rats with the various steroids produced no significant effects on the hydroxylation of biphenyl, or on any of the other drug-metabolizing enzymes or cytochrome P450. Phenobarbitone, which is one of the most potent inducers of drug-metabolizing enzymes, did produce a significant induction of these enzymes under the same conditions, but the induction was not very marked, probably because mature animals were used (Kato and Takanaka, 1968). It cannot be stated that these steroids are not inducers of drug metabolism because
this is usually tested for in immature animals, but it can be said that in mature female rats they do not produce any induction of drug-metabolizing enzymes using the normal three-day test for induction. (It should be remembered that it is in mature females that these steroids are generally used.)

One significant point is that mestranol pretreatment for both three and eighteen days increased the liver weight. Consequently the total activity of the various enzymes was increased, although the specific activity remained the same. This effect was not produced in combination with ethynodiol.

Chronic administration of ethynodiol diacetate together with mestranol by intraperitoneal injection produced a significant increase in the hydroxylation of biphenyl, but did not affect any of the other enzymes or cytochrome P450 content. This effect could not be attributed to either of the components alone, and is similar to the observation that chronic administration of medroxyprogesterone acetate with ethinyloestradiol produces an increase in the hepatic microsomal enzymes responsible for the hydroxylation of aniline, the N-demethylation of aminopyrine and the O-demethylation of p-nitroanisole (Jori et al. 1969), although this was shown to be due to the progestogen. However, this is in contrast with the observation that norethynodrel with mestranol treatment of rats decreases the hydroxylation of hexobarbitone (Juchau and Fouts, 1966) in liver microsomes.

Prolonged oral administration of ethynodiol diacetate alone at a much smaller dose decreased body weight gain and the 4-hydroxylation of biphenyl by liver preparations but did not affect any of the other parameters. Similar treatment with norethynodrel produced a similar effect on body weight but did not affect any of the other
parameters. With prolonged oral administration of chlormadinone acetate, however, the liver weight and 4-hydroxylation of biphenyl were decreased but none of the other parameters was affected. Thus the only reaction to be affected was the aromatic hydroxylation. It would seem unlikely that such a small dose of steroid could produce inhibition by competition with the enzyme substrate, especially as the dose is only given once daily. However, the steroids may be retained in the body on prolonged administration to different extents, and then released very slowly, or alternatively produced their effect in some other manner.

Contrasting results have been shown here; the administration of a mixture of progestogen and oestrogen have been shown to stimulate hydroxylation of biphenyl, while administration of a different progestogen alone has been shown to inhibit the hydroxylation. These results contrast with those of Juchau and Fouts (1966) who showed that the administration of a mixture of progestogen or oestrogen caused inhibition of the hydroxylation of hexobarbitone, but that the progestogen alone produced a stimulation of the hydroxylation.

The results obtained in this chapter, therefore, show that although both naturally occurring and synthetic progestogens and oestrogens produce inhibition of drug-metabolizing enzymes in vitro in large concentrations, pretreatment of mature female rats with them for prolonged periods does not produce any generalized effect.
CHAPTER VII

EFFECTS OF PREGNANCY AND CONTRACEPTIVE STEROIDS ON THE PHARMACOLOGICAL ACTIVITY OF HEXOBARBITONE
Introduction

The previous two chapters have been concerned with the effect of pregnancy and oral contraceptives on drug metabolism at the molecular level. It is obviously desirable to investigate these effects in the intact animal as well, and this has been done by studying the duration of action of hexobarbitone, and the in vivo metabolism of phenacetin (see Chapter eight).

The duration of narcosis following administration of hexobarbitone affords a convenient in vivo measure of hepatic microsomal enzyme function since the duration of action is primarily dependent on the rate of microsomal detoxication of the drug in the liver (Conney, 1967). Thus the effect of pregnancy and pretreatment with naturally occurring and semi-synthetic steroids on the response to hexobarbitone in rats is described in this chapter.

There has been one previous report of the effect of pregnancy on the duration of pentobarbitone anaesthesia in rats (King et al., 1963) and one recent report (Feuer and Liscio, 1969). Both of these show that pregnancy increases the duration of action of pentobarbitone, indicating that the metabolism of pentobarbitone has been inhibited. The reasons for the increase are not known, although it has been suggested that sex hormones play a role.

The duration of action of hexobarbitone is much longer in adult female rats than in adult males (Quinn et al., 1958), a difference that only becomes apparent at the age of five to six weeks, when there is an abrupt decrease in the action of the drug in male rats due to increased activity of the hexobarbitone metabolizing enzyme(s). It was therefore suggested that the male hormones were responsible for this change at puberty.
Oestrogen, but not progesterone, treatment of male rats decreases the activity of liver microsomal systems that metabolize hexobarbitone (Quinn et al, 1958; Juchau and Fouts, 1966). The lower levels of cytochrome P450 in liver preparations from pregnant rats (Chapter five) might suggest that the metabolism of hexobarbitone would be inhibited and, therefore, its duration of action longer in pregnant rats. However, the fact that the total hepatic content of cytochrome P450 is the same in pregnant and non-pregnant rats would suggest that there would be no difference in duration of action of hexobarbitone.

It was of interest therefore to investigate the effect of pregnancy on hexobarbitone sleeping time, and the effect of treatment of mature female rats with progestogens and oestrogens.

Materials and Methods

The rats used were all of the Wistar albino strain and were kept under similar conditions to those described in Chapter two. Pregnancy was established using methods described in Chapter five.

In the acute and subacute pretreatment studies the steroids were administered in ethyl oleate (0.5 ml) intraperitoneally at doses of 10 - 20 mg/kg for progestogens and 0.5 - 1 mg/kg for oestrogens; controls received ethyl oleate alone. In acute studies animals received a dose of hexobarbitone at various intervals after a single dose of steroid. Subacute pretreatment studies involved treating rats with a daily dose of steroid for three days and then administration of the hexobarbitone twenty-four hours after the final dose. In the chronic studies the rats received a daily dose of the steroid (10 mg progesterone/kg, i.m., 2 mg oestradiol/kg, i.m., 5 mg ethynodiol diacetate/kg, i.p.)
or 0.5 mg mestranol/kg, i.p.) for eighteen days and were given the hexobarbitone twenty-four hours after the final injection of steroid.

Hexobarbitone was administered as a solution of the sodium salt in water. Initially hexobarbitone sodium as "Cyclonal sodium" (May and Baker) was used, but later the solution of the sodium salt was prepared by dissolving hexobarbitone base (May and Baker) in an equivalent amount of sodium hydroxide solution. All solutions of hexobarbitone sodium were prepared freshly for each experiment.

The duration of action of hexobarbitone, otherwise known as hexobarbitone sleeping time, was determined as follows. Rats were weighed immediately before dosing and given a dose of 100 mg/kg (females) and 200 mg/kg (males) dissolved in water by intraperitoneal injection. The time of injection was taken as the start of the sleeping time although this was not strictly correct as the animal did not lose its righting reflex until some two to three minutes later; however, it was impractical, when injecting large numbers of animals, to determine the point of loss of righting reflex accurately. Once the animal had lost consciousness it was laid on its back on the bench under an electric light to keep it warm. During the period of anaesthesia the tail of each animal was pinched every five minutes for the first thirty minutes, then every two minutes until the righting reflex was restored. If this stimulus was not given it was found that the rats tended to "sleep" longer and more irregularly. The righting reflex was considered to be restored when the animal could "right" itself three immediately consecutive times after being laid on its back. The difference between the time of injection and this time was taken as the hexobarbitone sleeping time.
Results

Effect of pregnancy

The duration of action of hexobarbitone given on a weight basis in non-pregnant rats and in pregnant rats at different stages of pregnancy was investigated and the results are shown in Table 7.1.

Table 7.1
Duration of action of hexobarbitone in non-pregnant and pregnant rats at different stages of pregnancy

<table>
<thead>
<tr>
<th>Length of pregnancy (days)</th>
<th>No. of animals</th>
<th>Sleeping time (mins.)</th>
<th>Percentage difference from non-pregnants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-pregnant</td>
<td>22</td>
<td>53 ± 3</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>7</td>
<td>46 ± 4</td>
<td>- 13</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>54 ± 2</td>
<td>+ 2</td>
</tr>
<tr>
<td>13</td>
<td>6</td>
<td>64 ± 2</td>
<td>+ 21</td>
</tr>
<tr>
<td>15</td>
<td>6</td>
<td>68 ± 3</td>
<td>+ 28</td>
</tr>
<tr>
<td>17</td>
<td>6</td>
<td>87 ± 4</td>
<td>+ 64</td>
</tr>
<tr>
<td>20</td>
<td>6</td>
<td>113 ± 2</td>
<td>+113</td>
</tr>
<tr>
<td>1 day post partem</td>
<td>3</td>
<td>56 ± 3</td>
<td>+ 6</td>
</tr>
</tbody>
</table>

Significantly different from controls / p = 0.01; * = 0.001

The results show that in three-day pregnant animals there is a slight decrease in the sleeping time but this is not significant. There is no difference in sleeping time between non-pregnant animals and two ten-day pregnant animals tested. However, there is a progressive
increase in sleeping time thereafter until the twentieth day of pregnancy when the increase is 113%. One day post partem the sleeping time is back to that of non-pregnant animals.

During pregnancy the rat increases in weight quite considerably. The mean increase in body weight in six rats is shown in Table 7.2.

Table 7.2

Body weight increase during pregnancy in the rat

<table>
<thead>
<tr>
<th>Days Pregnant</th>
<th>Weight (g)</th>
<th>Percentage increase above initial (g)</th>
</tr>
</thead>
<tbody>
<tr>
<td>0</td>
<td>271 ± 7</td>
<td>-</td>
</tr>
<tr>
<td>3</td>
<td>274 ± 8</td>
<td>1</td>
</tr>
<tr>
<td>6</td>
<td>284 ± 7</td>
<td>5</td>
</tr>
<tr>
<td>10</td>
<td>295 ± 8</td>
<td>9</td>
</tr>
<tr>
<td>13</td>
<td>305 ± 9</td>
<td>13</td>
</tr>
<tr>
<td>15</td>
<td>316 ± 8</td>
<td>17</td>
</tr>
<tr>
<td>17</td>
<td>330 ± 10</td>
<td>22</td>
</tr>
<tr>
<td>20</td>
<td>361 ± 5</td>
<td>33</td>
</tr>
<tr>
<td>1 day post partem</td>
<td>304 ± 7</td>
<td>12</td>
</tr>
</tbody>
</table>

The table shows that it is over the last week that most of the weight gain occurs. This is probably due to the growth of the foetuses since it is at this stage that they begin increasing in size. The gain in weight before this is due to the mother increasing in size. Thus, after the mother has littered, the body weight returns to what it was at the thirteenth day and not what it was initially.
The sleeping time and body weight, therefore, both increase during pregnancy. Fig. 7.1 shows the relative percentage increases in body weight and sleeping time with increased duration of pregnancy. During the first half of pregnancy the sleeping time does not increase although the body weight does, indicating that the mother is able to metabolize the hexobarbitone at the same rate as the non-pregnant rat. During the second half of pregnancy, however, the sleeping time increases, and at a greater rate than the weight increases, indicating that the mother is unable to metabolize the increased amount of hexobarbitone at the same rate as the non-pregnant. Is the increase in sleeping time simply due, then, to the increased amount of hexobarbitone that the animal receives due to its increased weight, or is it due to some other factor such as inhibition of the hepatic drug-metabolizing enzymes? To investigate this further the dose/response for hexobarbitone in normal animals was studied. Four groups of eight non-pregnant female rats were given doses (50, 100, 115 and 130 mg/kg) of hexobarbitone, and the sleeping times were determined as previously described. The doses were chosen to correspond to the amounts given to the pregnant animals; thus as the full-term pregnant animal was given a 30% increase in total dose, to to a 30% increase in dose was used in these experiments with non-pregnant animals. The results are shown in Table 7.3.

Table 7.3
Dose-response for hexobarbitone in female rats

<table>
<thead>
<tr>
<th>Dose of hexobarbitone (mg/kg)</th>
<th>Sleeping time* (mins.)</th>
</tr>
</thead>
<tbody>
<tr>
<td>50</td>
<td>11 ± 1</td>
</tr>
<tr>
<td>100</td>
<td>69 ± 4</td>
</tr>
<tr>
<td>115</td>
<td>83 ± 9</td>
</tr>
<tr>
<td>130</td>
<td>113 ± 6</td>
</tr>
</tbody>
</table>

* The results are the mean ± S.E.M. for eight animals in each group.
Fig. 7.1 Relative percentage increases in body weight and hexobarbitone sleeping time during pregnancy in the rat.
The results show that there is an almost linear relationship between dose and sleeping time over the dose range used. However, for an increase of 30% in the dose from 100-130 mg/kg, the sleeping time increases by 63%.

The question then arises as to whether the pregnant rat is as capable as the non-pregnant in metabolizing the same total dose of hexobarbitone. An experiment was carried out, therefore, whereby pregnant rats were given a dose of hexobarbitone of 100mg/kg based on their weight before mating. Controls were weighed at the same time and given a dose on the same basis. The results are shown in Table 7.4.

**Table 7.4**

Duration of action of hexobarbitone in non-pregnant and pregnant rats dosed on a basis of their original non-pregnant weight

<table>
<thead>
<tr>
<th>Days pregnant</th>
<th>No. of animals</th>
<th>Sleeping time</th>
<th>Percentage difference from non-pregnants</th>
</tr>
</thead>
<tbody>
<tr>
<td>Non-pregnant</td>
<td>5</td>
<td>47 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>7</td>
<td>3</td>
<td>43 ± 1</td>
<td>- 8</td>
</tr>
<tr>
<td>10</td>
<td>2</td>
<td>42 ± 2</td>
<td>- 10</td>
</tr>
<tr>
<td>15</td>
<td>3</td>
<td>33 ± 6</td>
<td>- 30</td>
</tr>
<tr>
<td>20</td>
<td>3</td>
<td>44 ± 5</td>
<td>- 6</td>
</tr>
</tbody>
</table>

The results show that at all stages of pregnancy the pregnant rat is just as capable, and in some cases more capable, of metabolizing the same total dose of hexobarbitone. Thus, in the 15-day pregnant animal the sleeping time is reduced by 30%, but in the 20-day pregnant animal it is not reduced.
Effect of pretreatment with steroids

Table 7.5 shows the effect of acute pretreatment of both male and female rats with progesterone on hexobarbitone sleeping times. The dose of progesterone used was 10 mg/kg; both male and female rats were given a dose of 100 mg/kg of hexobarbitone.

Table 7.5
Effect of acute progesterone pretreatment on hexobarbitone sleeping time in male and female rats

<table>
<thead>
<tr>
<th>Pretreatment period</th>
<th>Sex</th>
<th>Sleeping time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>None</td>
<td>M</td>
<td>23 ± 1.2 (6)</td>
</tr>
<tr>
<td>1 hour</td>
<td>M</td>
<td>23 ± 0.3 (3)</td>
</tr>
<tr>
<td>24 hours</td>
<td>M</td>
<td>22 ± 1.2 (3)</td>
</tr>
<tr>
<td>None</td>
<td>F</td>
<td>81 ± 5 (6)</td>
</tr>
<tr>
<td>1 hour</td>
<td>F</td>
<td>86 ± 3 (6)</td>
</tr>
<tr>
<td>24 hours</td>
<td>F</td>
<td>81 ± 4 (4)</td>
</tr>
</tbody>
</table>

Figures in parentheses refer to the number of rats in each group.

* Statistically different from controls p = 0.02.

The results show that pretreatment of male rats with progesterone for one or twenty-four hours does not alter the hexobarbitone sleeping time. With female rats the results are different; one hours pretreatment resulted in an increased sleeping time, but twenty-four hours pretreatment produced no difference.
Similar acute pretreatment studies were carried out using the semi-synthetic progestogen norethynodrel. A dose of 20 mg/kg was given in dimethyl sulphoxide, and the results are shown in Table 7.6.

**Table 7.6**

**Effect of acute norethynodrel pretreatment on hexobarbitone sleeping times in male and female rats**

<table>
<thead>
<tr>
<th>Pretreatment period</th>
<th>Sex</th>
<th>Sleeping time (minutes)</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td>Controls</td>
</tr>
<tr>
<td>None</td>
<td>M</td>
<td>23 ± 1</td>
</tr>
<tr>
<td>1 hour</td>
<td>M</td>
<td>19 ± 4</td>
</tr>
<tr>
<td>24 hours</td>
<td>M</td>
<td>24 ± 1</td>
</tr>
<tr>
<td>None</td>
<td>F</td>
<td>81 ± 5</td>
</tr>
<tr>
<td>1 hour</td>
<td>F</td>
<td>72 ± 3</td>
</tr>
<tr>
<td>24 hours</td>
<td>F</td>
<td>73 ± 3</td>
</tr>
<tr>
<td>48 hours</td>
<td>F</td>
<td>76 ± 9</td>
</tr>
</tbody>
</table>

Figures in parentheses refer to the number of animals in each group.
Statistically different from controls ≠ p = 0.05; ∆ p = 0.001

Pretreatment of male rats one hour previously with norethynodrel caused an increase in the sleeping time of 80%, but 24 hours pretreatment produced no difference. Similar results were obtained with female rats although the increase in sleeping time after one hour's treatment was slightly greater than in the males (90%). The effect of a single dose of norethynodrel 48 hours before hexobarbitone caused a decrease in sleeping time of 40% in the female rats indicating that the metabolism had been increased.
The effect of pretreatment of female rats with a single dose of various contraceptive hormones dissolved in ethyl oleate twenty-four hours before hexobarbitone is shown in Table 7.7. The hormones used were chlormadinone acetate (20 mg/kg), norethynodrel (20 mg/kg), and mestranol (1.0 mg/kg).

Table 7.7

Effect of twenty-four hours pretreatment of female rats with contraceptive steroids on the duration of action of hexobarbitone

<table>
<thead>
<tr>
<th>Steroid</th>
<th>No. of animals</th>
<th>No. of animals losing righting reflex</th>
<th>Sleeping time (mins.)</th>
<th>Percentage difference from controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>12</td>
<td>12</td>
<td>85 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>Chlormadinone</td>
<td>6</td>
<td>5</td>
<td>80 ± 4</td>
<td>- 6</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>6</td>
<td>5</td>
<td>58 ± 5</td>
<td>- 32%</td>
</tr>
<tr>
<td>Mestranol</td>
<td>6</td>
<td>6</td>
<td>62 ± 5</td>
<td>- 26%</td>
</tr>
</tbody>
</table>

* Statistically different from controls  p = 0.01

/ The sleeping time is the mean of those animals which lost their righting reflex.

The results show that twenty-four hours after a dose of norethynodrel or mestranol the hexobarbitone sleeping time is significantly decreased, but after a dose of chlormadinone, it is not significantly different from controls. The results for norethynodrel differ from those given in Table 7.6, where no difference in sleeping time after twenty-four hours was observed. Those results were obtained at St. Mary's, while these were obtained in Surrey. The other difference was that
the norethynodrel in the first instance was given in
dimethyl sulphoxide, and in the second in ethyl oleate.

The effects of pretreatment of female rats with
various hormones for three days on the duration of
action of hexobarbitone are shown in Table 7.8. The
steroids used were progesterone (20 mg/kg), oestradiol
(0.5 mg/kg), chlormadinone acetate (20 mg/kg),
norethynodrel (20 mg/kg) and mestranol (1.0 mg/kg).

Table 7.8

Effect of three days pretreatment of female rats with
progestogens and oestrogens on the duration of action
of hexobarbitone

<table>
<thead>
<tr>
<th>Steroid</th>
<th>No. of animals</th>
<th>No. of animals losing righting reflex</th>
<th>Sleeping time (mins.)</th>
<th>Percentage difference from controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>12</td>
<td>12</td>
<td>86 ± 4</td>
<td>-</td>
</tr>
<tr>
<td>Progesterone</td>
<td>6</td>
<td>6</td>
<td>85 ± 5</td>
<td>-2</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>6</td>
<td>6</td>
<td>87 ± 5</td>
<td>+3</td>
</tr>
<tr>
<td>Chlormadinone acetate</td>
<td>6</td>
<td>5</td>
<td>73 ± 5</td>
<td>-15</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>6</td>
<td>3</td>
<td>59 ± 3</td>
<td>-31&lt;sup&gt;※&lt;/sup&gt;</td>
</tr>
<tr>
<td>Mestranol</td>
<td>6</td>
<td>6</td>
<td>54 ± 2</td>
<td>-38&lt;sup&gt;※&lt;/sup&gt;</td>
</tr>
</tbody>
</table>

<sup>※</sup> Significantly different from controls: p = 0.01
<sup></sup> The sleeping time is the mean of those animals which lost their righting reflex.

The results in Table 7.8 show that progesterone and
chlormadinone acetate do not produce any significant
difference in sleeping time from the controls, while
norethynodrel and mestranol produce similar decreases in
sleeping time, which are significant. Oestradiol does not alter the sleeping time. It should be noted that with the norethynodrel the number of animals that did not lose their righting reflexes was quite high. This might indicate that induction was greater in these animals, or that norethynodrel alters the sensitivity of the nervous system to hexobarbitone.

The effect of three days pretreatment of male rats with these hormones is shown in Table 7.9. The hormones used were progesterone (10 mg/kg), chlormadinone acetate (10 mg/kg), norethynodrel (10 mg/kg) and mestranol (1 mg/kg). The dose of hexobarbitone used was 200 mg/kg.

Table 7.9

Effect of three days pretreatment of male rats with progestogens and oestrogens on the duration of action of hexobarbitone

<table>
<thead>
<tr>
<th>Steroid</th>
<th>No. of animals</th>
<th>No. of animals losing righting reflex</th>
<th>Sleeping time (mins.)</th>
<th>Percentage difference from controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls</td>
<td>12</td>
<td>10</td>
<td>42 ± 6</td>
<td>-</td>
</tr>
<tr>
<td>Progesterone</td>
<td>6</td>
<td>6</td>
<td>46 ± 2</td>
<td>+9</td>
</tr>
<tr>
<td>Chlormadinone acetate</td>
<td>6</td>
<td>5</td>
<td>43 ± 2</td>
<td>+2</td>
</tr>
<tr>
<td>Norethynodrel</td>
<td>6</td>
<td>5</td>
<td>52 ± 3</td>
<td>+24</td>
</tr>
<tr>
<td>Mestranol</td>
<td>6</td>
<td>6</td>
<td>85 ± 8</td>
<td>+102†</td>
</tr>
</tbody>
</table>

* The sleeping time is the mean of those animals which lost their righting reflex.
† Significant difference from controls p = 0.001.

The results show that neither progesterone nor chlormadinone acetate have any effect on the duration of
action of hexobarbitone in male rats at the dose used here. Norethynodrel, however, causes a small but insignificant increase in sleeping time, while mestranol causes a very large significant increase.

The final results in this chapter are concerned with the prolonged treatment (18 days) of female rats with progestogens and oestrogens both separately and combined. The natural hormones used were progesterone (2.5 mg per animal daily) and oestradiol (0.5 mg per animal daily) and combined at the same doses. The semi-synthetic hormones used were ethynodiol diacetate (1.0 mg per animal daily) and mestranol (0.1 mg per animal daily) and combined at the same doses. The effects of such treatments on the duration of action of hexobarbitone are shown in Table 7.10.

Table 7.10
Effect of eighteen days pretreatment of female rats with progestogens and oestrogens on the duration of action of hexobarbitone

<table>
<thead>
<tr>
<th>Steroid</th>
<th>No. of animals</th>
<th>Sleeping time</th>
<th>Percentage difference from controls</th>
</tr>
</thead>
<tbody>
<tr>
<td>Controls A</td>
<td>5</td>
<td>72 ± 7</td>
<td>-</td>
</tr>
<tr>
<td>&quot;</td>
<td>6</td>
<td>81 ± 2</td>
<td>-</td>
</tr>
<tr>
<td>Progesterone</td>
<td>5</td>
<td>66 ± 3</td>
<td>-8</td>
</tr>
<tr>
<td>Oestradiol</td>
<td>6</td>
<td>68 ± 4</td>
<td>-6</td>
</tr>
<tr>
<td>Progesterone + Oestradiol</td>
<td>4</td>
<td>69 ± 3</td>
<td>-4</td>
</tr>
<tr>
<td>Ethynodiol diacetate</td>
<td>6</td>
<td>58 ± 2</td>
<td>-28 **</td>
</tr>
<tr>
<td>Mestranol</td>
<td>6</td>
<td>69 ± 6</td>
<td>-15</td>
</tr>
<tr>
<td>Ethynodiol diacetate + Mestranol</td>
<td>6</td>
<td>57 ± 4</td>
<td>-30 **</td>
</tr>
</tbody>
</table>

* Significantly different from controls: p = 0.001
A = controls for progesterone and oestradiol animals
B = " " ethynodiol diacetate and mestranol animals.
The results show that the sleeping times are not altered by prolonged treatment with progesterone or oestradiol either alone or combined. Treatment with mestranol does not significantly alter the sleeping time although it is slightly reduced, while treatment with ethynodiol diacetate, either alone or in combination with mestranol significantly decreases the sleeping time.

Discussion

It has been shown that in the 20-day pregnant rat the duration of action of hexobarbitone is more than doubled when compared with non-pregnant rats, when the animals are dosed on a weight basis. This finding agrees with those of King et al (1963) and Feuer and Liscio (1969), that pentobarbitone anaesthesia is prolonged in pregnant rats. It has also been shown that the pregnant rat increases in weight during pregnancy, and it therefore receives a greater amount of hexobarbitone than the non-pregnant when dosed on a weight basis. Is the increased sleeping time, therefore, simply due to the fact that the pregnant animal receives a greater total of hexobarbitone, or is it due to some other factor such as inhibition of its metabolism? Taking the 20-day pregnant rat as a whole, its increase in weight, and therefore the increase in the dose of hexobarbitone it receives, is approximately 30%, and this increases the sleeping time by over 100%. When a non-pregnant rat is given a similar increase in dose, the sleeping time is only increased by 63%, i.e. about half this amount. This would indicate that the greater amount of hexobarbitone that the pregnant rat receives could be responsible for some, but not all, of the increase in sleeping time. However, it was shown in Chapter five that in the 20-day pregnant rat the liver weight increases in proportion to the body weight. If, therefore, the specific activity (the activity of 1 gm. of
liver) for hexobarbitone metabolism of the liver from the pregnant rat was the same as that of the non-pregnant, then the hexobarbitone should be metabolized at a similar rate and the sleeping time should be the same when animals are dosed on the basis of body weight. The fact that it is not, suggests that the specific activity of the liver with regard to hexobarbitone metabolism is decreased in pregnant rats. This agrees with the findings in Chapter five that the hydroxylation of biphenyl and cytochrome P450 content are decreased in the livers of 20-day pregnant rats when the results are expressed as a function of unit liver weight. These findings suggest that the increased sleeping time in pregnancy could be due to the impaired ability of the liver to metabolize hexobarbitone, and not simply to the increased amount of hexobarbitone that the pregnant rat receives.

Could the impaired liver function be responsible for an increase of over 100% in the sleeping time? On the face of it this would require the metabolism to be inhibited by over 50%. It should be remembered, however, that there is a threshold dose (the dose response data obtained did not use a dose as low as this threshold) of hexobarbitone, and it is only the dose above this threshold that is effective in causing anaesthesia. Thus, merely doubling the dose of hexobarbitone from 50 mg/kg to 100 mg/kg increases the sleeping time sixfold. Therefore, in doing the reverse, i.e. inhibiting the metabolism, it would not be necessary to inhibit the metabolism by 50% to double the effective dose above the threshold. Therefore the decrease of 26% observed in cytochrome P450 in chapter five may be sufficient to produce the 113% increase in sleeping time. Nevertheless other possible causes should be borne in mind.

The sleeping time was only increased during the second half of pregnancy, the greatest increase being over the last seven days. The body weight, though,
increases all the time during pregnancy, although there is a more rapid growth during the last seven days. This would indicate that over the first half of pregnancy, when the increase in weight is due mainly to the growth of maternal and placental tissues, the liver maintains its metabolizing ability, but during the second half, and more particularly the last seven days, when the increase in weight is due to the growth of the foetuses, the liver is unable to maintain its metabolizing ability. Now the foetuses are separated from the mother by the placental barriers, and although the hexobarbitone will diffuse across the placental barrier it will probably not do so as rapidly as it can diffuse around the mother's body. Therefore the effective dose in the mother's body will be slightly increased, so contributing to the increased duration of action, though this is unlikely to account for all the increase. However, it does raise one other important point, and that is the distribution of the hexobarbitone in the pregnant rat. If the distribution of the hexobarbitone is similar in the pregnant and non-pregnant animal, then the increased duration of action is probably due to inhibition of its metabolism. If the distribution is not similar, then this may contribute to the increased duration of action.

Although it has been shown that the duration of action of hexobarbitone is increased in pregnant rats when the hexobarbitone is given on a weight basis, when the same total dose is given to 20-day and non-pregnant rats there is no difference in sleeping time, indicating that the total capacity of the liver for hexobarbitone metabolism is unaltered by pregnancy. With 15-day pregnant rats dosed similarly, the sleeping time is less than the non-pregnants. These results also agree with the findings in Chapter five that the total capacity of the liver for hydroxylation of biphenyl and for cytochrome P450
content is similar in the 20-day pregnant and non-pregnant rat, but slightly greater in the 15-day pregnant rat.

It has been established for some time now that certain steroids employed in high doses have an anaesthetic action (Selye 1941; Pan and Laubach 1964), and progesterone has this action. It seemed possible, therefore, that the increase in duration of action of hexobarbitone could be due to an additive effect of the progesterone and hexobarbitone, or similarly progesterone lowering the threshold level of hexobarbitone. Progesterone levels are, in fact, very high during pregnancy, but in the pregnant rat progesterone levels are maximal at the fourteenth to fifteenth day of gestation (Eto et al, 1962; Hashimoto et al, 1968) and thereafter decline steadily until parturition. It seems unlikely, then, that the increased duration of action of hexobarbitone is due to the anaesthetic action of progesterone, as there is an inverse relationship between the levels of the progesterone and the sleeping time.

Juchau and Pouts (1966) found that progesterone inhibited hexobarbitone metabolism when added in vitro at concentrations of $10^{-5}M$ to incubations of rat liver homogenate. They also found that pretreatment of young male rats with progesterone (50 mg/kg) for one hour or twenty-four hours caused inhibition of the in vitro hydroxylation of hexobarbitone. These observations might suggest that progesterone increases the duration of action of hexobarbitone by inhibiting its metabolism, and one could therefore implicate it as having this role in pregnancy. However, the same argument to that applied in the preceding paragraph can be forwarded. If progesterone were the cause, one would expect maximal inhibition, and therefore longest duration of action of hexobarbitone, about the fifteenth day of pregnancy.
The effect of pretreatment of male and female rats with progesterone was carried out to see whether it did have any effect on the duration of action of hexobarbitone. Pretreatment of male rats did not alter the sleeping time. These results do not agree with those of Juchau and Pouts (1966), but they were using younger rats and a larger dose (50 mg/kg) of progesterone. Pretreatment of female rats was studied for one hour, twenty-four hours, three days and eighteen days; only the one hour's pretreatment caused any significant alteration in hexobarbitone sleeping time. This would indicate that the presence of progesterone in the body does increase the hexobarbitone sleeping time by one of the methods mentioned above, but that for this effect to become apparent the level needs to be very high indeed. Why does progesterone pretreatment for one hour cause inhibition in the females but not in the males? Now male rats metabolize hexobarbitone more rapidly than females, consequently the sleeping time of the former induced by the same dose of hexobarbitone is less. The rate of metabolism in the male, as it is so rapid, may not be the limiting factor which determines the duration of anaesthesia, so that any inhibition produced by progesterone is not manifested. Alternatively, the progesterone may also be metabolized more rapidly so that any effect would disappear after one hour.

It is not possible to state categorically that the inhibition of hexobarbitone metabolism during pregnancy is not due to progesterone, because although eighteen days chronic treatment does not increase the sleeping time, only one dose level has been used, and that was given once daily, albeit by intramuscular injection. The inhibition of metabolism in pregnancy could, of course, be due to oestrogen, or may require a combination of oestrogen and progesterone. However, neither oestradiol nor progesterone plus oestradiol affected hexobarbitone sleeping time in female rats dosed for eighteen days.
It should be pointed out here that during pregnancy these hormones are being produced continuously. It is not possible to do this in pretreatment studies, although intramuscular injections do give a prolonged release, but necessarily at low levels. Therefore, the effect of the continuous presence of steroids in pregnancy may be similar to that produced by one hour's pretreatment with progesterone. Nevertheless the results do suggest that it is not these steroids that are responsible for the increased sleeping time during pregnancy.

The explanation suggested in Chapter five fits in with the results found here. There it was suggested that during the later stages of pregnancy when the maternal organism has to provide the material for a very rapid protein synthesis, this is channelled away from producing drug-metabolizing enzymes and cytochrome P450. This is possibly brought about by increased genetic repression produced by growth hormones. Now the most rapid growth, and therefore presumably most rapid production of growth hormone occurs over the last seven days of pregnancy, and this corresponds to the period over which duration of action of hexobarbitone increases. It is possible, therefore, that the growth hormone produced during pregnancy is responsible for the decreased metabolism of hexobarbitone.

Whether the natural steroids of pregnancy are responsible for the effect on the duration of action of hexobarbitone or not, what are the effects of steroids used as oral contraceptive agents?

Norethynodrel pretreatment of both male and female rats for one hour resulted in the duration of action of hexobarbitone being almost doubled. This finding is in agreement with that of Juchau and Fouts (1966) who found that the hexobarbitone hydroxylase activity of the liver of young male rats was decreased one hour after a dose of
norethynodrel. Jori et al (1969) have also reported that the pentobarbitone anaesthesia of female rats dosed two hours previously with norethynodrel is increased. The effect has been suggested to be due to inhibition of hepatic metabolism by norethynodrel, since it has been shown competitively to inhibit hexobarbitone metabolism in vitro (Juchau and Fouts, 1966).

Twenty-four hours pretreatment of male or female rats with norethynodrel dissolved in dimethyl sulphoxide did not affect hexobarbitone sleeping time; a single dose forty-eight hours before the hexobarbitone did, however, decrease the sleeping time in female rats. Pretreatment of female rats with norethynodrel dissolved in ethyl oleate, however, decreased the sleeping time after twenty-four hours. It would seem that the rate of absorption was different from the two solvents. These results also agree with those of Juchau and Fouts (1966) since they found that twenty-four hours pretreatment with norethynodrel increased the hexobarbitone hydroxylase activity of the liver. Jori et al (1969) did not find any decrease in the pentobarbitone anaesthesia twenty-four hours after norethynodrel.

Based on their results on hexobarbitone and zoxazolamine hydroxylation, Juchau and Fouts (1966) came to the conclusion that norethynodrel stimulated drug metabolism, but only after an initial inhibition. The results obtained in this chapter using an in vivo technique would agree with this. Thus norethynodrel may act like certain other drug-metabolizing enzyme inhibitors such as SKF 525-A, which also enhance hepatic microsomal enzyme activity after an initial inhibition (Anders and Mannerling, 1966; Kato et al, 1964). The results obtained in Chapter six however, should be borne in mind since there, using other enzyme systems, no stimulatory phase was observed with norethynodrel treatment.
Pretreatment of female rats with chlormadinone for twenty-four hours did not alter the sleeping time. Chlormadinone is a progestogen but, unlike norethynodrel which is more closely related to testosterone structurally, it is more closely related to progesterone. It has been shown here that progesterone treatment of female rats for twenty-four hours does not affect duration of action of hexobarbitone, and it is known that the treatment of female rats with testosterone increases the metabolism of hexobarbitone (Quinn et al., 1958), so these results are not altogether unexpected. However, treatment for twenty-four hours of female rats with the oestrogen, mestranol significantly decreases the sleeping time, and this is perhaps a little unexpected, especially as hexobarbitone metabolism is decreased in male rats treated with oestrogen (Quinn et al., 1958).

Treatment of female rats for three days with chlormadinone, norethynodrel or mestranol produced similar effects to the twenty-four hour treatment. The average chlormadinone-treated sleeping time was slightly less than the controls, but was not significant. Pretreatment of male rats for three days with the steroids produced somewhat different results. Mestranol increased the duration of action of hexobarbitone by 100%, a result that agrees with previous findings that oestrogen treatment of male rats decreases hexobarbitone metabolism (Quinn et al., 1958). Norethynodrel also increases the sleeping time, but this effect is not as marked as that of mestranol. It is possible that the small amount of mestranol that is present in the commercially available norethynodrel is sufficient to produce this effect. Neither progesterone nor chlormadinone affected the sleeping time in male rats. The norethynodrel results do not agree with the in vitro results of Juchau and Fouts (1966) who found that in male rats three days pretreatment stimulated the metabolism of hexobarbitone.
Chronic treatment of female rats with norethynodrel was not possible as the supply became exhausted. Instead a very closely related synthetic steroid, ethynodiol, as its diacetate was used, which like norethynodrel is a derivative of testosterone. Chronic pretreatment of rats with a dose of ethynodiol lower than that used for norethynodrel in acute studies, produced a significant decrease in sleeping time. The same effect was obtained when ethynodiol was given in combination with mestranol. Mestranol itself did not have any significant effect on the sleeping time, but the average time was slightly decreased. These results suggest that ethynodiol diacetate induced the hepatic drug-metabolizing enzymes responsible for metabolizing hexobarbitone. These results are therefore in agreement with the findings in Chapter six that chronic pretreatment of rats with ethynodiol induces hydroxylation of biphenyl. Jori et al. (1969) have made similar findings with the synthetic progestogen, medroxyprogesterone acetate, but which is more closely related to progesterone than testosterone. They found that thirty days treatment with this steroid either alone or in combination with ethinyloestradiol, decreased the pentobarbitone anaesthesia time, indicating that the metabolism was increased. However, they also measured concentrations of pentobarbitone in the brain and found that after medroxyprogesterone acetate treatment the sleeping time and brain concentrations ninety minutes after pentobarbital administration were not statistically correlated as in the control animals. This suggests that progestational compounds affect pentobarbitone activity by several mechanisms in addition to the metabolic effect, including that repeated administration of steroids may increase the sensitivity of the central nervous system towards pentobarbitone. However, the same authors
found that chronic administration of medroxyprogesterone acetate significantly increased hepatic microsomal hydroxylation of aniline, N-demethylation of aminopyrine and O-demethylation of p-nitroanisole. The doses used in these experiments and those of Juchau and Fouts (1966), however, were all very high indeed. Whether lower doses produce similar effects is not known.

The findings of this chapter, therefore, show that during the third trimester of pregnancy in the rat the hexobarbitone sleeping time is increased. The total capacity of the pregnant rat to metabolize a given dose of hexobarbitone is not, however, any different from the non-pregnant. Although these effects of pregnancy may be due to the high levels of circulating steroids, pretreatment of rats with similar endogenous steroids for various lengths of time does not alter the sleeping time. Pretreatment of female rats with certain synthetic steroids, however, decreases the hexobarbitone sleeping time, probably by increasing its metabolism in the liver.
CHAPTER VIII

IN VIVO METABOLISM OF PHENACETIN IN THE RAT AND HUMAN
Introduction

Some in vivo studies on the effects of pregnancy and oral contraceptives on drug metabolism have been undertaken in this chapter. The drug chosen for study was phenacetin (p-ethoxyacetanilide or acetophenetidin) an analgesic which in recent years has fallen out of favour because of the toxic side effects of kidney damage and methaemoglobinaemia produced by long-term administration. To some extent it has been superseded as an analgesic by paracetamol (N-acetyl-p-aminophenol), its major metabolite which has a similar analgesic action to phenacetin but is reported to have less toxic effect.

The metabolism of phenacetin has been well studied in man, dog, rabbit and cat. Brodie and Axelrod (1949) showed that in man the major route of metabolism is by O-dealkylation to give N-acetyl-p-aminophenol which is then conjugated chiefly as the glucuronide; Smith and Williams (1949) found a similar picture in rabbits. The cat, however, is unable to conjugate the N-acetyl-p-aminophenol as the glucuronide, but excretes the N-acetyl-p-aminophenol as an unknown conjugate (Welch et al. 1966). Phenacetin is also metabolized to a lesser extent by deacetylation to p-phenetidine which, it has been suggested, is responsible either directly or indirectly for producing methaemoglobinaemia (Brodie and Axelrod 1949). More recently a cysteine conjugate S-(acetamido-4-hydroxyphenyl)-cysteine and the corresponding mercapturic acid have also been reported as minor metabolites of phenacetin in humans (Jagenburg and Toczko, 1964). Phenacetin is also hydroxylated to a minor extent in the position ortho to the N-acetyl to give 2-hydroxyacetophenetidine (Klutch et al., 1966) and can also be hydroxylated at the amino group to give N-hydroxyacetophenetidine (Klutch and Bordun, 1968).
The pathways of phenacetin metabolism may be summarized as follows:

Phenacetin is therefore an example of a drug that can be metabolized by several different pathways. It can also be given in high doses so that a quantitative study of its metabolism is facilitated by the relatively high amounts of metabolites that are produced.
Brodie and Axelrod (1949) have described colorimetric methods for the determination of phenacetin and p-phenetidine, and also for N-acetyl-p-aminophenol (Brodie and Axelrod, 1948). More recently Klutch and Bordun (1968) have developed selective thin-layer chromatographic methods for the estimation of the various possible transformation products of phenacetin present in urine or other biological material. The advantage of the chromatographic methods is their sensitivity and specificity for a compound present in a multi-component mixture, but they are not as quantitative as the chemical methods described. The same authors have briefly described gas chromatographic techniques for more accurate quantitative determinations of the metabolites of phenacetin.

In the present study it was at first envisaged that the metabolism of phenacetin would be studied with isotopically labelled material. $^{14}$C-ring labelled aniline was available, but the synthesis of $^{14}$C-phenacetin from $^{14}$C-aniline proved unsuccessful. The possibility of labelling either the acetyl or ethyl groups was considered but was not eventually carried out. The method finally adopted was a gas-liquid chromatographic technique modified from that described by Klutch and Bordun (1968).

The investigations carried out included some aspects of the quantitative study of phenacetin metabolism in pregnant and non-pregnant rats and in human volunteers taking oral contraceptives.

**Materials and Methods**

**Materials**

Phenacetin m.p. 135°C, p-phenetidine, N-acetyl-p-aminophenol m.p. 167°C, p-aminophenol m.p. 184°C, and diphenylamine m.p. 53°C, were all obtained from British Drug Houses and were used as supplied. The phenacetin
administered to human subjects was in tablet form complying with the specifications of "Phenacetin Tablets" in the British Pharmaceutical Codex, 1963. Each tablet contained 300 mg of phenacetin. 2-Hydroxyphenacetin was a gift from Dr. L. Prescott.

Silicone rubber (SE30) (Pye-Unicam Ltd.), Celite 80-100 grade (Pye-Unicam Ltd.), Carbowax 20M (Hopkin & Williams) and β-glucuronidase (Sigma, bovine liver) were all used as supplied.

All solvents used were of analytical grade (British Drug Houses).

Gas chromatography of phenacetin and its metabolites

The instrument used was a Pye Unicam Series 104 gas chromatograph fitted with a dual flame ionisation/electron capture detector head. The splitter was removed from the detector so that the instrument was used as a flame ionisation detector. The recorder was a Honeywell model.

5 ft. glass columns, 5 mm. internal diameter (Pye Unicam Ltd.) were used, and were packed as follows: the stationary phase was dissolved in methylene chloride (about 40 ml), and the required amount of supporting medium (Celite, 80-100 grade, about 10 g.) was added to the solution. After well mixing, the methylene chloride was removed under reduced pressure in a rotary evaporator using a very slow rate of rotation to minimize production of 'fines'. When all of the solvent had been removed, the temperature of the water bath was raised to boiling and evaporation under reduced pressure continued for 30 minutes to thoroughly dry the packing material. The packing material was then allowed to cool in a dessicator before sieving and packing the 80-100 mesh fraction into the column. To fill the column a small wad of glass fibre was placed in the shorter arm before attaching a connector. A vacuum line was then affixed to the connector and the glass fibre plug sucked to the end of
the column. A small amount of packing material was then fed into the other end of the column and, with assistance from continuous vibration from a "Whirlimixer", was sucked along the tube. In this manner uniform packing was achieved. Each new column was conditioned in the chromatography oven at \(25^\circ C\) above the proposed maximum operating temperature, with a small flow of nitrogen passing through it, for at least 24 hours before use.

The column packings used were based on those used by Klutch and Bordun (1968). For separating mixtures of phenacetin, \(p\)-phenetidine and \(p\)-aminophenol they used a 6 ft. column packed with a mixture of SE.30 (1\%) - Carbowax 20 M (1\%) coated on Anakrom AS, 80-90 mesh, while for separating phenacetin and \(N\)-acetyl-\(p\)-aminophenol they used a 4 ft. column packed with SE.30 (0.5\%) - Carbowax 20 M (0.5\%) on the same support material. The method used by these authors therefore required two columns. As I only had a single head flame ionisation detector such a method would have involved continual changing of columns. I therefore endeavoured to modify their method so that separation and estimation of the metabolites of phenacetin could be achieved on one column. Three different packings were therefore tried, SE.30 and Carbowax 20 M mixed at the following concentrations - 1\% of each, 0.6\% of each and 0.3\% of each. The columns were all 5 ft. in length and the support material was Celite.

In the initial investigations all work was carried out isothermally at various temperatures between 140\(^\circ\)C and 200\(^\circ\)C with a detector oven temperature of 250\(^\circ\)C (constant). However, in order to obtain separation of all the metabolites on the same column, temperature programming was used later. At some of the higher levels of sensitivity the base line tended to drift during
temperature programming. The carrier gas used for all
determinations was oxygen-free nitrogen at a flow rate
of 60 ml/minute. Normally 1 yl volumes of solutions of
the compounds in ethyl acetate were injected into the
column. No flash heater was available for use so 11.5 cm
long needles were used to inject the samples.

The retention times of phenacetin, p-phenetidine,
p-aminophenol, N-acetyl-p-aminophenol, 2 hydroxyacetophenetidine and diphenylamine are shown in Table 8.1.

<table>
<thead>
<tr>
<th>Table 8.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Gas-liquid chromatography of phenacetin and its metabolites</td>
</tr>
</tbody>
</table>

<table>
<thead>
<tr>
<th>Compound</th>
<th>Retention time (mins) with the following stationary phases</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>A</td>
</tr>
<tr>
<td>Phenacetin</td>
<td>18.0</td>
</tr>
<tr>
<td>p-Phenetidine</td>
<td>1.5</td>
</tr>
<tr>
<td>p-Aminophenol</td>
<td>8.5</td>
</tr>
<tr>
<td>2-Hydroxyacetophenetidine</td>
<td>-</td>
</tr>
<tr>
<td>N-acetyl-p-aminophenol</td>
<td>-</td>
</tr>
<tr>
<td>Diphenylamine</td>
<td>-</td>
</tr>
</tbody>
</table>

A: 1% W/w SE. 30 - 1% W/w Carbowax 20M on Celite, 5 ft. 180°C.
B: 0.6% W/w SE. 30 - 0.6% W/w Carbowax 20M on Celite, 5 ft. 200°C.
C: 0.3% W/w SE. 30 - 0.3% W/w Carbowax 20M on Celite, 5 ft. 200°C.
D: 0.3% W/w SE. 30 - 0.3% W/w Carbowax 20M on Celite, 5 ft. temperature programmed from 135°C to 205°C. Initial period 1.0 min., rate of increase 32°C per min., final period 20 min.

The column and conditions chosen for routine use was D.
Diphenylamine was used as an internal standard since it possessed a suitable retention time that did not interfere with the other components. The p-aminophenol had a retention time very close to that of the diphenylamine but was not detectable in the urine (see later) so that the closeness of retention times did not matter. The retention time of N-hydroxyacetophenetidine could not be determined as none was available. Although Klutch and Bordun (1968) could not separate N-hydroxyacetophenetidine from phenacetin they could only detect the N-hydroxy-metabolite after hydrolysis with β-glucuronidase, so it would not interfere with the estimation of phenacetin.

The retention times of the individual compounds were not affected by mixing with the other compounds. A chromatogram of a mixture is shown in Fig. 8.1. The peak for N-acetyl-p-aminophenol is seen to tail a little; however, the height of the peak was shown to be proportional to amount, so its use is satisfactory. The sensitivity towards N-acetyl-p-aminophenol is not very great, but the amount to be detected in various urine samples is large so that again the method is satisfactory.

**Extraction of phenacetin and its metabolites from urine**

Known amounts of N-acetyl-p-aminophenol (200 μg), phenacetin (20 μg), phenetidine (20 μg) and p-aminophenol (40 μg) were dissolved in a portion of urine (4 ml). The urine solution was then extracted as follows. To 4.0 ml of urine, 2.5 g of sodium chloride and 1.0 ml of 0.2 M phosphate buffer pH 7.4 were added and the mixture was shaken for 15 minutes in 50 ml. stoppered tubes with 40 ml of diethyl ether containing 1.5% V/v isoamyl alcohol. A 20 ml. portion of the ether was removed and evaporated to dryness under a stream of air in a conical tube immersed in a water-bath at 30°C. The residue was taken up in a 100 μl of ethyl acetate containing 5 μg of diphenylamine. 1 μl samples were
Fig. 8.1.

Gas chromatogram of phenacetin and some of its metabolites

Instrument: Pye 104
Detector: Flame ionisation, 250°C.
Column: 5' x 5 mm. glass. 0.3% SE30 plus 0.3% Carbowax 20M on Celite.
Temperature: Programmed 135°C-205°C. Initial period 1 min. Rate of increase 32°C per min. Final period 20 min.

Retention time (min)

Detector response
then injected on to column D in the gas chromatograph using the conditions described above. The diphenylamine acts as an internal standard. Quantitative measurements made by comparison of peak heights with the heights of standard solutions indicated that the percentage recoveries were 88% for N-acetyl-p-aminophenol, 75% for phenacetin, 55% for p-aminophenol and 16% for p-phenetidine. The low recovery of p-phenetidine was found to be due to loss during evaporation of the solvent. By adding one drop of concentrated hydrochloric acid to the 20 ml of ether extract before evaporation this loss was prevented. The p-phenetidine was then present as the hydrochloride (and so also was the p-aminophenol); the free base was released by passing a stream of NH₃ over the residue for 15 seconds. This increased the recovery of phenetidine to 70% and of p-aminophenol to 65%, while the recovery of phenacetin and N-acetyl-p-aminophenol remained unaltered. These recoveries were considered satisfactory enough to use this method of extraction from the urines of animals and humans fed a dose of phenacetin. No 2-hydroxyacetophenetidine was available at this stage.

Thus, one single extraction with ether was found to give a satisfactory method of extracting phenacetin and its major metabolites from urine samples in the concentrations anticipated.

In rat experiments 4.0 ml of urine were taken through the procedure, but in human experiments 8.0 ml samples of urine were used, and all the quantities used in the extraction procedure were doubled.

Analysis for total N-acetyl-p-aminophenol (i.e. free and conjugated) was carried out as follows: a 0.2 ml portion of rat urine was buffered with 0.5 ml of 0.1 N acetate buffer, pH 5.0, containing 1000 units of
β-glucuronidase, and the mixture was incubated at 37°C for 24 hours. The mixture was then diluted to 4.0 ml with water, 1.0 ml of 0.2 M phosphate buffer pH 7.4 and 2.5 g. of sodium chloride were added and the extraction was carried out as described above using 40 ml of ether containing 1.5% v/v isoamyl alcohol. For human urine 1.0 ml was treated with 5000 units of β-glucuronidase in 1.0 ml of acetate buffer, and the extraction carried out as for rat urine.

Dosage and collection of urine

Each human subject was instructed to collect a sample of urine at 9.00 a.m. on the day of the experiment. This served as control urine. Each then took four tablets, each containing 300 mg. of phenacetin (1200 mg. total dose) and collected the urine for 24 hours. Samples were kept in a cool place and refrigerated at the earliest opportunity. Two male subjects, three control female subjects and three female subjects taking oral contraceptives, two on "Minovlar" (norethisterone acetate and ethinyl oestradiol) and one on "Lyndiol" (lynestrenol and mestranol), took part in the experiments.

In the rat experiments phenacetin was administered as a suspension (10 mg/ml) in 10% Tween 80, either intraperitoneally by injection or orally by intubation, at a dose of 100 mg/kg. The animals were then placed individually in metabolism cages and the urine collected for 24 hours. The cages were rinsed with water to remove adhering urine. Urine collected for the 24-hour period prior to phenacetin administration was used as control urine. All rats used were female Wistar albinos, and pregnancy was established using the technique described in chapter five.
Results and Discussion

Table 8.2 shows the pattern of urinary excretion of the major metabolites of phenacetin in control male and female human subjects and in females taking oral contraceptive agents. The results are expressed as a percentage of the dose given.

**Table 8.2**

**Pattern of urinary excretion of phenacetin in humans**

<table>
<thead>
<tr>
<th>Group</th>
<th>Subject</th>
<th>Phenatecin</th>
<th>p-Phenetidine</th>
<th>Free N-acetyl-p-aminophenol</th>
<th>Conjugated N-acetyl-p-aminophenol</th>
</tr>
</thead>
<tbody>
<tr>
<td>Males</td>
<td>M.G.N.</td>
<td>0.27</td>
<td>0.02</td>
<td>2.5</td>
<td>69</td>
</tr>
<tr>
<td></td>
<td>W.E.L.</td>
<td>0.22</td>
<td>0.02</td>
<td>4.1</td>
<td>42</td>
</tr>
<tr>
<td>Female controls</td>
<td>L.E.F.</td>
<td>0.26</td>
<td>0.01</td>
<td>3.5</td>
<td>73</td>
</tr>
<tr>
<td></td>
<td>J.M.C.</td>
<td>0.20</td>
<td>0.01</td>
<td>2.8</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>E.D.N.</td>
<td>0.39</td>
<td>0.10</td>
<td>5.8</td>
<td>12</td>
</tr>
<tr>
<td>On oral contraceptives</td>
<td>L.B.*</td>
<td>0.48</td>
<td>0.40</td>
<td>19.5</td>
<td>30</td>
</tr>
<tr>
<td></td>
<td>J.W.†</td>
<td>0.52</td>
<td>0.05</td>
<td>4.4</td>
<td>40</td>
</tr>
<tr>
<td></td>
<td>A.N.‡</td>
<td>0.47</td>
<td>0.05</td>
<td>2.5</td>
<td>68</td>
</tr>
</tbody>
</table>

* Minovlar    † Lyndiol

It should be mentioned to begin with that obviously no firm conclusions can be drawn from the small number of subjects used. **Results** for the controls agree well with those obtained by Brodie and Axelrod (1949), although the amount of p-phenetidine is lower than they found. The low recovery of conjugated N-acetyl-p-aminophenol in one male subject could well be due to a sample being missed during
the 24 hours. The exceptionally low excretion of conjugated N-acetyl-p-aminophenol in one control female is unlikely to be due to this reason; it was learned after the experiment that this subject was suffering from amenorrhoea, but it is unlikely that this would produce such a difference. The urine was particularly dark and contained a lot of sediment. All three subjects taking oral contraceptives showed an increased excretion of unchanged phenacetin, and possibly also of p-phenetidine. In one case there was a large increase in the excretion of unconjugated N-acetyl-p-aminophenol. In the two subjects taking Minovlar there also appeared to be a decreased excretion of conjugated N-acetyl-p-aminophenol. This was not the case with the subject taking Lyndiol. No urine samples were collected after the first 24 hours so whether the remainder of the dose is excreted after this time or whether some other metabolite is produced is not known. One might suggest that the metabolism of phenacetin by dealkylation and the subsequent conjugation of N-acetyl-p-aminophenol with glucuronic acid is inhibited. These findings are thus similar to those described by Crawford and Rudofsky (1966) who found that the amount of unchanged pethidine and promazine in the urine of human subjects on the pill is greater than in controls. However, the number of findings described here is small, and confirmation or otherwise will have to await larger numbers of observations. Moreover, the effects described need not be due to alterations in metabolism, and could be attributable to altered excretion or absorption.

The pattern of urinary excretion of phenacetin and its metabolites by pregnant and non-pregnant rats has been determined in animals dosed both intraperitoneally and orally. The results are shown in Tables 8.3 and 8.4 respectively.
Table 8.3

Pattern of urinary excretion of phenacetin and its metabolites in pregnant and non-pregnant rats dosed intraperitoneally

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage of dose excreted as:</th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unchanged Phenacetin</td>
<td>Phenetidine</td>
<td>Free N-acetyl-p-amino-phenol</td>
<td>Conjugated N-acetyl-p-amino-phenol</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>0.25 0.33 0.28 0.15</td>
<td>0.06 0.21 0.50 0.05</td>
<td>4.5 14.9 2.5 1.3</td>
<td>2.4 0.9 22.6 11.2</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>0.25 ± 0.04</td>
<td>0.21 ±0.10</td>
<td>5.8±3.1</td>
<td>9.3±5.0</td>
</tr>
<tr>
<td>20-day pregnant</td>
<td>0.09 0.18 0.17</td>
<td>0.00 0.20 0.18</td>
<td>0.8 7.9 1.2</td>
<td>2.3 11.7 11.4</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>0.15 ± 0.03</td>
<td>0.13±0.06</td>
<td>3.3±2.2</td>
<td>8.4±3.1</td>
</tr>
</tbody>
</table>

The results obtained after intraperitoneal administration were not very consistent, and only a small percentage of the total dose was recovered. This could be due to insufficient hydrolysis of conjugates of N-acetyl-p-aminophenol by the β-glucuronidase, possibly due to the presence of an inhibitor of the β-glucuronidase in the rat urine. Alternatively, since the recovery from human urine was good but from rat urine it was poor, it is possible that in the rat the phenacetin is excreted in the bile as rats are good biliary excreters. Tests for complete hydrolysis of the glucuronide could not be
carried out as no reference N-acetyl-p-aminophenol glucuronide was available. A comparison between the amount of conjugated N-acetyl-p-aminophenol excreted by the pregnant and non-pregnant rats is, therefore, probably not justified, but nevertheless the ranges are approximately the same. As far as the unconjugated metabolites were concerned, there appeared to be little difference between the twenty-four hour excretion of p-phenetidine and N-acetyl-p-aminophenol in pregnant and non-pregnant rats. The amount of unchanged phenacetin excreted was slightly lower in the pregnant animals. No metabolites were detectable in the second twenty-four hour urine samples.

Table 8.4

Pattern of urinary excretion of phenacetin and its metabolites in pregnant and non-pregnant rats dosed orally

<table>
<thead>
<tr>
<th>Group</th>
<th>Percentage of Dose excreted as:</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Unchanged Phenacetin</td>
</tr>
<tr>
<td>Non-pregnant</td>
<td>0.06</td>
</tr>
<tr>
<td></td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.07</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>Mean ± S.E.M.</td>
<td>0.07±0.01</td>
</tr>
<tr>
<td>20-day pregnant</td>
<td>0.10</td>
</tr>
<tr>
<td></td>
<td>0.06</td>
</tr>
<tr>
<td>Mean</td>
<td>0.08</td>
</tr>
</tbody>
</table>
The results obtained after oral administration were rather more consistent, but the overall recovery was again low. There did not appear to be any difference between the pattern of excretion of the pregnant animals and the non-pregnants.

These results in pregnant rats do not indicate that the metabolism of phenacetin is significantly altered during pregnancy. Thus, there is certainly no evidence of inhibition of metabolism in vivo as one might expect from the in vitro studies. However, the rate of metabolism could be inhibited and the total metabolism still be completed within twenty-four hours. It would therefore be worthwhile measuring the concentration of phenacetin and its metabolites in the blood and urine, at different time intervals after dosage to determine the rates of metabolism.

Conclusion

The method described by Klutch and Bordan (1968) for the extraction and gas chromatographic separation of phenacetin and its metabolites, which required the use of two separate columns, has been modified to give a simplified quantitative method for the estimation of these compounds in urine, which involves the use of a single column. Thus, phenacetin, p-phenetidine and N-acetyl-p-phenetidine and N-acetyl-p-aminophenol present together in a sample of urine can be extracted into ether, and separated on the same column. 2-Hydroxyphenacetin can be separated on this column, but its extraction has not been studied. p-Aminophenol can also be separated on the column, but it is unstable and cannot be estimated in urine samples. The behaviour of N-hydroxyphenacetin under these conditions has not been investigated as none was available.

The method applied to humans and rats dosed with phenacetin has shown that women taking oral contraceptives may show a mild degree of inhibition of phenacetin metabolism, while pregnant rats do not show any difference to non-pregnant animals in their pattern of excretion of phenacetin and its metabolites.
CHAPTER IX.

GENERAL DISCUSSION
The object of this final chapter is to try to correlate the in vitro results from Chapters five and six with the in vivo results from Chapters seven and eight, and also to suggest further work which would give a deeper insight into the effect of pregnancy and oral contraceptives on drug metabolism.

It was shown in Chapter five that drug-metabolizing enzymes responsible for aromatic hydroxylation and glucuronidation, but not for nitroreduction, are inhibited in the full-term pregnant rat liver when measured in vitro and expressed in terms of unit liver weight; similarly the microsomal cytochrome P450 is decreased. The in vivo demonstration that the duration of action of hexobarbitone is increased in full-term pregnant rats shows that there is agreement between the in vitro and in vivo results. Contrary to these findings, no evidence was found from the pattern of twenty-four hour urinary excretion of phenacetin and its metabolites that the metabolism of this drug was inhibited in the full-term pregnant rat. However, only the overall twenty-four hour picture was obtained during which time metabolism could have been delayed, yet still completed. It would be of interest to compare the rates of phenacetin metabolism in pregnant and non-pregnant rats by measuring the blood and urine levels of phenacetin and its metabolites at different time intervals after a single dose. An alternative explanation for the lack of effect of pregnancy on phenacetin metabolism could be that as with nitroreduction, the metabolism of phenacetin is, in fact, not altered by pregnancy. However, the major route of metabolism is by dealkylation which takes place by a hydroxylation reaction and might be expected, therefore, to be similar to biphenyl and hexobarbitone.
The activity of the drug-metabolizing enzyme systems responsible for aromatic hydroxylation, nitro-reduction and glucuronidation together with cytochrome P450 and microsomal protein content of livers from 15-16 day pregnant rats showed no significant difference from non-pregnant rats although the cytochrome P450 and the hydroxylase were slightly decreased. The duration of action of hexobarbitone was, though, significantly increased in 15-16 day pregnant rats, but the increase was not as great as with the full-term rats so that both in vitro and in vivo results agree qualitatively.

Although both in vitro and in vivo studies showed that pregnancy in the rat has an inhibitory effect on drug metabolism, it is important to note that there is also an increase in liver weight so that the total liver activity of drug-metabolizing enzymes in the pregnant rat is equal to the non-pregnant. Hence, when the same total dose of hexobarbitone is given to full-term pregnant rats there is no difference in sleeping time. With the 15-16 day pregnant rats, where the total liver cytochrome P450 is increased, the duration of action of the same total dose of hexobarbitone is decreased. Here again there is direct agreement between in vitro enzymic studies and in vivo studies.

In vitro studies demonstrated that there is no drug-metabolizing enzyme activity or cytochrome P450 present in rat placentae and foetal livers. Although no direct in vivo experiments were carried out with these tissues, it would be expected that any activity in them might lead to a more rapid breakdown of hexobarbitone and, therefore, a decreased duration of action, which is not the case. It can be concluded that neither the placentae nor foetal livers supplement the drug-metabolizing activity of the maternal liver.
Previous studies (Hsia et al 1963) had suggested that the high levels of circulating hormones during pregnancy might be responsible for impaired conjugation reactions during pregnancy, and this idea was extended to cover metabolic transformations when it was shown that steroid hormones could competitively inhibit microsomal drug-metabolism, (Tephly and Mannering, 1964; 1968; Juchau and Fouts, 1966; Wada et al 1968). The effect of in vitro addition of natural oestrogens and progestogens on the enzymes studied in the pregnant rat was investigated and it was shown that both types of steroid could inhibit these reactions, probably competitively, but only at relatively high concentrations. In vivo studies involving pretreatment of female rats for varying lengths of time with progesterone and oestradiol, both alone and combined, did not, however, produce any inhibitory effects on microsomal drug metabolism, nor alter the duration of action of hexobarbitone, except one hour's pretreatment with progesterone which increased significantly the hexobarbitone anaesthesia. This could have been due to impaired metabolism of the hexobarbitone but could also have been due to the anaesthetic action of progesterone itself. The pretreatment studies did not appear, therefore, to support the suggestion that the high levels of circulating steroid hormones in pregnancy are responsible for the inhibition of drug metabolism. However, only one dose level of the steroids was used and the dosage was peridioic rather than continuous so that a consistently high level of circulating steroid would not have been maintained as in pregnancy. This effect might be overcome by using liver perfusion techniques where a high level of steroid could be maintained in the perfusate.

Thus, the very high levels of steroid required to produce inhibition in vitro together with the failure of
pretreatment studies with steroids to demonstrate any inhibition of drug metabolism suggest that the inhibitory effect of pregnancy on drug metabolism may not be due directly to inhibition from steroids. This idea is given support by the fact that in late pregnancy in the rat there is a decrease in the cytochrome P450 concentration in the liver and this could account for the impaired drug metabolism.

A determination of the kinetic constants of the enzyme systems in pregnant and non-pregnant rats would shed some light on the cause of the inhibitory effect of pregnancy. If the apparent Michaelis constant ($K_m$) for the enzyme reaction differs between the pregnant and non-pregnant rat, it would show that a competitive mechanism of inhibition was operating and would suggest that the circulating steroids were responsible. If, on the other hand, $K_m$ remained the same and $V_{\text{max}}$ for the system decreased, this would indicate that the amount of enzyme present was limiting the reaction. Since the completion of this work Guarino et al. (1969) have described the effect of pregnancy in rats on the kinetic constants for the hepatic microsomal hydroxylation of aniline and the demethylation of ethylmorphine. They showed that in 20-day pregnant rats there is a significant decrease in $V_{\text{max}}$ for both aniline hydroxylase and ethylmorphine, but the apparent $K_m$s for both the enzymes did not differ between the 20-day pregnant and non-pregnant rat. Moreover, there is a decrease in microsomal cytochrome P450 in the 20-day pregnant rat liver and there is a quantitative correlation between this decrease and $V_{\text{max}}$ for the two enzymes. These effects were not apparent in 6 and 14-day pregnant rats. These results, therefore, substantiate the findings of this thesis that the inhibitory effect of pregnancy on hepatic drug-metabolizing enzymes in the rat is probably due to the
decreased amount of cytochrome P450 in the liver and probably not due to competitive inhibition from steroid hormones.

It is still possible that the steroid hormones produced during pregnancy are responsible for the decrease in cytochrome P450. However, pretreatment of female rats for varying lengths of time with progesterone and oestradiol both alone and combined, albeit at only one dose level, did not produce any decrease in cytochrome P450 in the liver. Further evidence that the steroid hormones are not responsible comes from the fact that the peak levels of these hormones during pregnancy in the rat do not correspond with the peak of the inhibitory effects, in fact steroid levels are raised throughout pregnancy, but the inhibitory effect on drug metabolism is only apparent during late pregnancy.

Impaired drug metabolism has been reported in many states characterized by rapid growth, and it is suggested that a common factor such as growth hormone may cause this effect. Such a factor may be responsible for the inhibitory effect of pregnancy on drug metabolism since this state is also characterized by rapid growth. Moreover, it is only over the final few days of pregnancy that the inhibition develops, and it is over this time that the level of growth hormone increases most. Recently Wilson (1969 a and 1969 b) has identified somatotropin (growth hormone) as the pituitary hormone responsible for decreasing liver metabolism of hexobarbitone, ethylmorphine and aminopyrine in rats. Forty-eight hours after a single subcutaneous injection of 1.73 units of somatotropin (but not after adrenocorticotropic hormone or prolactin) the metabolism of these three drugs is inhibited by approximately 25% (Wilson 1969 a), which is similar to the degree of inhibition produced by pregnancy in the rat. Thus it seems possible that the increased
level of growth hormone may be responsible for the inhibitory effect of pregnancy on drug metabolism. It will be interesting to determine whether growth hormone causes a decrease in cytochrome P450 levels in the liver; it would seem most probable that it would act by interfering with protein synthesis in some way.

Since iron is a vital component of haemoproteins it is possible that a deficiency of this element during pregnancy may result in a decrease in liver cytochrome P450. It would, therefore, be of considerable interest to investigate the hepatic drug-metabolizing enzyme activities in pregnant and non-pregnant rats fed on diets containing a deficiency and an excess of iron.

What is the therapeutic significance of the finding that pregnancy in the rat has an inhibitory effect on drug metabolism? The first point is the question of extrapolating from one species to another. Indeed it has been shown here that the rabbit shows a different pattern of response in the effect of pregnancy on drug metabolism to the rat. It is naturally difficult to investigate drug metabolism in the pregnant human in view of possible adverse effects on the foetus, but where it is essential to administer drugs their metabolism could be studied and compared with controls. Secondly, with many drugs it is not the metabolism that is the rate limiting step in determining the duration of action; other factors such as absorption, distribution and excretion may be more important in limiting drug action. Finally, even if the pregnant human does show similar effects to the pregnant rat and the rate of metabolism does control the drug action, it should be remembered that the total ability of the pregnant rat to metabolize a drug was the same as the non-pregnant, so as long as the same total dose of drug is given to the pregnant human as the non-pregnant, there will probably be little difference in therapeutic response.
The synthetic steroids used as oral contraceptive agents produced inhibition of microsomal hydroxylation in a similar manner to the natural steroids of pregnancy when added in vitro to rat liver preparations. Pretreatment studies with these steroids for three days showed no inhibitory or stimulatory effect on drug-metabolizing enzymes. Pretreatment one hour before with norethynodrel, however, did show an inhibitory effect on aromatic hydroxylation, but no decrease in cytochrome P450. Eighteen days treatment with a mixture of progestogen and oestrogen caused an increase in aromatic hydroxylation and a decrease in duration of action of hexobarbitone but no increase in microsomal cytochrome P450. Prolonged oral treatment of rats with a low dose of two progestogens caused a decrease in aromatic hydroxylation, but had no effect on cytochrome P450; a third progestogen similarly studied did not produce the decrease. The duration of action of hexobarbitone did not always correlate with the in vitro findings. Thus, one hour's pretreatment with norethynodrel did prolong the duration of action, but all other pretreatments decreased the duration of action suggesting that the metabolism of hexobarbitone had been stimulated. The general trend of the effect of oral contraceptive steroids on drug metabolism appeared to be an initial inhibition followed by stimulation. The content of cytochrome P450, however, was not altered by any pretreatment with oral contraceptive steroids.

As with the pretreatment studies with the natural steroids, the synthetic steroids were only given periodically and, therefore, high blood levels would not be maintained. However, this is how the oral contraceptive pill is normally taken, and it represents a major difference between the steroid picture during pregnancy and in women using the pill. The fact that one hour's pretreatment of rats with
norethynodrel caused inhibition of metabolism might suggest that its basic action is inhibitory, and it would, therefore, be worth investigating its effect using the perfused liver.

The in vivo studies with phenacetin were only carried out on a small number of human subjects, but these gave an indication of slight impairment of metabolism. It may be that the effect of oral contraceptives on drug metabolism depends on the rate of metabolism of the component steroids such that if a steroid is slowly metabolized it may exert its effect for longer. Since there is a large range of oral contraceptives on the market it is possible that some might produce inhibition, others no effect, and still others stimulation of drug metabolism. Further studies involving measuring rates of metabolism and excretion of phenacetin and other drugs and their metabolites need to be undertaken.

It is certainly apparent that the effect of oral contraceptive steroids on drug metabolism in the rat is not the same as the effect of pregnancy. This is best illustrated by the fact that there is no decrease in microsomal cytochrome P450 and that the duration of action of hexobarbitone is decreased in the steroid treated rat, whereas in the pregnant rat there is a decrease in the microsomal cytochrome P450 and an increase in the duration of action of hexobarbitone.

In conclusion it can be stated that during late pregnancy in the rat there is a decreased concentration of cytochrome P450 in the liver which is accompanied by a consequent decrease in the activity of some, but not all, drug-metabolizing enzymes; these decreases are compensated for by an increase in size of the liver, and are not supplemented by any activity in the placenta.
or foetal liver. These in vitro effects are reflected in vivo by the duration of action of hexobarbitone, but do not alter the pattern of metabolism of phenacetin. The effect of late pregnancy has not been reproduced by pretreatment of rats with natural steroids nor by synthetic steroids used as oral contraceptive agents. The latter, in fact, seem to produce the opposite effect, i.e. a stimulation of drug metabolism, at least in the rat.
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