STUDIES ON CHEMICALLY-INDUCED LIVER ENLARGEMENT

AND HEPATIC NODULAR LESIONS IN THE RAT

by

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Abstract

Long-term feeding studies in rats have been carried out with a number of compounds known to produce liver enlargement in this species, and sequential measurements of hepatic enzymes have been correlated with histochemical, histological and ultrastructural changes in the liver.

Administration of butylated hydroxytoluene (BHT) or safrole for 1 week in the diet at levels of 0.4 and 0.25% respectively produced liver enlargement and induction of the microsomal drug metabolising enzymes. During continued administration of BHT for up to 75 weeks this response was maintained unaltered and without production of liver injury. However, in the case of safrole, drug metabolising enzyme activity fell to around control levels by week 8 but the liver remained enlarged. Cytopathological changes, indicated histochemically by autophagy and depression of glucose-6-phosphatase, were observed at this stage. These became progressively more severe leading to focal necrosis and the development around week 60 of nodular hepatic lesions. A similar sequence of events was initiated after only 1 week in rats fed a diet containing 1.0% Ponceau MX, a compound producing marked liver enlargement but only weak stimulation of drug metabolising enzyme activity. Thus, where liver enlargement is unaccompanied by drug metabolising enzyme induction, or where such enzyme induction is only transitory, gross pathological changes may develop in the enlarged liver.

The nodular hepatic lesions produced by safrole and Ponceau MX have been compared with hyperplastic liver nodules induced by the hepatocarcinogen 2-acetylaminofluorene and found to differ in some aspects of their biochemical and biological characteristics.
Finally, some in vitro studies were conducted to explore the unusual interaction of safrole with hepatic microsomal cytochrome P450. The stable binding to cytochrome P450 of a species apparently formed during the metabolism of safrole could be reversed by certain alternative substrates of cytochrome P450 with the production of novel spectral changes.
TO MY PARENTS AND
TO JULIET
Acknowledgements

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Finally, I am indebted to Miss Kathy Ratcliffe for typing this thesis and to the Science Research Council for financial support.
All experience is an arch wherethro'
Gleams that untravelled world, whose margin fades
For ever and for ever when I move.

Tennyson (Ulysses)
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CHAPTER ONE

INTRODUCTION
## INTRODUCTION

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1.1 Chemically-Induced Liver Enlargement

The liver is affected by the administration of foreign compounds more frequently than any other organ of the mammalian body (Rowe et al., 1959; Weil and McCollister, 1963). Thus, a very large number of compounds, differing widely in pharmacological activity and chemical structure, produce an increase in the weight of the liver when administered to experimental animals (Barka and Popper, 1967; Golberg, 1966; Kunz et al., 1966a; Silvestrini et al., 1966; Smyth et al., 1952). Such liver enlargement may or may not be associated with, or followed by, histopathological alterations depending upon the type of compound, the dose, the age and species of animal and the mode and frequency of administration. Accordingly, the response of the liver to foreign compounds may range from enlargement without histological change to advanced structural changes, such as cirrhosis or hyperplastic nodules, and neoplasia. The induction of frank liver cancer by such compounds as dimethylnitrosamine (Magee and Barnes, 1956) or aflatoxins (Lancaster et al., 1961), may present few problems of interpretation from a pathological, and perhaps even a toxicological point of view. However, the interpretation of other chemically-induced hepatic effects, including some pathological changes such as hyperplasia following cell damage or death, remains a controversial issue (Bonser and Roe, 1970; Grasso et al., 1969; 1974). Hyperplastic liver nodules are often preceded by liver enlargement without overt histological abnormalities but the mechanism of their formation and their toxicological significance is largely unknown. It was against this background that the work recorded in this thesis was undertaken. The observations made are in part relevant to liver enlargement per se and it is therefore appropriate to briefly review some aspects of this subject.
1.2 Types of Liver Enlargement

Chemically-induced liver enlargement most frequently derives from changes in the parenchymal cells (hepatocytes) despite the fact that these comprise only 50-60% of the total cell population of normal mammalian liver (Steiner et al., 1966). These changes may involve an increase in the size of individual hepatocytes (hypertrophy), an increase in the number of hepatocytes as a result of mitotic division (hyperplasia), an increase in ploidy - also considered to represent hyperplasia (Barka and Popper, 1967) - or a combination of these processes to varying extents according to the particular compound administered (Koransky and Schulte-Hermann, 1970). The contribution of each or all of these processes to liver enlargement may vary for a particular compound depending upon the dose level and mode and frequency of administration. Less frequently the liver may enlarge as a result of hypertrophy of the reticuloendothelial system with storage of macromolecular material, e.g. iron dextran (Golberg, 1966), or of disproportionate proliferation of non-parenchymal cells, e.g. bile ductular cells as in ethionine intoxication (Farber, 1956).

1.3 Metabolism of Foreign Compounds

Many of the compounds known to produce liver enlargement are metabolised, at least to some extent, by a non-specific, NADPH- and oxygen-dependent enzyme system localised in the endoplasmic reticulum of the hepatocytes. This enzyme system catalyses the oxidation of a wide range of foreign compounds, mediating such reactions as aromatic and aliphatic hydroxylation, N-, O-, and S-dealkylation, N- and S-oxidation, deamination and dehalogenation (Gillette, 1971; Gillette and Brodie, 1970; Parke, 1968). The overall reaction sequence may be
summarised as

$$RH + NADPH + H^+ + O_2 \rightarrow ROH + NADP + H_2O$$

one atom of the oxygen molecule being incorporated into the substrate
and the other into water (Hayashi, 1962). Hence the use of the terms
mixed function oxidase (Mason, 1957) or monooxygenase (Hayashi, 1962)
in describing this enzyme system, which is frequently referred to
simply as the the drug metabolising enzyme system. Despite the
chemical diversity of the substrates for this system, it appears that a
single, integrated electron transport chain is involved, rather than a
multiplicity of different enzymes, and some progress has been made in
elucidating the mechanisms involved in mixed function oxidation.

It is firmly established (Cooper et al., 1965; Diehl et al., 1969)
that the introduction of oxygen into the substrate is mediated by a b-
type haemoprotein termed cytochrome P450 (Omura and Sato, 1964).
According to current theory, substrates combine with the oxidised form
of cytochrome P450 and this binding may be visualised in liver slices
or microsomal suspensions by difference spectrophotometry (Cinti and
Schenkman, 1972; Schenkman et al., 1967). The spectral change produced
by most substrates comprises a maximum at about 390 nm and a minimum at
about 425 nm: this is termed a type I spectrum. A limited number of
compounds, usually containing nitrogen, give rise to a spectral maximum
at about 430 nm and a minimum at about 390 nm: this is termed a type II
spectrum. These and other less usual forms of substrate interaction
spectra are the subject of an extensive literature (e.g. Mannering,
1971; Remmer et al., 1969; Schenkman, 1970; Schenkman et al., 1972b,
1973; Temple, 1972). Spectrally detectable binding is not, however, a
prerequisite for metabolism (Chaplin and Mannering, 1970; Leibman and
Estabrook, 1971) and conversely, some compounds produce interaction spectra but are not metabolised (Burke, 1972). In the presence of NADPH, metabolism proceeds with the reduction of the substrate-cytochrome P450 complex. The flavoprotein NADPH-cytochrome c reductase is known to be involved in this electron transfer (Masters et al., 1973) but whether this or a separate entity constitutes NADPH-cytochrome P450 reductase remains uncertain. The reduced complex next reacts with oxygen to form a reduced cytochrome P450-substrate-oxygen complex. It is thought that a second electron is then introduced, either from NADPH via cytochrome c reductase or from NADH via cytochrome b5 reductase, resulting in the formation of an active oxygen intermediate which decomposes to liberate the oxidised substrate and oxidised cytochrome P450 (Baron et al., 1973; Sasame et al., 1973). The role of cytochrome P450 in drug metabolism has recently been reviewed (Gillette et al., 1972).

1.4 Induction of Foreign Compound Metabolism

Many compounds, when administered to experimental animals or man, elicit an increase in the activity of the drug metabolising enzymes (Conney, 1967; Conney et al., 1971; Ernster and Orrenius, 1965). This phenomenon, in addition to its intrinsic interest from a mechanistic point of view and as a model for the study of substrate-initiated enzyme induction, has important practical implications since rate of metabolism is a prime determinant of duration and intensity of pharmacological action. In view of the multi-step process involved, the increase in metabolic activity may result from one or more of a number of possible changes. Thus, administration of the widely studied compound phenobarbitone increases the amounts of both cytochrome P450
and NADPH-cytochrome c reductase (Orrenius et al., 1965) and the rate of reduction of cytochrome P450 (Gram et al., 1968). In addition the magnitude of both type I and type II substrate interaction spectra is increased (Remmer et al., 1969). The $V_{\text{max}}$ for type I substrate metabolism is increased without change in the $K_{m}$ (Rubin et al., 1964) whilst in the case of the type II substrate aniline, the $V_{\text{max}}$ is increased but affinity for the substrate is decreased (Guarino et al., 1969). In contrast, administration of 3-methylcholanthrene increases the amount of cytochrome P450 (Hildebrandt et al., 1968) but not the rate of its reduction (Gram et al., 1968) or the activity of NADPH-cytochrome c reductase (Hernandez et al., 1967). The magnitude of the type II spectral change is increased whereas the type I spectrum is either unaffected or decreased (Remmer et al., 1969). Moreover, whilst phenobarbitone induction does not change the pH at which the two maxima of the cytochrome P450-ethylisocyanide spectrum are of equal magnitude, 3-methylcholanthrene treatment produces a decrease in the pH at which this equilibrium point occurs (Sladek and Mannering, 1966). This change is paralleled by a shift in the absorption maximum of the cytochrome P450-carbon monoxide complex from 450 to 448 nm (Kuntzman et al., 1969) or 446 nm (Hildebrandt et al., 1968) and is considered to reflect the synthesis of a modified haemoprotein (Mannering, 1971).

These differences in the action of phenobarbitone and 3-methylcholanthrene on various component functions of the drug metabolising enzyme system are reflected in their respective effects on substrate metabolism. Thus, phenobarbitone, the effects of which are typical of the majority of inducing agents (Sher, 1971), stimulates most pathways of drug metabolism whereas polycyclic aromatic hydrocarbons, typified by 3-methylcholanthrene, stimulate a more limited group of
reactions (Conney, 1967). The induction of drug metabolism elicited by steroids - both naturally occurring and synthetic (Hamrick et al., 1973; Parke, 1972) - and by polychlorinated biphenyls (Alvares et al., 1973; Bickers et al., 1974) has certain features in common with the effects both of phenobarbitone and 3-methylcholanthrene. Many other factors, genetic, hormonal, dietary and environmental are known to influence the activity of the drug metabolising enzymes (e.g. Anders, 1971; Flynn et al., 1972; Gillette, 1971; Gillette et al., 1972; Kuntzman, 1969; Vessell et al., 1973) and the stimulatory effect of some inducers on hepatic blood flow (Ohnhaus et al., 1971) and bile flow (Klaassen, 1969) may also be important in determining the overall rate of drug elimination in the intact animal. In this context, factors affecting the rate of drug metabolism in extrahepatic tissues may also be important: this topic has been reviewed by Lake et al. (1973) and, with particular reference to the gut, by Hartiala (1973). Finally, it may be noted that compounds such as phenobarbitone, whilst stimulating a broad spectrum of drug metabolising enzyme activity, may not stimulate, or may even depress the activity of other microsomal enzymes such as glucose-6-phosphatase and ATP-ase (Ernster and Orrenius, 1965; Platt and Cockrill, 1967).

1.5 Proliferation of Smooth Endoplasmic Reticulum

Induction of the drug metabolising enzymes is usually accompanied by proliferation of the smooth endoplasmic reticulum within the hepatocytes (Burger and Herdson, 1966; Jones and Fawcett, 1966; Meldolesi, 1967), a response which appears to result both from enhanced membrane synthesis and decreased membrane degradation (Holtzman and Gillette, 1968; Jick and Shuster, 1966; Shuster and Jick, 1966). The new membranes are believed to arise from a
'budding-off' of smooth-surfaced elements from the cisternal ends of the rough endoplasmic reticulum (Staubli et al., 1969). Frequently the increase in smooth endoplasmic reticulum is dose-related and correlates with the degree of enzyme induction (Botham et al., 1970; Conney, 1967). In fact, Staubli et al. (1969) have reported that in rats treated with phenobarbitone, the increase in the specific surface of the smooth endoplasmic reticulum is linearly related to the increase in drug metabolising enzyme activity. However, preparation of isolated smooth and rough endoplasmic reticulum has shown that drug metabolising enzyme activity is not confined to the smooth fraction (Holtzman et al., 1968). Moreover, in the case of polycyclic hydrocarbons, enzyme induction is accompanied only by marginal increases in the amount of smooth endoplasmic reticulum (Fouts and Rogers, 1965). Equally, stimulation of smooth endoplasmic reticulum proliferation is not the sole perogative of enzyme inducers since several compounds are known to produce marked proliferation without increasing enzymic activity (Kunz et al., 1966b). Many hepatotoxins and carcinogens can also elicit an increase in smooth endoplasmic reticulum (Meldolesi, 1967; Ortega, 1966; Svoboda and Higginson, 1968) although the effects of such compounds are not restricted to this fraction of the liver cell (Du Boistesselin, 1966; Rouiller, 1964). There is disagreement as to whether the smooth membranes so induced are identical with (Stenger, 1970), or different from (Arcasoy et al., 1968), those induced by compounds regarded as non-hepatotoxic, e.g. phenobarbitone.

1.6 Liver Enlargement with Enzyme Induction

Enlargement of the liver with proliferation of the smooth endoplasmic reticulum may be brought about by a variety of chemicals
in the absence of any other histological or ultrastructural changes (Barka and Popper, 1967; Fouts and Rogers, 1965; Wilson et al., 1970). Where it is accompanied by induction of the drug metabolising enzymes, this response has been viewed as a physiological adaptation to increased metabolic demand - a so-called 'hyperfunctional' liver enlargement (Gilbert and Golberg, 1965). The significance of this change in relation to the concept of the no-effect level in toxicological assessment has been discussed by a number of authors (Hutterer et al., 1969; Platt and Cockrill, 1967, 1969; Popper, 1966; Wilson et al., 1970; Wright et al., 1972). It may be argued that the response is per se a beneficial one since it results in accelerated metabolism, and hence excretion, of the administered compound. In this context Gilbert and Golberg (1967) showed that after administration of butylated hydroxytoluene (BHT) for 5 days the total capacity of the rat for the metabolism of BHT increased from 42 to 330 mg/kg body weight/day. This induction of metabolism was paralleled by a sharp fall in the body fat concentration of BHT (Gilbert and Golberg, 1965). After pretreatment with low doses of dieldrin, Hutterer et al. (1969) showed that rats could survive previously lethal doses without ill effect. DDT, likewise, is known to stimulate its own metabolism (Morello, 1965) and to elicit a dose-related increase in liver weight (Hoffman et al., 1970). These effects appear to be readily reversible after short-term administration of the inducing compound and this finding has strengthened the concept of an adaptive, hyperfunctional liver response (Golberg, 1966). However, whilst there other examples of arguably beneficial effects of enzyme induction, a number of situations are known in which a hyperfunctional liver response may have adverse consequences for the animal. This is mentioned in Chapter 8.
1.7 Liver Enlargement without Enzyme Induction

Enlargement of the liver may not always be associated with increased drug metabolising enzyme activity. Thus, administration of carbon disulphide to rats results in an increase in liver weight with increased total liver protein content but decreased drug metabolic activity (Bond and De Matteis, 1969). Chloroform, halothane and methoxyflurane cause marked proliferation of the smooth endoplasmic reticulum without enhancing enzyme activity (Kunz et al., 1966b). A similar dissociation of liver enlargement from increased drug metabolising enzyme activity is seen with coumarin and 2,4-xylidine: both of these compounds give rise to early histochemical, but not histological, changes indicative of hepatotoxicity (Grasso et al., 1974) and, on prolonged administration, to the development of gross pathological changes in the liver (Hazleton et al., 1956; Lindstrom et al., 1963). In the case of dieldrin, initial hyperfunctional liver enlargement gives way, on continued administration of the compound, to a situation in which increased liver weight and proliferation of the smooth endoplasmic reticulum are associated with a return of drug metabolising enzyme activity to control levels (Hutterer et al., 1969). These changes occur while the liver remains histologically normal but prolonged administration of dieldrin is known to produce histopathological changes in the rat liver (Deichmann et al., 1970). This type of liver response where increased liver size and protein content precede the ultimate development of liver injury has been referred to as 'anabolic hepatotoxicity' (Madhaven et al., 1970; Popper, 1966). Madhaven et al. emphasise the distinction between 'anabolic' and 'catabolic' hepatotoxicity, the latter produced by such compounds as dimethylnitrosamine and aflatoxin, being characterised by an early inhibition of protein synthesis, a rapid decline in drug metabolising enzyme activity and no increase in liver weight.
1.8: Nature of the Proposed Project

The depressive effects of potent hepatotoxins and carcinogens on drug metabolising enzyme activity are well known (Baldwin and Barker, 1965; Barker and Smuckler, 1972; Miller et al., 1958; Trams et al., 1961). Less attention has been directed to compounds possessing a lower order of hepatotoxicity, whose effects may be manifest only after prolonged administration of high doses. The findings mentioned above, showing that liver enlargement produced by such compounds is unaccompanied by any sustained induction of drug metabolising enzyme activity, raise the possibility that the inadequacy or failure of this enzyme response may be important in the subsequent development of pathological changes in the liver. Such a hypothesis is a corollary of the view that liver enlargement accompanied by drug metabolising enzyme induction is an adaptive response of benefit to the liver.

For the present study, therefore, four model compounds were selected, all of which were known to produce liver enlargement, and, at least initially, induction of the drug metabolising enzymes, but which ultimately give rise to widely differing pathological changes in the liver. Sequential observations have been made during the prolonged administration of these compounds to rats with the aim of attempting to correlate biochemical and histochemical changes with morphological alterations in the liver. The compounds selected are described below, literature relevant to each being briefly reviewed in the appropriate experimental chapters.

Butylated hydroxytoluene - a synthetic, hindered phenolic antioxidant which produces a typical hyperfunctional liver enlargement without apparently giving rise to any pathological changes in the liver, even after life-span administration.
Safrole and Ponceau MX - a naturally occurring food flavour and a synthetic azo food colour respectively, prolonged administration of which in high doses leads to the development of hepatic lesions variously described as nodular hyperplasia and benign and malignant neoplasia.

2-Acetylaminoﬂuorene - a model carcinogen known to produce a high incidence of hepatocellular carcinoma, the development of which is preceded by the early appearance of hyperplastic liver nodules.

Such a study, in addition to its academic interest, was considered important in view of the widespread occurrence of enzyme inducers in the human environment, the paucity of data concerning the long term effects on drug metabolising enzyme activity of compounds not acutely toxic to the liver, and the current debate as to the toxicological significance of liver nodules. Resolution of the nature of the latter is an important practical problem since several compounds which give rise to this lesion have extensive utility, e.g. DDT (Fitzhugh and Nelson, 1947), safrole (Long et al., 1963), carbon tetrachloride (Confer and Stenger, 1966; Reuber and Glover, 1967) and Ponceau MX (Grasso et al., 1969).
CHAPTER TWO

MATERIALS AND METHODS
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w) Histology
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y) Statistics
2.1 Materials

a) Chemicals Administered to Rats

Butylated hydroxytoluene (Topanol OC), pharmaceutical grade, was a gift from I.C.I. Ltd., Heavy Organic Chemicals Division, Billingham, Teeside. Safrole was purchased from Hopkin and Williams Ltd., Chadwell Heath, Essex and the stated purity was > 98%. Ponceau MX was a gift from Williams (Hounslow) Ltd., Hounslow, Middlesex. All of the dye used was from the same batch and conformed to British Standard N° 3671:1963 for Ponceau MX for use in foodstuffs. 2-Acetylanilinofluorene (M.pt. 192 - 196°C) was obtained from Ralph Emmanuel Ltd., Wembley, Middlesex. 2,4-, 2,5- and 2,6-Xyldine (4-amino-1,3-dimethyl, 2-amino-1,4-dimethyl, and 2-amino-1,3-dimethylbenzene respectively) were purchased from Koch Light Laboratories Ltd., Colnbrook, Bucks. Hexobarbitone, B.P. grade, was obtained from May and Baker Ltd., Dagenham, Essex; sodium phenobarbitone, reagent grade, from BDH Ltd., Poole, Dorset and 3-methylcholanthrene from Sigma (London).

2,4-, 2,5- and 2,6-Xyldine were redistilled under reduced pressure to give colourless products. All other compounds were used without further purification.

b) Substrates

Aniline hydrochloride, biphenyl and sodium succinate were obtained from BDH Ltd., cytochrome c (Type IIA), glucose-6-phosphate dipotassium salt and sodium β-glycerophosphate (Grade 1) from Sigma and ethylmorphine hydrochloride from May and Baker Ltd. Biphenyl was recrystallised from absolute ethanol, and sodium succinate from water. Other substrates were used as purchased.

For the studies in Chapter 7, substrates were obtained as follows: benzene, toluene, ethylbenzene, butylbenzene, hexylbenzene, isopropylbenzene,
hexane, cyclohexane, carbon tetrachloride, 4-methoxyphenol, 4,4'-bipyridyl and nicotinamide from B.D.H. Ltd.; naphthalene and allylbenzene from Koch Light Laboratories Ltd.; butylated hydroxyanisole from May and Baker Ltd.; N,N'-dimethylaniline and eugenol from Hopkins and Williams Ltd.; 4-phenylanisole, piperonal, piperonyl butoxide and aminopyrine from Phase Separations Ltd., Deeside Industrial Estate, Queensferry, Flintshire; 4,4'-dimethylbiphenyl from Ralph Emmanuel Ltd. The octyl carbamate and dodecyl carbamate were synthesized by Dr. B. Houston in these laboratories. Imipramine was kindly donated by Biorex Laboratories Ltd., London and SKF 525A kindly donated by Smith, Kline and French Ltd., Welwyn Garden City, Herts.

c) Enzymes and Cofactors

Nicotinamide adenine dinucleotide phosphate (NADP), its reduced form NADPH, reduced nicotinamide adenine dinucleotide (NADH) and glucose-6-phosphate (disodium salt) were initially obtained from Boehringer (London) Ltd. and subsequently from P-L Biochemicals (International Enzymes Ltd., London). Glucose-6-phosphate dehydrogenase (Type XII) and Protease VII were purchased from Sigma.

d) Other Chemicals

2- and 4-Hydroxybiphenyls, 4-aminophenol and formaldehyde were obtained from B.D.H. Ltd., bovine serum albumin and 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride from Koch Light, and tetranitro blue tetrazolium and Fast Blue RR salt from Sigma. Most other chemicals were obtained from B.D.H. Ltd. and were usually of reagent grade.

Gases were supplied by B.O.C. Ltd., Crawley and, with the exception of ethanol (99.9% and 96%) from James Burroughs Ltd., London, solvents were obtained from B.D.H. Ltd. Water was glass-distilled and stored in polypropylene containers.
2.2. Animals

a) Protocol of Usage

Wistar albino rats (Porton strain, random bred in closed colony) were supplied by the University of Surrey Animal Breeding Unit. For one series of experiments with 2-acetylamino fluorene male rats were used; for all other experiments female rats were used.

For the chronic dietary administration of test compounds, five week old (80-100g) female rats were randomly assigned to control and treated groups and housed in polypropylene cages with aluminium lids (26" x 13.5" x 8"), 5 rats per cage, on "Sterolit" bedding (W.P. Usher and Company, Ltd., London). Prior to administration of the test diet, all rats were maintained on Spillers Laboratory Animal Diet N°2 for 7 days to allow acclimatisation to the powder form of diet. At all times animals were allowed access to diet and tap water ad libitum and bedding was changed at weekly intervals. Measurements of body weight (individual) and food intake (per cage) were made at regular intervals. Food intake was monitored over periods of 3 consecutive days and accurate measurement was facilitated by the use of stainless steel food pots, specially designed to minimise diet spillage, which were kindly loaned by the British Industrial Biological Research Association, Carshalton, Surrey.

Where compounds were administered other than in the diet, rats were treated as above but were maintained on Spillers Laboratory Animal Diet N°1 (pelleted form of N°2) and food intake was not measured.

The temperature and relative humidity of the animal room was regulated to 22°C and 50% respectively and lighting was time-switched on at 06.30 and off at 18.30 hours.
b) Preparation of Diets

For dietary administration, compounds were mixed into the stock powdered diet using a Hobart food mixer (model A 200). BHT, safrole and xylidine diets were prepared by adding the compounds dissolved in a quantity of groundnut oil ("Saladin", Van den Burghs & Jurgens Ltd., London) such as to raise the fat content of the diet by 2% (w/w). The groundnut oil was free from known additives (A. Rahim, personal communication, 1971). Control diet was prepared by adding 2% (w/w) groundnut oil to the stock powdered diet. Ponceau MX and 2-acetylaminofluorene diets were prepared by dry-mixing the compounds into control diet. In the case of the carcinogen, 2-acetylaminofluorene, mixing was carried out by shaking in a tightly sealed glass jar.

Diets were normally prepared at weekly intervals in 4 or 8 kg amounts and were mixed for 15 minutes at the slowest speed of the Hobart mixer. Oil solutions were initially mixed (2 - 3 minutes) into 25% of the total quantity of diet and this premix subsequently dispersed into the remainder. On account of their volatility, safrole and the xylidine isomers were added to the diet at a level 10% higher than the desired concentration (see Borchert et al., 1973b; Hagen et al., 1967) and were mixed for 10 minutes. All diets were stored in tightly sealed dark glass jars at room temperature.

The distribution of Ponceau MX in the diet was shown to be even by reading aqueous extracts of aliquots of the diet at 505nm: even distribution was also shown for the 2-acetylaminofluorene diet, ethanol extracts being read at 288nm. The other diets were not checked for uniformity of mixing.

c) Administration of Compounds Other than in the Diet

Compounds were administered by intraperitoneal injection as follows unless stated otherwise:-

Sodium Phenobarbitone - 80 mg/kg daily for 4 days as a 5% (w/v) aqueous
solution.

3-Methylcholanthrene - 30 mg/kg daily for 2 days as a 1% (w/v) solution in groundnut oil.

Safrole - 150 mg/kg daily for 4 days as a 7.5% (w/v) solution in groundnut oil.

Control animals normally received an equal volume of solvent but in the case of comparative experiments where all three treatments were used, only groundnut oil controls were run. Animals were killed 16 hours after the final injection.
2.3. Methods

a) Preparation of Liver Fractions

Rats were fasted overnight and killed by cervical dislocation between 08.00 and 10.00 hours. Livers were rapidly excised and rinsed in ice-cold 1.15% (w/v) KCl. After removal of any adhering fat or connective tissue the liver was blotted dry and weighed. A homogenate was prepared in 2 volumes of ice-cold 1.15% KCl using four return strokes of a size C Potter-Elvejhem type, teflon-glass homogeniser (A.H. Thomas and Co., Philadelphia, U.S.A.) power driven at 2,950 r.p.m. The homogeniser was rinsed with a further volume of 1.15% KCl and the resultant 25% homogenate centrifuged in polypropylene tubes at 10,000 g av. for 20 minutes at 20° C (M.S.E. "High Speed 18", 8 x 50 ml angle rotor, 11,000 r.p.m.). The supernatant, referred to as the microsomal supernatant and comprising the microsomal and soluble cell fractions, was used for the determination of a number of enzyme activities. Further centrifugation at 105,000 g av. for 60 minutes or 157,000 g av. for 40 minutes at 20° C (M.S.E. "Superspeed 50", 8 x 25 ml angle rotor at 40,000 r.p.m. or 10 x 10 ml rotor at 50,000 r.p.m.) yielded a clear supernatant, which was retained for assay of glucose-6-phosphate dehydrogenase, and a microsomal pellet which, after rinsing to remove residual supernatant, was resuspended with two return strokes in a size B homogeniser, in a medium and to a protein concentration as detailed under specific procedures. To obtain a "washed" microsomal fraction, in which contamination by haemoglobin and trapped soluble enzymes and cofactors is reduced to a minimum, the pellet was resuspended in 1.15% KCl and recentrifuged at 105,000 or 157,000 g av. for 60 or 40 minutes respectively. The supernatant was discarded and, after rinsing, the pellet was resuspended as described above.

All of the above procedures were performed as rapidly as possible and at a temperature of 0 - 40° C. Prior to use, all fractions were kept at 00° C.
b) Measurement of Protein Concentration

The method of Lowry et al. (1951) was used. To 0.5 ml aliquots of tissue suitably diluted in 0.5N NaOH, was added 5 ml of freshly prepared Lowry reagent (2% Na₂CO₃, 1% CuSO₄·5H₂O, 2% Na K tartrate 100:1:1 by vol.). After at least 10 minutes, 0.5 ml of Folin-Ciocalteu Phenol reagent (diluted 1:1 with water) was added and immediately vortex mixed. After allowing 30 minutes for development, the blue colour was measured at 720 nm in a Cecil model CE 272 u.v. spectrophotometer (Cecil Instruments Ltd., Cambridge). All determinations were carried out in duplicate and blanks (0.5N NaOH) and standards (50 - 150 µg bovine serum albumin in 0.5N NaOH) were treated identically.

Where microsomes were suspended in the presence of glycerol, the procedure was modified since traces of glycerol were found to markedly enhance the intensity of the colour. A dilution of the glycerol buffer was prepared in exactly the same manner as the dilution of the microsomal suspension. 0.5 ml of this solution was then added to the standards, blanks and other samples not containing glycerol, and 0.5 ml 0.5N NaOH added to the glycerol-containing samples prior to addition of the Lowry reagent.

c) In Vitro Enzyme Assays: General Considerations

The following points apply to all the in vitro enzyme assays performed.

1. All incubation systems were prepared in duplicate on ice. Where subsequent extraction of the reaction product was necessary, teflon-stoppered test tubes were used.
2. Incubations were carried out in an atmosphere of air in a shaking incubator (Mickle Laboratory Engineering Co., Gomshall, Surrey) with a shaking rate of around 100 oscillations per minute.
3. An M.S.E. "Mistral 6L" centrifuge equipped with a 6X IL swing-out rotor was used for all centrifugations.

4. For all assays based on colourimetric measurement of the reaction product there was a linear relationship between optical density and amount of product formed over a concentration range greater than the extremes of variability encountered. Reaction rates were checked for linearity over the incubation time used.

5. Enzyme activities and protein and cytochrome concentrations were normally expressed per gram wet weight liver. In certain cases measurements were also expressed per milligram of microsomal protein and per total liver per 100 grams body weight.

d) Succinate Dehydrogenase

This enzyme activity was measured as succinate - 2-(p-iodophenyl)-3-(p-nitrophenyl)-5-phenyl-tetrazolium chloride (INT) reductase, according to the method of Pennington (1961). A dilution of liver whole homogenate (1:100 in 1.15% KCl) was used in the following incubation system:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer ml.</td>
<td>0.3</td>
<td>0.5</td>
</tr>
<tr>
<td>Substrate ml.</td>
<td>0.2</td>
<td>-</td>
</tr>
<tr>
<td>Whole homogenate ml.</td>
<td>0.5</td>
<td>0.5</td>
</tr>
</tbody>
</table>

Incubation volume = 1.0 ml.

Buffer: Sodium phosphate 0.2M pH 7.4 containing 1.25 mg INT/ml.
Substrate: Sodium succinate 0.3M at pH 7.4

After incubation for 8 minutes at 37° C the reaction was terminated by addition of 1.5 ml of ice-cold 10% (w/v) trichloroacetic acid. At this stage tubes were routinely stored at -10° C for 48 hours. 5 ml of ethyl acetate was then added and the tubes shaken vigorously. After
centrifugation for 10 minutes at 2,000 r.p.m. to separate the phases, the reduced INT was measured in the ethyl acetate phase at 490 nm in the Cecil spectrophotometer. Enzyme activity was calculated using an extinction coefficient for reduced INT of 20.1 mM$^{-1}$ cm$^{-1}$ (Pennington, 1961).

e) Glucose-6-Phosphate Dehydrogenase

The assay system of Löhr and Waller (1963) was utilised, in which the reduction of NADP is followed at 340 nm. No correction for 6-phosphogluconate dehydrogenase activity was made.

Into each of two 1 cm quartz cuvettes, the following additions were made:

<table>
<thead>
<tr>
<th></th>
<th></th>
<th>1.9</th>
<th>0.5</th>
<th>0.1</th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl 50 mM pH 8.1 ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADP solution 4 mM ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>105,000 g liver supernatant ml.</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

The cuvettes were placed in the forward cell compartment of a Pye-Unicam SP 1800 dual beam u.v. spectrophotometer at ambient temperature. After recording the basal absorption, 0.5 ml of buffer was added to the reference cuvette and the reaction started by addition of 0.5 ml 20mM glucose-6-phosphate to the test cuvette from an Oxford Sampler (Boehringer Ltd., London). Liquid is expelled from this instrument sufficiently forcefully to provide adequate mixing without removing the cuvette from the spectrophotometer. The increase in absorption at 340 nm was followed and from the initial, linear phase of the reaction, glucose-6-phosphate dehydrogenase activity was calculated using an extinction coefficient for reduced NADP of 6.22 mM$^{-1}$ cm$^{-1}$ (Dawes, 1967).
f) **Ethylmorphine N-Demethylase**

N-demethylation is a typical reaction of the microsomal mixed function oxidase system, the formaldehyde produced being conveniently measured by the method of Nash (1953). The following incubation system, based on that of Holtzman *et al.* (1968), was used:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer ml.</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Semicarbazide solution ml.</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Cofactor solution ml.</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Substrate ml.</td>
<td>0.2</td>
<td>0.2*</td>
<td>0.2*</td>
</tr>
<tr>
<td>Microsomal supernatant ml.</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Standard ml.</td>
<td>-</td>
<td>-</td>
<td>0.1*</td>
</tr>
</tbody>
</table>

* denotes added after the incubation. Incubation volume = 1.8ml

Buffer: Tris-HCl, 0.3M pH 7.4

Semicarbazide solution: 2% (w/v) aqueous solution adjusted to pH 7.0 with NaOH.

Cofactor solution: each 0.2 ml contained NADP 2 μmol, glucose-6-phosphate 20 μmol and magnesium, as MgCl₂, 10 μmol.

Substrate: Ethylmorphine hydrochloride, 75 mM aqueous solution

Standard: aqueous formaldehyde approximately 5 μmol/ml, standardised using an extinction coefficient of 8 mM⁻¹ cm⁻¹ for the HCHO-Nash reagent complex.

Incubation was for 10 minutes at 37° C and the reaction was terminated by addition of 1 ml 15% (w/v) zinc sulphate after placing the tubes into ice. After addition of substrate and standard to the appropriate tubes, 1 ml of saturated barium hydroxide was added to each tube and the contents mixed. All tubes were then centrifuged at 2,000 r.p.m. for 15 minutes at 4° C.
of clear supernatant was pipetted into clean tubes and 2 ml of freshly prepared Nash reagent (0.4% (v/v) acetylacetone in 4 M ammonium acetate) added. After heating at 37° C for 40 minutes with shaking, the resultant yellow colours were measured at 412 nm in the Cecil spectrophotometer.

g) Aniline 4-Hydroxylase

Aniline is hydroxylated at the nitrogen atom and at the ring 2-, 3- and 4- positions. The major metabolite is 4-aminophenol and this can be measured by the indophenol reaction. The following incubation system, based on that of Guarino et al. (1969), was used:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
<th>Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer ml.</td>
<td>0.7</td>
<td>0.7</td>
<td>0.6</td>
</tr>
<tr>
<td>Cofactor solution ml.</td>
<td>0.2</td>
<td>0.2</td>
<td>0.2</td>
</tr>
<tr>
<td>Substrate ml.</td>
<td>0.5</td>
<td>0.5*</td>
<td>0.5*</td>
</tr>
<tr>
<td>Microsomal supernatant ml.</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>Standard ml.</td>
<td>-</td>
<td>-</td>
<td>0.1*</td>
</tr>
</tbody>
</table>

* denotes added after the incubation. Incubation volume = 1.9 ml.

Buffer and Cofactor solutions: as for f) above

Substrate: Aniline hydrochloride, 40 mM aqueous solution adjusted to approx. pH 7 with NaOH.

Standard: 4-aminophenol, 5 μmol/ml in 0.01N HCl.

Incubation was for 15 minutes at 37° C and the reaction was terminated by placing the tubes into ice and adding to each, approximately 1g solid NaCl. After addition of substrate and standard as appropriate, 12 ml of diethyl ether containing 1.5% (v/v) isoamyl alcohol was added to each tube and all tubes extracted for 20 minutes on a rotary shaker. Any emulsion formed was broken by centrifugation at 2,000 r.p.m. for 5 minutes. 10 ml of the ether phase was then pipetted into clean tubes, 4 ml of 1% (w/v) phenol in 0.5M
tripotassium orthophosphate added and the tubes extracted for 10 minutes on the rotary shaker. After standing for at least 30 minutes, the aqueous phase was read at 620 nm in the Cecil spectrophotometer. It is preferable not to aspirate off the ether phase: if this is done, fairly rapid fading of the blue colour in the aqueous phase ensues.

h) Biphenyl 4- and 2- Hydroxylase

Biphenyl undergoes hydroxylation at a number of positions, but in the rat the major metabolite is 4-hydroxybiphenyl. Smaller amounts of 2-hydroxybiphenyl are produced and both isomers can be measured simultaneously since at pH 5.5, they exhibit different fluorescence spectra. An assay based on that of Creaven et al. (1965) was used, comprising the following incubation system:

<table>
<thead>
<tr>
<th></th>
<th>Test</th>
<th>Blank</th>
<th>2-OH Standard</th>
<th>4-OH Standard</th>
</tr>
</thead>
<tbody>
<tr>
<td>Buffer ml.</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>1.15% KCl ml.</td>
<td>0.5</td>
<td>0.5</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Cofactor solution ml.</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
<td>0.25</td>
</tr>
<tr>
<td>Substrate ml.</td>
<td>0.25</td>
<td>0.25*</td>
<td>0.25*</td>
<td>0.25*</td>
</tr>
<tr>
<td>Microsomal supernatant ml.</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
<td>0.5</td>
</tr>
<tr>
<td>2-hydroxybiphenyl standard ml.</td>
<td>-</td>
<td>-</td>
<td>0.5*</td>
<td>-</td>
</tr>
<tr>
<td>4-hydroxybiphenyl standard ml.</td>
<td>-</td>
<td>-</td>
<td>-</td>
<td>0.5*</td>
</tr>
</tbody>
</table>

* denotes added after incubation. Incubation volume: 2.0 ml.

Buffer: Tris-HCl, 0.05 M pH 8.1

Cofactor solution: each 0.25 ml contained NADP 2 µmol, glucose-6-phosphate 10 µmol, MgCl₂ 10 µmol.

Substrate: Biphenyl 12 mM in 1.15% KCl containing 2.5% (w/v) Tween 80 (polyoxyethylene sorbitan monooleate)
Standards: 2-hydroxybiphenyl 12 µg/ml in 10% (v/v) aqueous ethanol
4-hydroxybiphenyl 60 µg/ml " " " " "

After incubation at 37° for 15 minutes the reaction was terminated by
addition of 0.5 ml 2N HCl. Substrate and standard were added to the appropriate tubes. After addition of 8 ml n-heptane, all tubes were extracted for 15 minutes on the rotary shaker: any emulsion formed was broken by centrifugation at 2,000 r.p.m. for 15 minutes. 2 ml of the heptane was then transferred to clean tubes, 5 ml of 0.1 N NaOH added and the tubes extracted for a further 15 minutes on the rotary shaker. After centrifugation at 2,000 r.p.m. for 15 minutes, the heptane layer was aspirated off at the water pump. Hydroxybiphenyls in the NaOH phase were stable in the dark at 4° C for at least 48 hours.

2 ml of the NaOH phase was pipetted into a fluorimeter cuvette and the pH brought to 5.5 by addition of 0.5 ml 0.5N succinic acid. 2- and 4-Hydroxybiphenyls were determined using a Perkin Elmer MPF 3 spectrophotofluorimeter at 290 nm (excitation) and 420 nm (emission) and 282 nm (excitation) and 340 nm (emission) respectively (Instrumental readings).

It is necessary to read the 4-hydroxybiphenyl standard and all test samples and blanks at the wavelength maxima of both 2- and 4-hydroxybiphenyl, since 4-hydroxybiphenyl, under the conditions employed, fluoresces slightly at the wavelength maxima of 2-hydroxybiphenyl. A correction factor for subtraction from the 2-hydroxy readings of all test samples and blanks is derived as follows:-

$$\left( \frac{4\text{-OH biphenyl reading} \times \text{conc. 4-OH biphenyl standard}}{4\text{-OH biphenyl standard reading}} \right)_{\lambda_{\text{max} \text{ 4OH biphenyl}}} \times \left( \frac{4\text{-OH biphenyl standard reading}}{\text{conc. 4-OH biphenyl standard}} \right)_{\lambda_{\text{max} \text{ 2-OH biphenyl}}}$$
i) NADPH-Cytochrome c Reductase

An assay system based on that of Gigon et al. (1969) was used, in which the absorption of reduced cytochrome c is followed at 550 nm.

Into each of the two 1 cm glass cuvettes, the following additions were made:

<p>| | |</p>
<table>
<thead>
<tr>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td>Tris-HCl 0.02M in 1.15% KCl, pH 7.4 ml.</td>
<td>0.5</td>
</tr>
<tr>
<td>Potassium cyanide 3 mM ml.</td>
<td>1.0</td>
</tr>
<tr>
<td>Cytochrome c 0.15 mM ml.</td>
<td>1.0</td>
</tr>
<tr>
<td>Microsomal suspension, 25% in Tris/KCl as above ml.</td>
<td>0.1</td>
</tr>
</tbody>
</table>

The cuvettes were placed in the forward cell compartment of the SP 1800 spectrophotometer at ambient temperature. After zeroing the recorder, 0.2 ml Tris/KCl was added to the reference cuvette and the reaction started by the addition of 0.2 ml of NADPH regenerating system* to the test cuvette from an Oxford Sampler (see 2.3e). The increase in absorption at 550 nm was followed and from the initial, linear phase of the reaction, cytochrome c reductase activity was calculated using an extinction coefficient for reduced cytochrome c of 27.7 mM$^{-1}$ cm$^{-1}$ (Mahler and Cordes, 1966).

* The NADPH regenerating system was prepared in Tris-HCl 0.02M/1.15% KCl, pH 7.4 and its composition was as follows:-

- NADP 7.5 mM
- Glucose-6-phosphate 50 mM
- MgCl$_2$ 25 mM
- Glucose-6-phosphate dehydrogenase 5 units/ml
Formation In Vitro of the 455 nm Absorbing Species from Safrole

The metabolic transformation of safrole to a species which absorbs maximally at 455 nm is discussed in Chapters 4 and 7. The reactions involved are not clearly understood but the process can be followed by measuring the rate of increase in absorption at 455 nm on addition of safrole to an NADPH-reduced microsomal suspension. However, the species formed has a maximum at 427 nm in addition to that at 455 nm and the two are in pH-dependent equilibrium. With increasing pH the magnitude of the 455 nm maximum is relatively increased. Since the relationship between the apparent amount of species formed and the rate of its formation was not necessarily direct, the "optimum pH" of 8.0 as determined, may therefore have little absolute significance. Rate measurements were made as described below.

Microsomal suspension, 20 – 25 mg protein/ml in 0.1M sodium phosphate buffer pH 8.0, was diluted to a concentration of 0.5 – 1.0 mg protein/ml with the same buffer but which had previously been saturated with oxygen. To 2.5 ml of this diluted suspension in a 1 cm glass cuvette was added 10 µl 100mM NADPH solution (final cuvette NADPH concentration 0.4 mM). The cuvette was closed with a Subaseal stopper (Gallenkamp Ltd., London) and placed in the primary cell compartment (thermostatted at 30° C) of a Perkin-Elmer model 356 dual wavelength, double beam spectrophotometer set in the dual wavelength mode with sample and reference beams at 455 and 490 nm respectively and the range selector at 0.03 A full-scale deflection. After establishment of a stable baseline absorption, the reaction was started by injection through the stopper of 5 µl 250 mM safrole in absolute ethanol (final cuvette safrole concentration 0.5 mM). The cuvette was rapidly shaken, replaced in the spectrophotometer within 5 seconds and the increase in absorption at 455 nm monitored until maximal (1 – 5 minutes). The rate
of change in absorption was calculated from the initial, linear phase of the reaction and both this, and the total absorbance change were expressed per mg protein.

The rate of reaction was shown to be linearly related to the microsomal protein concentration over a range 0.5 - 1.5 mg protein/ml. It is preferable to start the reaction by the addition of safrole rather than NADPH so that the basal absorption is recorded in the presence of NADPH. If this is not done, the increase in absorption at 455 nm immediately upon addition of NADPH to microsomes from safrole-treated rats (see 7.3.1a) makes measurement of the basal absorption impossible.

k) Cytochrome b₅

The method of Omura and Sato (1964) was slightly modified. 2.5 ml microsomal suspension (1 - 3 mg protein/ml in 0.2M sodium phosphate buffer pH 7.4) was pipetted into each of two 1 cm glass cuvettes, a small amount (1 - 2 mg) of solid NADH added to the sample cuvette and the reduced minus oxidised difference spectrum recorded between 390 and 500 nm using the SP1800 spectrophotometer. Cytochrome b₅ content was calculated using an extinction coefficient of 185 mM⁻¹ cm⁻¹ for the extinction difference between 409 and 427 nm. (Omura and Sato, 1964).

1) Cytochrome P450: Carbon Monoxide Ligand

In its reduced form the cytochrome P450 - CO complex absorbs strongly at 450 nm. Measurement of this spectral maximum permits quantitation of the cytochrome (Omura and Sato, 1964).

2.5 ml aliquots of microsomal suspension (as k) above) were pipetted into each of two 1 cm glass cuvettes and reduced with a few mg of sodium dithionite. CO was bubbled through the contents of the sample cuvette for
30 seconds and the difference spectrum then recorded between 390 and 500 nm using the SP1800 spectrophotometer. Cytochrome P450 content was calculated from the extinction difference between 450 and 490 nm. In the absence of an accepted extinction coefficient for rat liver cytochrome P450, the original value calculated for rabbit liver cytochrome P450 of 91 mM$^{-1}$ cm$^{-1}$ (Omura and Sato, 1964) was utilised.

m) Cytochrome P450: Ethylisocyanide Ligand

Ethylisocyanide combines with reduced cytochrome P450 to produce a spectrum with maxima at 430 and 455 nm which are in pH dependent equilibrium. The spectrum was generated according to the method of Imai and Sato (1967).

2.5 ml aliquots of microsomal suspension (1-2 mg protein/ml in 0.2M sodium phosphate buffer pH 7.6) were pipetted into each of two 1 cm stoppered glass cuvettes and reduced with a few mg of sodium dithionite. After closing the cuvettes with Subaseal stoppers, 50 µl ethylisocyanide (1 mM aqueous solution) was injected into the sample cuvette, an equal volume of water added to the reference cuvette and the difference spectrum recorded between 390 and 500 nm in the SP1800 spectrophotometer. The extinction difference between 430 and 490 nm and 455 and 490 nm was measured.

n) Cytochrome P450-Substrate Interaction Spectra

These spectra were generated essentially according to the method of Schenkman et al. (1967).

2.5 ml washed microsomal suspension (1 - 2 mg protein/ml in 0.1M sodium phosphate buffer pH 7.6) was pipetted into each of two 1 cm stoppered glass cuvettes. The cuvettes were closed with the subaseal stoppers, placed in the forward cell compartment of the SP1800 spectrophotometer (at ambient temperature) and a baseline of equal light absorbance recorded between 350
and 500 nm with the recorder set to 0.2A full scale deflection. Substrate solution was then injected into the sample cuvette and an equal volume of solvent injected into the reference cuvette using a 10 μl syringe. After adjusting the recorder pen to baseline at 500 nm the difference spectrum was recorded over the baseline between 350 and 500 nm. For investigation of the kinetics of substrate interaction the desired range of substrate concentrations was achieved by making sequential additions of substrate solution (and solvent) into the same 2.5 ml aliquots of microsomal suspension. After each pair of additions the cuvette contents were mixed and the recorder pen adjusted to baseline at 500 nm – a baseline for each substrate concentration having been recorded prior to the first addition of substrate. Substrate concentrations utilised for each compound are detailed in the relevant chapters. Stock substrate solutions were prepared either in water or absolute ethanol at a concentration such that the maximum required cuvette substrate concentration could be achieved by the addition of no more than 20 μl (total) of stock solution. The assumption was made that ethanol, which itself interacts with cytochrome P450 at high concentrations, did not, at the concentrations involved, exert any effect on the interaction of the substrate under study.

For investigation of the effect of one compound on the interaction spectrum of another, equal microlitre amounts of the modifier solution were injected into both sample and reference cuvettes before recording the baselines.

Spectral changes were quantitated as the sum of the absorption difference between the maximum and the baseline and the baseline and the minimum at each substrate concentration. The spectral dissociation constant ($K_s$) and the maximum absorption change ($AOD_{max}$) – analogous to the $K_m$ and $V_{max}$ respectively of Michaelis-Menton enzyme kinetics – were calculated from double reciprocal plots according to Lineweaver and Burk (1934). Straight lines were fitted to the experimental points using an Olivetti Programma
101 programmed for linear regression analysis. Where biphasic plots were obtained, straight lines were initially fitted by eye to determine the "break-point" in the data.

o) Isooctane Extraction of Microsomes
The procedure of Leibman and Estabrook (1971) was slightly modified.

To a 3.5 ml aliquot of microsomal suspension (30 mg protein/ml in 0.1M sodium phosphate buffer pH 7.6) was added 3.5 ml isoctane (2,2,4-trimethylpentane, spectroscopic grade) and the mixture stirred for 30 minutes in air at 4°C. Replicates in which 3.5 ml phosphate buffer replaced the isoctane were used as controls. Initially, centrifugation at 105,000 g av. for 60 minutes at 4°C was employed to re-sediment the extracted microsomal pellet. However, increasing the centrifugation conditions to 157,000 g av. for 60 minutes was found to improve substantially the recovery of microsomal protein.

p) Digestion of Microsomes with "Protease VII"
The action of certain purified proteolytic enzymes is effective in bringing cytochrome b₅, but not cytochrome P450, into solution. Loss of cytochrome P450 as P420 can be minimised by the use of high concentrations of glycerol (Ichikawa and Yamano, 1967). In this study, "Protease VII" (derived from Bacillus subtilis) was used in a method developed from those of Mitani et al. (1971); and Nishibayashi and Sato, (1968).

Washed microsomes were suspended to a concentration of 4 - 6 mg protein/ml in 0.1M Tris-HCl buffer pH 7.4 containing 20% v/v glycerol. "Protease VII" (5 mg/ml in the Tris/glycerol buffer) was added to 5 ml aliquots of microsomal suspension in 25 ml conical flasks to give a concentration of 40 μg enzyme/mg of protein. The volume was made to 6 ml with buffer and the flasks flushed with nitrogen (O₂ free) for 30 seconds
prior to sealing with rubber bungs. The flasks were then incubated at 0° C for 10 hours after which the digested microsomes were re-sedimented by centrifugation at 157,000 g av. for 60 minutes at 2° C. Control incubates, to which no "Protease VII" was added, were also taken through the above procedure. After centrifugation the supernatants were carefully withdrawn using a Pasteur pipette and the pellets resuspended in Tris/glycerol buffer to a concentration of 2 - 3 mg protein/ml. Both fractions were assayed for cytochrome b₅, 455 nm absorption maximum (where applicable) and cytochrome P₄₅₀. Up to 90% of the cytochrome b₅ (but no cytochrome P₄₅₀) was usually recovered in the supernatant whilst recoveries of 70 - 90% of the cytochrome P₄₅₀ content in the pellet were normal. Increasing the incubation time did not substantially increase the solubilisation of cytochrome b₅ and tended to decrease the recovery of cytochrome P₄₅₀.

q) Hexobarbitone Sleeping Time

The duration of sleep after a single dose of hexobarbitone is dependent primarily on the rate at which the drug is metabolised. Measurement of sleeping time therefore provides an assessment of drug metabolising enzyme activity in the intact animal.

Diet was withdrawn from the rats at 16.00 hours. At 11.00 hours the following day control and treated rats were injected intraperitoneally with a 5% (w/v) aqueous solution of sodium hexobarbitone at a dose level of 100 mg/kg bodyweight. Sleeping time was measured as the time between loss and regaining of the righting reflex. The time from injection to loss of righting reflex - the induction time - was also measured. Righting reflex was defined as the ability of an animal to right itself three times within 30 seconds.
r) Enzyme Histochemistry: General Considerations

In addition to the in vitro biochemical assays, a number of enzyme activities were examined histochemically in cryostat sections of liver from the same rats. Enzyme histochemistry is valuable in providing qualitative data on intralobular and intracellular alterations in enzyme activity and distribution. This can be particularly useful in establishing correlations with histological changes.

Small pieces of liver (~4 mm cube) from the centre of the left lobe were frozen onto microtome chucks standing in a shallow dish of ethanol/solid carbon dioxide mixture. These tissue blocks could be stored at -20°C for at least 48 hours without detectable loss of enzyme activity. Sections were cut at the required thickness in a Slee Type HR cryostat at -20°C and transferred directly onto glass microscope slides. These were stored in the cryostat (for up to 2 hours) prior to incubation, taking care to avoid dehydration of the tissue. Some of this work was carried out in the Histochemistry Section of the British Industrial Biological Research Association at Carshalton, in which case tissue was initially frozen onto chucks cooled in liquid nitrogen. The incubation conditions described for each assay were adequate for 6 - 8 slides with 3 sections per slide.

s) Glucose-6-Phosphatase

The method of Wachstein and Meisal (1956) was used in which inorganic phosphate released from the substrate is "captured" by lead ions in the medium and precipitated as lead phosphate. Treatment with ammonium sulphide converts this to the readily visible black lead sulphide.

Sections of liver (10μ) were incubated at 37°C for 30 minutes in 50 ml of solution containing Tris-maleate buffer pH 6.7, 40 mM; glucose-6-phosphate (dipotassium salt) 1.5 mM and lead nitrate 4.5 mM.
After incubation, slides were washed in distilled water and then taken through the following sequence of reagents:

1) Dilute ammonium sulphide solution (4 drops BDH stock solution in 50 ml water): leave until no further darkening takes place (approx. 1 minute)
2) Tap water below pH 6.5
3) Formaldehyde solution 6% (v/v) for 2 minutes
4) Tap water.

Sections were mounted in glycerol jelly and examined in a light microscope, sites of glucose-6-phosphatase activity being stained dark brown. Control incubations from which the substrate was omitted were also run through the procedure.

t) Succinate Dehydrogenase
Reduction of tetranitro blue tetrazolium (TNBT) in this system (Nachlas et al., 1957) results in the production of insoluble blue formazan.

Sections (10μ) were incubated at 37° C for 15 minutes in 50 ml of solution containing potassium phosphate buffer pH 7.8, 100 mM, sodium succinate 85 mM and TNBT 0.1% (w/v). The TNBT was dissolved in the buffer/substrate solution immediately prior to use by warming the solution to 60° C, filtering and cooling to 37° C. After incubation, sections were washed in three changes of distilled water, mounted in glycerol jelly and examined in the light microscope. Sites of succinate dehydrogenase activity were evident as blue formazan deposits. Control incubations from which the substrate was omitted were also run through the procedure.

u) Aniline Hydroxylase
The simultaneous capture method of Gangolli and Wright (1971) was used
in which the primary reaction product, 4-aminophenol, is coupled with Fast Blue RR salt to produce an insoluble dye complex.

Sections (20μ) were incubated at 37° C for 2 hours, sections being transferred after 1 hour to a freshly prepared substrate solution. This procedure is necessitated by the instability of Fast Blue RR salt under the conditions of the assay. The incubation medium contained the following:-

- Sodium phosphate buffer pH 7.4 100 mM
- MgCl₂ 3.0 mM
- Nicotinamide 3.6 mM
- NADP 0.1 mM
- Glucose-6-phosphate (disodium salt) 1.0 mM
- Aniline hydrochloride 2.2 mM
- Glucose-6-phosphate dehydrogenase 0.1 units/ml.

It was prepared in 35 ml amounts in each of which 20 mg Fast Blue RR salt was dissolved immediately prior to use. After incubation sections were washed in three changes of distilled water, mounted in glycerol jelly and examined in the light microscope. A brown deposit identified sites of aniline hydroxylase activity. Control incubations from which the substrate was omitted were also run but in this case the incubating solution was not changed after 1 hour.

v) Acid Phosphatase

The method of Gomori (1952) was used. As in the case of s), inorganic phosphate is captured by lead ions and converted to lead sulphide. For this assay however, formalin-fixed tissue is used, fresh frozen tissue giving poor results.

Small strips of liver (approx. 3 x 10 mm) were fixed at 4⁰ C for
24 hours in formol sucrose (sodium phosphate buffer 60 mM pH 7.4 containing 10% (v/v) formaldehyde and 7.5% (w/v) sucrose) and then for a further 24 hours in gum sucrose (1% (w/v) gum acacia in 30% (w/v) sucrose). The tissue was then frozen using an MSE "Pelcool" freezing stage unit and sections cut at 5 - 7μ on a freezing microtome. Sections were collected into ice-cold gum sucrose prior to incubation, free-floating at 37°C for 15 minutes. The incubation medium was prepared by adding 10 ml 155 mM sodium β-glycerophosphate to 100 ml of 100 mM sodium acetate buffer pH 5.0 containing 4.8 mM lead nitrate. This mixture was pre-incubated at 37°C for 1 hour and filtered immediately before use. After incubation sections were washed in distilled water, transferred to dilute ammonium sulphide solution (see s) and finally washed again in distilled water. They were then transferred to glass microscope slides and mounted in glycerol jelly. Sites of acid phosphatase activity were visible in the light microscope as deposits of black lead sulphide. Control incubations were not routinely run.

w) Histology

Small strips of liver from the left and median lobes were fixed in buffered formalin (10% (v/v) formaldehyde in 0.05 M sodium phosphate buffer), dehydrated in alcohol, cleared in xylene and embedded in paraffin wax. Sections approx. 5μ in thickness were cut on a base-sledge microtome (M.S.E. Ltd.) and stained with haematoxylin and eosin (Drury and Wallington, 1967).

x) Electron Microscopy

1mm cubes of liver were fixed for 1 hour at 4°C in 1% osmium tetroxide in sodium cacodylate buffer 0.1M, pH 7.4. The tissue was rapidly dehydrated through graded alcohols and embedded in Epon 812.
Sections 1μ thick were stained with toluidine blue and examined under the light microscope. Selected, centrilobular areas were then sectioned on an LKB ultramicrotome and stained with uranyl acetate and lead citrate. The sections were examined and photographed in an A.E.I. EM6B electron microscope. For these procedures reference was made to Glauert (1967).

y) Statistics

For all parameters of which multiple determinations were made, results were expressed as the arithmetic mean ± the standard error of the mean (SEM). Significant differences between means (minimum of 3 individual values) were established using Student's t-test. An Olivetti Programma 101, programmed for linear regression analysis, was utilised for the determination of correlation coefficients.
CHAPTER THREE

BIOCHEMICAL AND MORPHOLOGICAL CHANGES IN THE LIVER DURING CHRONIC DIETARY ADMINISTRATION OF BUTYLATED HYDROXYTOLUENE TO RATS
3.1 Introduction

The phenolic antioxidant butylated hydroxytoluene (2,6-di-tert-butyl-4-methylphenol, BHT) is widely used in processed food products, cosmetics and pharmaceuticals as a preservative for unsaturated lipids and other materials subject to spoilage by oxidation. Its industrial applications include the stabilisation of aviation fuels, lubricating oils, isoprene rubber and polyolefine packaging materials. From the latter, BHT may be transferred to food products and similarly, its use in animal feeds may result in residues in the edible tissues of farm animals. Currently the unconditional acceptable daily intake of BHT for humans is 0 - 0.5 mg/kg (F.A.O./W.H.O., 1967) although in practice, intake is probably far less than this: Gilbert and Martin (1967) estimated the daily intake of BHT in the U.K. at 1 mg/head, 0.01 - 0.02 mg/kg.

The ubiquity of BHT in the human environment - Collings and Sharratt (1970) found a mean BHT content of 0.23 ppm in 11 samples of human subcutaneous adipose tissue - has led to extensive investigation of its activity and disposition in biological systems (see reviews by Hathway, 1966 and The Lancet, 1965). Administration of high doses (50 - 500 mg/kg) of BHT to experimental animals produces enlargement of the liver and induction of the drug metabolising enzymes (Allen and Engblom, 1972; Botham et al., 1970; Creaven et al., 1966; Deichmann et al., 1955; Feuer et al., 1965; Gaunt et al., 1965 a,b; Gilbert and Golberg, 1965, 1966, 1967; Gilbert et al., 1969; Grantham et al., 1973; Johnson and Hewgill, 1961; Nievel, 1969). According to Gaunt et al. (1965 a,b) these changes were more marked in female than in male rats, although they were fully reversible in both sexes during 14 days of "recovery" on control diet. No change in either phenylbutazone plasma half-life or amidopyrine excretion was detected after administration of 140 mg BHT daily for 14 days to human males (Sharratt et al., 1970). This dose level - approximately 2 mg/kg - is some 25 times less than that required to produce enzyme induction in the rat.
The induction-sensitive step in the metabolism of BHT by the rat appears to be oxidation of the 4-methyl group to the hydroxymethyl derivative (Gilbert and Golberg, 1966) which is then rapidly oxidised to the carboxylic acid by a soluble enzyme (Wright et al., 1965). This metabolite appears in the urine and the bile, principally as an ester glucuronide, but its faecal excretion and that of the other major metabolite in the rat - S-(3,5-di-tert-butyl-4-hydroxybenzyl)-N-acetylcysteine - is delayed since both undergo enterohepatic circulation (Daniel et al., 1968). This delay probably accounts for the long half-life (7 - 10 days) found by Daniel and Gage (1965) after repeated administration of BHT to the rat. Shaw and Chen (1972) have isolated a hydroperoxide (4-hydroperoxy-4-methyl-2,6-di-tert butylcyclohexa-2,5-dienone) after incubation of BHT with rat liver microsomes. Formation of significant quantities of this material in vivo would be potentially hazardous in view of the high reactivity of most hydroperoxides. According to Daniel et al. (1968) man excretes an oxidation product of BHT in which all three alkyl substituents have been attacked, namely, 4-carboxy-2-[1-carboxy-1-methylethyl]-6-[1-formyl-1-methylethyl]phenol. This metabolite was not detected by Holder et al. (1970 and its existence therefore remains questionable.

The enzyme induction and liver enlargement produced by BHT is accompanied by a proliferation of hepatocyte smooth endoplasmic reticulum (Lane and Leiber, 1967), the extent of which is dose dependent (Botham et al., 1970). Liver enlargement primarily due to hypertrophy is suggested since BHT elicited either no change (Botham et al., 1970) or only a slight increase (Lane and Leiber, 1967) in mitotic activity. Different results were obtained by Schulte-Hermann et al. (1971) who contended that hyperplasia played a significant role in BHT-induced liver enlargement. This hyperplastic, but not the hypertrophic,
response to BHT was blocked by coadministration of the drug metabolising enzyme inhibitors SKF 525A and CFT 1201 (Schulte-Hermann et al., 1972).

Chronic administration of BHT to rats at dose levels sufficient to produce liver enlargement has not been associated with any histopathological change either in the liver or any other organ (Brown et al., 1959; Deichmann et al., 1955; Feuer et al., 1965; Gaunt et al., 1965a). In young monkeys, hepatic nucleolar fragmentation - apparently unaccompanied by inhibition of RNA synthesis - was observed after administration of 500, but not 50 mg/kg BHT for 28 days (Allen and Engblom, 1972). Of 18 mice, 6 developed bile duct hyperplasia after administration for 12 months of a diet containing 0.75% BHT (Clapp et al., 1973) - an effective dose level some 50,000 times the likely human exposure. Marino and Mitchell (1972) found that mice injected intraperitoneally with BHT at dose levels between 250 and 2500 mg/kg developed lung damage. This was apparently reversible and no other organs were effected. A systematic study of the effects of BHT in mice is in progress (see Thorpe and Walker, 1973).

In this chapter, some aspects of the liver response to BHT - which produces liver enlargement unaccompanied by histopathological change - have been studied to provide a "positive control" with which the liver response to the other compounds studied (see 1.8) could be compared.
3.2 Materials and Methods

a) Chronic Administration of BHT to Rats

Female rats were maintained on a diet containing 0.4% (w/w) BHT as described in 2.2. This dietary level was selected with reference to published work (see 3.1) as being equivalent to a dose level known to produce marked induction of the drug metabolising enzymes.

At weeks 1, 8, 16, 32 and 75, 4 treated and 4 control rats were killed and the livers used for the assays described in 2.3. Enzyme histochemistry (2.3) was carried out on liver samples from rats killed at weeks 1, 8, 16 and 80. Liver samples from the rats treated for 1 and 80 weeks were subjected to histological examination and the 80 week samples were additionally examined in the electron microscope.

b) Reversibility of BHT-Induced Hepatic Changes

After 80 weeks, 8 treated rats were given control diet in place of the BHT diet. After 14 days, 4 of these rats were injected intraperitoneally once daily for 3 days with sodium phenobarbitone at a dose level of 100 mg/kg. On day 18, all of these rats were killed, together with 8 control rats 4 of which had been similarly treated with phenobarbitone. Rats not receiving phenobarbitone were injected with an equivalent volume of saline.

c) Spectrally Apparent Interaction of BHT with Cytochrome P450

The kinetic constants $K_s$ and $\Delta O D_{max}$ were determined as described in 2.3. BHT was added as a solution 100mM in absolute ethanol to provide the following final cuvette concentrations (mM): 0.04, 0.06, 0.08, 0.10, 0.12, 0.16, 0.24, 0.32, 0.40, 0.80.
3.3 Results

a) Animal Body Weights and Food Intake

Growth curves for the control and BHT-fed rats are shown in Fig. 3.1. The mean body weight of the treated rats was slightly, but significantly (after 4 weeks) less than that of the control rats throughout the experiment. There was no initial rejection of the BHT diet but the food intake of the treated rats remained slightly depressed (Table 3.2). The effects of BHT on body weight and food intake are summarised in Table 3.1 and the approximate daily intake of BHT in mg/kg, calculated from the food intake data, is shown in Table 3.2.

b) Biochemical Changes During Chronic Administration of BHT

These data, expressed in the form treated as % of control values, are shown in Fig. 3.2 and Table 3.3. The complete data with statistical treatment are included in the Appendix (Table A1).

Administration of BHT for 1 week produced increases - per gram of liver - of 50 - 100% in the activities of ethylmorphine N-demethylase, biphenyl 4- and biphenyl-2-hydroxylase and in the contents of cytochrome P450, cytochrome b5 and microsomal protein. There was a 30% increase in the activity of NADPH-cytochrome c reductase but the activity of aniline 4-hydroxylase was not significantly increased. The activity of glucose-6-phosphate dehydrogenase was slightly decreased whilst succinate dehydrogenase activity and total liver protein content were significantly depressed. Liver weight - and relative liver weight - was increased by 30%.

There was little change from this pattern of response at subsequent sampling intervals. Relative liver weight increased slightly and the activity of biphenyl 2-hydroxylase relative to that of the 4-hydroxylase appeared to
FIG. 3.1

Growth Curve for Rats Maintained on Control (●—●) or 0.4% BHT (▲—▲) Diet.

Points represent mean values. Significant differences between means are shown:

a = p<0.05  b = p<0.01  c = p<0.001
### TABLE 3.1
Mean Body Weight and Food Intake of BHT-treated rats as % of Control

<table>
<thead>
<tr>
<th></th>
<th>WEEKS ON DIET</th>
<th>12</th>
<th>26</th>
<th>52</th>
<th>75</th>
</tr>
</thead>
<tbody>
<tr>
<td>Body Weight</td>
<td></td>
<td>95</td>
<td>95</td>
<td>95</td>
<td>91</td>
</tr>
<tr>
<td>Food Intake*</td>
<td></td>
<td>96</td>
<td>97</td>
<td>96</td>
<td>99</td>
</tr>
</tbody>
</table>

* Total food intake during weeks 1-12, 13-26, 27-52 and 57-75 as % of Control

### TABLE 3.2
Mean Food Intake (g/rat/day) of Control and Treated Rats and Approximate Daily BHT Intake (mg/kg body weight)

<table>
<thead>
<tr>
<th></th>
<th>WEEKS ON DIET</th>
<th>1</th>
<th>2</th>
<th>4</th>
<th>6</th>
<th>8</th>
<th>10</th>
<th>12</th>
<th>14</th>
<th>16</th>
<th>18</th>
<th>20</th>
<th>22</th>
<th>24</th>
<th>26</th>
<th>28</th>
<th>30</th>
<th>32</th>
<th>35</th>
<th>38</th>
<th>40</th>
<th>52</th>
<th>57</th>
</tr>
</thead>
<tbody>
<tr>
<td>CONTROL</td>
<td></td>
<td>13.9</td>
<td>14.1</td>
<td>15.7</td>
<td>15.7</td>
<td>14.2</td>
<td>14.4</td>
<td>15.0</td>
<td>14.2</td>
<td>14.7</td>
<td>15.4</td>
<td>14.2</td>
<td>15.9</td>
<td>14.5</td>
<td>13.2</td>
<td>13.3</td>
<td>10.8</td>
<td>13.5</td>
<td>12.3</td>
<td>12.8</td>
<td>13.1</td>
<td>13.4</td>
<td>11.3</td>
</tr>
<tr>
<td>0.4% BHT</td>
<td></td>
<td>13.1</td>
<td>13.6</td>
<td>14.3</td>
<td>15.3</td>
<td>14.0</td>
<td>14.1</td>
<td>14.0</td>
<td>14.4</td>
<td>14.3</td>
<td>15.2</td>
<td>13.5</td>
<td>14.4</td>
<td>13.7</td>
<td>13.2</td>
<td>12.8</td>
<td>10.7</td>
<td>12.6</td>
<td>11.9</td>
<td>12.4</td>
<td>12.5</td>
<td>12.2</td>
<td>11.2</td>
</tr>
<tr>
<td>Approx. BHT intake</td>
<td></td>
<td>409</td>
<td>363</td>
<td>321</td>
<td>303</td>
<td>250</td>
<td>241</td>
<td>234</td>
<td>238</td>
<td>234</td>
<td>242</td>
<td>209</td>
<td>222</td>
<td>211</td>
<td>203</td>
<td>200</td>
<td>164</td>
<td>193</td>
<td>178</td>
<td>185</td>
<td>186</td>
<td>171</td>
<td>158</td>
</tr>
</tbody>
</table>
Measurements of Some Drug Metabolising Enzymes and Related Parameters at Intervals During Chronic Dietary Administration of BHT to Female Rats

Mean values for the treated rats are expressed as % of control values (4 rats per group). Significant differences are shown (Units per g liver only): a = p<0.05, b = p<0.01, c = p<0.001.

- • Units per g liver
- Δ Units per mg microsomal protein
- 0 Units per total liver per 100g body weight

**ETHYLMORPHONE N-DEMETHYLASE**

**ANILINE 4-HYDROXYLASE**

**BIPHENYL 4-HYDROXYLASE**
TABLE 3.3

Some Parameters Additional to those Shown in Fig. 3.2*

<table>
<thead>
<tr>
<th></th>
<th>WEEKS</th>
<th></th>
<th></th>
<th></th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>8</td>
<td>16</td>
<td>32</td>
<td>75</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>211c</td>
<td>516c</td>
<td>214c</td>
<td>400b</td>
<td>194b</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>78a</td>
<td>70</td>
<td>77b</td>
<td>69b</td>
<td>75</td>
</tr>
<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>90</td>
<td>64a</td>
<td>77b</td>
<td>64a</td>
<td>-</td>
</tr>
<tr>
<td>Total liver protein</td>
<td>93c</td>
<td>87c</td>
<td>102</td>
<td>100</td>
<td>92</td>
</tr>
</tbody>
</table>

* Results expressed as % of control in units per g liver
increase markedly. This increase in the % induction of biphenyl 2-hydroxylase is, however, misleading since the actual activity decreased in both treated and control rats but this decrease was greater in the control than in the treated group. The activity of aniline 4-hydroxylase showed a significant increase at weeks 8 and 16 but this was no longer evident at weeks 32 and 75.

When expressed per total liver per 100g body weight, increases in drug metabolising enzyme activity were greater than when expressed per gram of liver weight and the depression of succinate dehydrogenase was no longer evident. Similarly, the increased microsomal protein content resulted in specific activity of the drug metabolising enzymes considerably less than the increases per gram liver.

c) Reversibility of BHT-Induced Hepatic Changes

Since induction of the drug metabolising enzymes persisted during administration of BHT for 75 weeks, the remaining treated rats were utilised to determine whether these changes were readily reversible on withdrawal of the BHT, and whether the response of the drug metabolising enzymes to subsequent "re-induction" with phenobarbitone was in any way diminished as a result of chronic BHT-treatment.

The results of this experiment, expressed as % of respective control values, are shown in Table 3.4. After administration of BHT for 80 weeks, 18 days of "recovery" on control diet resulted in a decrease from the induced levels of drug metabolising enzyme activity observed at 75 weeks, but not all parameters returned to control levels. Thus, the activity of ethylmorphine N-demethylase and cytochrome c reductase and the content of cytochrome b5 remained significantly increased and smaller increases persisted in a number of other parameters. The depression of succinate dehydrogenase observed at all previous intervals (Table 3.3). was no longer evident on withdrawal of the BHT diet.
Table 3.4

Reversibility of BHT-Induced Hepatic Changes and Subsequent Effect of Phenobarbitone
(for details of animal treatments see 3.2b)

<table>
<thead>
<tr>
<th></th>
<th>BHT 75 Weeks</th>
<th>BHT 'Recovered'</th>
<th>BHT 'recovered' + phenobarbitone</th>
<th>Control + phenobarbitone</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Body Weight</strong></td>
<td>86a⁺⁺</td>
<td>89⁺</td>
<td>82⁺</td>
<td>92§</td>
</tr>
<tr>
<td><strong>Liver Weight</strong></td>
<td>121</td>
<td>101</td>
<td>102</td>
<td>104</td>
</tr>
<tr>
<td><strong>Relative Liver Weight</strong></td>
<td>142 b</td>
<td>112</td>
<td>127</td>
<td>113</td>
</tr>
<tr>
<td><strong>Succinate dehydrogenase</strong></td>
<td>72 b</td>
<td>110 a</td>
<td>103 ns</td>
<td>94 ns, 97 ns</td>
</tr>
<tr>
<td><strong>Ethylmorphine N-demethylase</strong></td>
<td>209 c</td>
<td>139 a</td>
<td>348 c</td>
<td>250 c, 325 c</td>
</tr>
<tr>
<td><strong>Aniline 4-hydroxylase</strong></td>
<td>90</td>
<td>113</td>
<td>176 c</td>
<td>157 b, 166 c</td>
</tr>
<tr>
<td><strong>Biphenyl 4-hydroxylase</strong></td>
<td>130 a</td>
<td>115</td>
<td>251 c</td>
<td>213 c, 240 c</td>
</tr>
<tr>
<td><strong>Biphenyl 2-hydroxylase</strong></td>
<td>194 b</td>
<td>75</td>
<td>546 c</td>
<td>748 c, 505 c</td>
</tr>
<tr>
<td><strong>NADPH-Cytochrome c reductase</strong></td>
<td>170 b</td>
<td>129 a</td>
<td>163 b</td>
<td>127 a, 148 b</td>
</tr>
<tr>
<td><strong>Cytochrome P450</strong></td>
<td>204 c</td>
<td>107</td>
<td>266 c</td>
<td>249 c, 236 c</td>
</tr>
<tr>
<td><strong>Cytochrome b5</strong></td>
<td>188 c</td>
<td>131 a</td>
<td>162 b</td>
<td>124 a, 140 a</td>
</tr>
<tr>
<td><strong>Microsomal protein</strong></td>
<td>131 a</td>
<td>105</td>
<td>120 b</td>
<td>114 b, 113</td>
</tr>
<tr>
<td><strong>Total protein</strong></td>
<td>92</td>
<td>101</td>
<td>108</td>
<td>107, 103</td>
</tr>
</tbody>
</table>

i) Mean values (4 rats per group) in units per gram liver, expressed as % of, and compared statistically to, untreated controls.

§) As 1) but expressed as % of, and compared statistically to, BHT 'recovered' values.

*) a = p<0.05        b = p<0.01         c = p<0.001
Phenobarbitone, administered to treated rats 14 days after withdrawal of the BHT diet, elicited a degree of drug metabolising enzyme induction very similar to that produced in untreated control rats (Table 3.4).

d) Interactions of BHT with Cytochrome P450

BHT produced a typical type 1 interaction spectrum when added to liver microsomes from female rats. Mean values (3 determinations) for the kinetic constants were:-

\[ K_s = 3.3 \pm 0.2 \times 10^{-4} \text{M} \]

\[ \Delta \text{OD}_{\text{max}} = 0.050 \pm 0.004 \]

e) Enzyme Histochemical Changes in the Liver During Chronic Administration of BHT

At each sampling interval the activity of glucose-6-phosphatase was slightly depressed and that of aniline hydroxylase slightly stimulated in the centrilobular areas. The depression of succinate dehydrogenase observed biochemically, could not be clearly discerned histochemically. The very large centrilobular cells observed at 80 weeks (see 3.3g) contained enlarged lysosomes, but no autophagic vacuoles were present and the normal, pericanalicular distribution of the lysosomes was preserved (Fig. 3.3).

f) Macroscopic Changes in the Liver

Apart from enlargement, no other gross changes were observed.
FIG. 3.3 Acid phosphatase (x 450)

A. Unreated central liver = normal pericanalicular distribution of lysosomes.

B. BHT week 80 - normal lysosomal distribution in enlarged centrlobular cells.
g) Histological Changes in the Liver

The only histological change noted was centrilobular cell enlargement. This was evident at week 1 and by week 80 was pronounced. However, liver from rats killed 18 days after withdrawal of the BHT diet at week 80 was indistinguishable from that of untreated control rats.

h) Ultrastructural Changes in the Liver

The effects of short-term administration of BHT on the ultrastructure of rat hepatocytes have been reported in detail (Botham et al., 1970; Lane and Leiber, 1967). For the present study therefore, observations were confined to tissue taken at week 80. This differed from control tissue only in a moderate proliferation of the smooth endoplasmic reticulum.
3.4 Discussion

Enlargement of the liver and induction of the drug metabolising enzymes following the administration of BHT to rats is a well documented response (e.g. Gilbert and Golberg, 1965; Grantham et al., 1973). The results obtained in this study are consistent with these findings but demonstrate, in addition, that the response is maintained during the continued dietary administration of BHT for up to 75 weeks. During this time there was no histochemical, histological or ultrastructural evidence of liver injury. This situation contrasts with the relatively transient stimulatory effects on drug metabolising enzyme activity of safrole, Ponceau MX, 2-acetylaminofluorene and a number of other compounds which produce pathological changes in the liver. The significance of this difference in response is discussed in Chapter 8.

Changes in drug metabolising enzyme activity during the prolonged administration of enzyme-inducing chemicals do not appear to have been extensively investigated. In an early study, Gilbert and Golberg (1965) found that liver weight and enzyme activity were very significantly increased after administration of BHT to female rats by oral intubation at a dose-level of 500 mg/kg/day for 12 weeks. At a lower dose level (0.1% in the diet, equivalent to approximately 50-80 mg/kg), these effects were no longer evident after 16 weeks (Gaunt et al., 1965a). In this case it seems likely that, with the increasing body weight of the rats, the effective intake of BHT in mg/kg body weight fell to below the threshold level required to produce drug metabolising enzyme induction (Gilbert and Golberg, 1965). This eventuality did not occur at the dose level of BHT utilised in the present study.
A number of studies have shown that phenobarbitone also causes persistent enzyme induction and liver enlargement when administered to rats and dogs for continuous periods of up to 12 weeks (Burns et al., 1963; Remmer, 1962), whilst an experiment recently completed at B.I.B.R.A. indicates that these effects may be maintained indefinitely with continued administration of the compound. Under these conditions phenobarbitone, like BHT, produced no pathological changes in the liver.

In contrast to the depression of glucose-6-phosphate dehydrogenase observed in the present study, Feuer et al. (1965) reported that this activity was unchanged or slightly increased at comparable dose levels of BHT, whilst higher doses (500 mg/kg) elicited a two-fold increase. Other enzyme inducers such as phenobarbitone or DDT may also enhance the activity of glucose-6-phosphate dehydrogenase (Platt and Cockrill, 1969). An explanation for the present result is not readily apparent. Decreased hepatic activity of succinate dehydrogenase in BHT-treated rats has been previously reported (Nikol'skaya, 1964; Placer et al., 1965). Placer et al. attributed this effect to an interference with mitochondrial electron transport by BHT acting as an electron acceptor. The presently observed return of succinate dehydrogenase activity to control level on withdrawal of the BHT diet is consistent with such a view. However, a significant effect of this type in vivo seems improbable in view of the ability of experimental animals to tolerate very high doses of BHT.

Gaunt et al. (1965b) reported that the increases in rat liver weight and drug metabolising enzyme activity elicited by 14 daily doses of 500 mg BHT/kg were fully reversible within a 14 day recovery period. Evidence is presented here that these changes are reversible even after 80 weeks although at this stage the process appears to proceed relatively
slowly. According to Gilbert and Golberg (1965), an equilibrium between intake and excretion of BHT is established as soon as the response of enzyme induction is complete and thereafter, tissue levels of BHT remain relatively constant. Consequently, in view of the maintenance of enzyme induction, it is unlikely that the slower reversibility after 80 weeks is the result of increased tissue concentrations of BHT. Nor can it be explained in terms of a prolongation of the enterohepatic circulation of metabolites since the major oxidation products of BHT in the rat are poor enzyme inducers (Gilbert and Golberg, 1966). More probably the slower reversibility results from changes in enzyme regulatory function, possibly involving the stabilisation of messenger or ribosomal RNA. There is some evidence for this in the case of phenobarbitone, after the prolonged administration of which a return to normal of the increased liver weight and drug metabolising enzyme activity may take several weeks or even months (Burns et al., 1963; Remmer, 1962). Thus, phenobarbitone is known to inhibit rat liver ribonucleases I, II and III (Seifert and Vacha, 1970) and recent evidence indicates that it may also inhibit the intranucleolar degradation of newly synthesised ribosomal precursor RNA (Smith et al., 1974). It is not known whether BHT exerts similar effects. However it seems clear that the prolonged administration (80 weeks) of BHT does not produce irreversible changes in enzyme regulatory function since, on cessation of treatment, the drug metabolising enzyme response to subsequent administration of phenobarbitone was in no way impaired.

The failure of any histopathological changes to develop in the liver is in keeping with the results of previous studies with BHT (see 3.1), as were the observed histological and ultrastructural changes. Even in the greatly enlarged centrilobular hepatocytes after 80 weeks no histochemical evidence of liver injury (Grasso et al., 1974) was apparent
and the cells returned to normal size on withdrawal of the BHT diet. This indicates that the BHT-induced cell enlargement possesses a significance quite different from that of the largely irreversible hepatic megalocytosis as produced, for example, by pyrrolizidine alkaloids (Schoental and Magee, 1959) or N-nitrosopyrrolidine (Hendy and Grasso, 1975).

In conclusion, the absence of any evidence of liver damage and the findings of maintained enzyme induction during prolonged administration of BHT, strengthen the view (Golberg, 1966) that the changes evoked by this compound are more likely to represent an adaptive, physiological response than a degenerative one.
CHAPTER FOUR

BIOCHEMICAL AND MORPHOLOGICAL CHANGES IN THE LIVER DURING CHRONIC DIETARY ADMINISTRATION OF SAFROLE TO RATS
Safrole (4-allyl-1,2-methylenedioxybenzene) is a constituent of a number of naturally occurring flavours. It is the principal component (80 - 90%) of oil of sassafras from the roots of Sassafras albidum, of Brazilian sassafras oil from Octea pretiosa and of the oil of Illicium parviflorum (Foote, 1938; Hickey, 1948). It is also a major component of certain oils from plants of the Asiarum, Asarum and Heterotropa genera and a minor component of the oils of nutmeg, cinnamon leaf, star anise, mace, camphor, California laurel and American wormseed (Jacobs, 1958; Saiki et al., 1967 a, b, c, d). Safrole is thus a natural ingredient of Sassafras tea and it was quite extensively used as a flavouring agent in soft drinks - particularly root beer - and in certain pharmaceutical preparations, until its use as such was prohibited by the United States Food and Drug Administration in 1960 (Long et al., 1961). Because of its occurrence in essential oils however, safrole is still ingested in small amounts by many humans. Currently, almost the entire annual production of safrole - about 1.4 million pounds - is utilised in the manufacture of the insecticide synergist piperonyl butoxide, and piperonal, an intermediate in the synthesis of other methylenedioxyphenyl synergists (Hennessey, 1970).

A case of severe human intoxication resulting from ingestion of oil of sassafras was reported as early as 1888 (Albright 1888, cited by Jacobs, 1958), whilst Heffter (1895) noted that in animals, large doses of safrole produced rapid respiratory paralysis and lower doses caused death from widespread fatty degeneration of heart, liver, kidneys and other organs. Other reports of human and animal toxicity have been briefly reviewed by Jacobs (1958).

More recent studies, with non-lethal dose levels, have shown that the toxic effects of safrole are manifest principally in the liver. Administration of 0.04 - 1.0% of safrole in the diet of male and female rats for 150 - 730 days produced hepatic adenomas and carcinomas (Abbott et al., 1961; Hagen et al.,
(1965, 1967; Homburger et al., 1961, 1962; Long et al., 1963). According to Long et al. (1963), 19 out of 37 rats fed 0.5% safrole for 470 days had liver tumors, 74% of which were malignant on the basis of histological criteria and the finding, in two rats (14%), of metastases to the lungs. Of these malignant tumors, 66% were diagnosed as hepatocellular carcinoma and the remainder as hepatoblastoma. Other histological features of these livers included diffuse or nodular cell enlargement, fatty change, bile duct proliferation and focal, cystic necrosis. After repeated oral administration, or after injection into infant animals, safrole is also hepatocarcinogenic in the mouse (Epstein et al., 1970; Innes et al., 1969). The histological changes produced by safrole are similar in both rat and mouse liver (Hagen et al., 1965).

The carcinogenicity of safrole in a tissue distant from the sites of administration suggests a requirement for metabolic activation. The number of potential metabolites of safrole is very large. Oxidative cleavage of the methylenedioxy ring appears to be a quantitatively important route of metabolism (Casida et al., 1966; Fishbein and Falk, 1969), such that the numerous oxidation products of the allyl side chain may occur either as derivatives of methylenedioxybenzene or catechol. The number of such derivatives is increased by the possibility of migration of the double bond from the 1,2- to the 2,3-position of the side chain (Solheim and Scheline, 1973; Williams, 1959). Reactions involving the allyl group of safrole have been described by Borchert et al. (1973a), Fishbein et al. (1967), Kamienski and Casida (1970), McKinney et al. (1972), Oswald et al. (1971) and Williams (1959), whilst Solheim and Scheline (1973) have discussed the metabolism of 4-methoxyallylbenzene in detail. The role of these metabolites in the carcinogenicity of safrole is unknown, although Borchert et al. (1973 a, b) have shown that 1'-hydroxysafrole - present to the extent of 1-3% in the urine of safrole-treated rats - is very much more carcinogenic to rats and mice than safrole itself. The proposed further metabolism of 1'-hydroxysafrole to form
a reactive ester metabolite accords with the currently favoured view that the ultimate carcinogenic forms of most, if not all, chemical carcinogens are strong electrophilic reactants, able to bind covalently to tissue macromolecules (Miller, 1970). Safrole itself has no such ability (Borchert et al., 1973a).

The apparent requirement for metabolic activation suggests an important role for cytochrome P450, since this is known to be involved in the metabolism of safrole (Casida et al., 1966; Kuwatsuka, 1969) and since safrole is known to be a powerful inducer of cytochrome P450-dependent drug metabolism (Parke and Rahman, 1970; Wagstaff and Short, 1971). However, the interaction of safrole with cytochrome P450 appears to differ from that of a typical enzyme-substrate relationship. Thus, Parke and Rahman (1971) noted that the redox difference spectrum of liver microsomes from safrole-treated rats contained an additional absorption maximum at 455nm. A similar absorption maximum was produced in vitro by incubation of piperonyl butoxide with mouse liver microsomes and NADPH (Philpot and Hodgson, 1971). Franklin (1971) showed that the formation of this maximum, on incubation of microsomes from phenobarbitone-treated rats with safrole, isosafrole, piperonyl butoxide or methylenedioxybenzene, required oxygen and NADPH. This suggested the involvement of cytochrome P450-dependent metabolism and it is currently considered that the 455nm absorption maximum represents the formation of a relatively stable complex between cytochrome P450 and a methylenedioxyphenyl metabolite (Franklin, 1972a,b; Ullrich and Schnabel, 1973). The formation of this complex is discussed in more detail in Chapter 7.

In the present study, sequential measurements of drug metabolising enzyme activity have been made during the chronic administration of safrole to rats, to determine whether any relationship exists between the diverse effects of safrole on this enzyme system and the development of pathological changes in the liver.
4.2 Materials and Methods

a) Chronic Administration of Safrole to Rats

Female rats were maintained on a diet containing 0.25% (w/w) safrole as described in 2.2. This dose level was chosen because it was high enough to produce induction of the drug metabolising enzymes and, according to Hagen et al. (1965), to give rise to gross pathological changes in the liver, but not high enough to result in extensive early mortality.

At weeks 1, 8, 16, 25, 42 and 75, 4 treated and 4 control rats were killed and the livers used for the assays described in 2.3. At week 85 all the remaining treated animals (8) were killed and from the livers of 5 of these rats, samples of nodular tissue were obtained. The tissue taken was of a similar texture and colour to the adjacent parenchyma and care was taken to discard any associated necrotic or cystic tissue. Where multiple nodules were present in any one liver the tissue was pooled: in each case a sample of the nodular tissue was taken for histological examination. Subcellular fractions were prepared and assays carried out as for normal liver tissue (2.3).

Enzyme histochemistry (2.3) was carried out on liver samples from rats killed at weeks 1, 8, 16 and 25 and on nodular, and adjacent, non-nodular liver from rats killed at 80 weeks. Liver samples from the same rats were also examined by light and electron microscopy (2.3).

b) Co-administration of Safrole and Phenobarbitone

Female rats - initial body weight 100-120g - were given 0.1% (w/v) sodium phenobarbitone in their drinking water for 1 week prior to, and during administration of the 0.25% safrole diet for either 1 or 8 weeks. Control rats received either safrole diet alone, phenobarbitone and control diet, or control diet alone. The phenobarbitone solution was freshly prepared on alternate days.
4.3 Results

a) Animal Body Weights and Food Intake

Growth curves for the control and safrole-fed rats are shown in Fig. 4.1. Administration of safrole markedly depressed the rate of body weight gain, the mean difference in body weight between treated and control groups being highly significant after 1 week, and the proportionate difference between the groups increased gradually throughout the experiment (Table 4.1). The early decreased growth rate could be correlated with the decreased food intake of the treated rats (Table 4.2), although the relative decrease in body weight of these rats after about week 50 was not associated with any further depression of food intake. On the first day of safrole feeding there was almost total diet rejection, but food intake stabilised by day 7. The effects of safrole on body weight and food intake are summarised in Table 4.1 and the approximate daily intake of safrole, in mg/kg body weight, calculated from the food intake data, is shown in Table 4.2.

Under the conditions of this experiment, administration of safrole did not result in increased mortality and there was a much lower incidence of respiratory disease amongst the treated rats than amongst the control rats.

b) Biochemical Changes During Chronic Administration of Safrole

These data are presented in the form treated as % of control values, in Fig. 4.2 and Table 4.3. The full data with statistical treatment are included in the Appendix (Table A2).

Administration of safrole for 1 week resulted in increases - per gram liver - of 80 - 210% in the activities of ethylmorphine N-demethylase, aniline and biphenyl 4-hydroxylases and NADPH - cytochrome c reductase and in the contents of cytochrome P450 and b5. The 2-hydroxylation of biphenyl was
FIG. 4.1

Growth Curve for Rats Maintained on Control (■■■) or 0.25% Safrole (▲▲▲) Diet.

Points represent mean values. Significant differences between means are shown: c = p<0.001
**TABLE 4.1**

Mean Body Weight and Food Intake of Safrole-Treated Rats as % of Control

<table>
<thead>
<tr>
<th></th>
<th>WEEKS ON DIET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Body Weight</td>
<td>83</td>
</tr>
<tr>
<td>Food Intake*</td>
<td>82</td>
</tr>
</tbody>
</table>

* Total food intake during weeks 1-12, 13-26 and 27-55 as % of Control

**TABLE 4.2** Mean Food Intake (g/rat/day) of Control and Treated Rats and Approximate Daily Safrole Intake (mg/kg body weight)

<table>
<thead>
<tr>
<th></th>
<th>WEEKS ON DIET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>CONTROL</td>
<td>13.9</td>
</tr>
<tr>
<td>0.25% SAFROLE</td>
<td>8.3</td>
</tr>
<tr>
<td>Approx. Safrole intake (mg/kg)</td>
<td>151</td>
</tr>
</tbody>
</table>
stimulated to a greater extent than the 4-hydroxylation. Microsomal protein content was increased by 25%, whilst there was no change in the total liver protein content or in the activity of succinate dehydrogenase. Glucose-6-phosphate dehydrogenase was significantly depressed. The relative, but not the absolute, liver weight was significantly increased.

The initial potent induction of drug metabolising enzyme activity was greatly reduced after administration of safrole for 8 weeks, although in terms of total liver activity, this decrease was partly offset by a 66% increase in relative liver weight. By 16 weeks induction was no longer apparent: in fact there was a significant depression of biphenyl 4-hydroxylase activity, even when expressed per total liver per 100g body weight. However, despite this decreased activity towards drug substrates, the levels of NADPH-cytochrome c reductase, cytochrome b5 and microsomal protein remained greatly elevated. Throughout this period of change in drug metabolising enzyme activity there was little change in the apparent content of the 455nm absorption maximum.

At week 42 there was some indication of a recovery of drug metabolic activity in the treated rats and by week 75, this was manifest in a significant increase in the activities of ethylmorphine N-demethylase and biphenyl 4-hydroxylase. Associated with these increases was some decrease in the content of the 455nm absorption maximum and in the very high levels of cytochrome b5 and NADPH-cytochrome c reductase. However, by week 85, there was a reversal of this recovery. The very marked increase in relative liver weight evident at both 75 and 85 weeks was associated with the presence of gross pathological change in the liver. Consequently, the calculation of activities per total liver per 100g body weight was not considered to be meaningful, due to the presence of considerable amounts of cystic and necrotic tissue in the livers of the treated rats killed at these intervals.
Measurements of Some Drug Metabolising Enzymes and Related Parameters at Intervals During Chronic Dietary Administration of Safrole to Female Rats.

Mean values for the treated rats are expressed as % of control values (4 rats per group). Significant differences are shown (units per g liver only): a = p<0.05, b = p<0.01, c = p<0.001

- - - - Units per g liver
△-----△ Units per mg microsomal protein
0-----0 Units per total liver per 100 g body weight

**ETHYLORPHINE N-DEMETHYLASE**

**ANILINE 4-HYDROXYLASE**

**BIPHENYL 4-HYDROXYLASE**
### TABLE 4.3

Some Parameters Additional to those Shown in Fig. 4.2.

<table>
<thead>
<tr>
<th></th>
<th>WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>540c</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>102</td>
</tr>
<tr>
<td>Glucose 6-phosphate dehydrogenase</td>
<td>73c</td>
</tr>
<tr>
<td>Total liver protein</td>
<td>102</td>
</tr>
<tr>
<td>455 nm Absorption Maximum</td>
<td>1.04†</td>
</tr>
<tr>
<td></td>
<td>2.5§</td>
</tr>
</tbody>
</table>

* Results expressed as % of control in units per g. liver
† Values are $\Delta \text{OD}_{490-455 \text{nm}}$ per g liver
§ Values are $\Delta \text{OD}_{490 455 \text{nm}}$ per g microsomal protein
c) Biochemical Investigations of Safrole-Induced Liver Nodules

Safrole did not induce the development of non-necrotic, solid liver nodules as readily as Ponceau MX (see 5.3) and, owing to difficulties in maintaining an adequate number of rats, nodular tissue suitable for biochemical analysis could only be obtained from 5 rats. Table 4.4 shows the results obtained, in the form nodular and non-nodular treated liver as % of control, and nodular as % of non-nodular treated liver. Full data are given in the Appendix (Table A6).

Results obtained from the nodular tissue tended to be very variable. In general, the nodules were characterised by levels of drug metabolising enzymes lower than those of the surrounding, non-nodular liver and considerably lower than those of the control liver. These decreases were less evident when results were expressed per mg microsomal protein. The total protein content and succinate dehydrogenase activity of the nodular liver was quite comparable with that of the control liver. Although lower, the relative activities of the various parameters in the nodule were in general similar to those in the non-nodular tissue. Exceptions in this respect were the relatively high activity of biphenyl 4-hydroxylase and the very low level of the 455 nm absorption maximum in the nodule.

d) Co-administration of Safrole and Phenobarbitone

These experiments were aimed to determine whether the fall in drug metabolising enzyme activity produced by safrole (4.3b) was prevented by co-administration of phenobarbitone, whether additional stimulation of the synthesis of cytochrome P450 resulted in the formation of relatively greater amounts of the 455nm absorption maximum, and whether this treatment influenced the development of early histopathological change in the liver (4.3k).

The results of these experiments are shown in Figs.4.3 and 4.4.
<table>
<thead>
<tr>
<th></th>
<th>Units per g liver</th>
<th>Units per mg microsomal protein</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NON-NODULAR*</td>
<td>NODULAR*</td>
<td>NON-NODULAR*</td>
</tr>
<tr>
<td></td>
<td>NODULAR AS %</td>
<td>OF NON-NODULAR†</td>
<td>NODULAR AS %</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
<td>OF NON-NODULAR†</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>106</td>
<td>59 a</td>
<td>83</td>
</tr>
<tr>
<td></td>
<td></td>
<td>56 a</td>
<td>69 a</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>73 b</td>
<td>54 b</td>
<td>58 a</td>
</tr>
<tr>
<td></td>
<td></td>
<td>74</td>
<td>65 a</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>.99</td>
<td>89</td>
<td>80</td>
</tr>
<tr>
<td></td>
<td></td>
<td>89</td>
<td>107</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>845 c</td>
<td>618 c</td>
<td>666 c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>73</td>
<td>736 c</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>230 b</td>
<td>137</td>
<td>174 b</td>
</tr>
<tr>
<td></td>
<td></td>
<td>59 a</td>
<td>158 a</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>139 b</td>
<td>34 c</td>
<td>106</td>
</tr>
<tr>
<td></td>
<td></td>
<td>25 c</td>
<td>41 b</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>301 c</td>
<td>100</td>
<td>226 c</td>
</tr>
<tr>
<td></td>
<td></td>
<td>33 c</td>
<td>116 a</td>
</tr>
<tr>
<td>455nm Absorption Maximum</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>17 c</td>
<td>25 c</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>132 a</td>
<td>85</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>64 b</td>
<td>-</td>
</tr>
<tr>
<td>Total protein</td>
<td>96</td>
<td>92 b</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>96</td>
<td>-</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>92</td>
<td>91 b</td>
<td>-</td>
</tr>
<tr>
<td></td>
<td></td>
<td>100</td>
<td>-</td>
</tr>
</tbody>
</table>

* Results expressed as % of, and compared statistically to values for untreated control rats
† Results compared statistically to values for non-nodular liver
a = p<0.05  b = p<0.01  c = p<0.001
Effect of Coadministration of Phenobarbitone with Safrole for 1 Week on Some Drug Metabolising Enzymes and Related Parameters

Mean values for the treated rats are expressed as % of control values (4 rats per group) in units per g liver.

Treatments are shown as: P = Phenobarbitone, S = Safrole
PS = Phenobarbitone + Safrole (see 4.2b)
Mean values for the treated rats are expressed as % of control values (4 rats per group) in units per g liver.

Treatments are shown as: P = Phenobarbitone, S = Safrole, PS = Phenobarbitone + Safrole (see 4.2b)
Co-administration of safrole and phenobarbitone for 1 week elicited greater increases in drug metabolising enzyme activity than did either compound alone. Increases in the activity of ethylmorphine N-demethylase and aniline 4-hydroxylase were virtually additive. However, the activity of biphenyl 2-hydroxylase after co-administration was only some 50%, and the content of the 455nm absorption maximum only 25%, of the respective values from rats given safrole alone.

After 8 weeks the drug metabolising enzyme activity of rats co-administered with safrole and phenobarbitone remained greatly elevated whilst in the rats given safrole alone there was a marked fall in activity as previously observed. Values for the co-administered group were comparable with those of the group given phenobarbitone alone with the exception of the content of cytochrome P450, which was significantly lower, and of cytochrome b5, which was significantly higher in the former group. Surprisingly, the activity of biphenyl 4-hydroxylase in the co-administered group was higher than in the group given phenobarbitone alone, despite the apparent inhibition of this activity in the group given safrole alone. In comparison with the results after 1 week, the content of the 455nm absorption maximum was increased in the co-administered group, the value being 46 as opposed to 25% of the value for the group given safrole alone.

e) Hexobarbitone Sleeping Times

These experiments were performed to determine whether the results of an in vivo measurement of drug metabolic activity correlated with the observations in vitro of an initial induction followed by an inhibition of drug metabolism, during chronic administration of safrole.

After administration of safrole for 1 week there was a highly significant decrease in sleeping time, whilst after 25 weeks, sleeping time was
### Table 4.5

**Effect of Safrole Pretreatment for 1 and 25 Weeks on Hexobarbitone Sleeping Time in Female Rats**

<table>
<thead>
<tr>
<th>Group</th>
<th>1 WEEK</th>
<th>25 WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Induction time</td>
<td>Sleeping time</td>
</tr>
<tr>
<td>Control</td>
<td>2.4 ± 0.2</td>
<td>60.4 ± 2.3</td>
</tr>
<tr>
<td>Safrole</td>
<td>2.6 ± 0.1</td>
<td>20.7 ± 3.0</td>
</tr>
</tbody>
</table>

* Times (minutes) are means of values from 6 animals ± SEM

† Mean significantly different from control: b = p<0.01  c = p<0.001
significantly prolonged (Table 4.5). Changes in the induction time correlated with these results.

f) Correlation of the Maintained Induction of NADPH-Cytochrome c Reductase with Enhanced in vitro Formation of the 455nm Absorption Maximum

Although the initial induction of drug metabolising enzyme activity was not maintained during chronic administration of safrole (4.3b), the activity of NADPH-cytochrome c reductase did remain at a greatly elevated level. The possibility that this, otherwise anomalous, result was associated with induction of the metabolism of safrole was therefore investigated by comparing the rate of formation of the 455nm absorption maximum from safrole in vitro, in microsomes from untreated rats and rats administered with safrole for 1, 8 and 16 weeks.

Table 4.6 shows that the percentage induction of formation of the 455nm absorption maximum and of the activity of cytochrome c reductase was very similar at each interval. However, although no extinction coefficient is available for the 455nm maximum, it appears that the rate of reaction leading to its formation is extremely slow - probably much slower than the activity of cytochrome c reductase.

g) Effect of 455nm Absorption Maximum on the Apparent Content of Cytochrome b₅

It seemed possible that the unexpectedly high level of cytochrome b₅ induced by safrole was in fact due to a contribution to the reduced b₅ spectrum (λ_max 427nm) of the 427nm maximum of the safrole metabolite-cytochrome P450 complex (see 7.1). This was investigated by selective solubilisation of cytochrome b₅ after Protease VII digestion (see 2.3) of liver microsomes from rats fed the 0.25% safrole diet for 8 weeks.
Table 4.6

Induction of Cytochrome c reductase and Enhanced Formation in vitro of the 455nm Absorption Maximum During Chronic Administration of Safrole to Rats

<table>
<thead>
<tr>
<th>Safrole Pretreatment</th>
<th>NADPH-Cytochrome c reductase Production in vitro of 455nm Absorption Maximum</th>
<th></th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Weeks</td>
<td>n.mol/mg*</td>
</tr>
<tr>
<td>Control</td>
<td>1</td>
<td>29.3 ± 1.0</td>
</tr>
<tr>
<td>Safrole</td>
<td></td>
<td>51.8 ± 1.7</td>
</tr>
<tr>
<td>Control</td>
<td>8</td>
<td>27.0 ± 0.8</td>
</tr>
<tr>
<td>Safrole</td>
<td></td>
<td>53.6 ± 1.7</td>
</tr>
<tr>
<td>Control</td>
<td>16</td>
<td>25.2 ± 1.1</td>
</tr>
<tr>
<td>Safrole</td>
<td></td>
<td>54.0 ± 1.9</td>
</tr>
</tbody>
</table>

* n.mol cytochrome c reduced per minute per mg microsomal protein
† ΔOD_{455-490nm} per hour per mg microsomal protein
§ Mean values ± SEM (3 rats per group) significantly different from control
b = p<0.01; c = p<0.001
Table 4.7

Distribution and Recovery of Cytochrome P450 and b, and the 455nm Absorption Maximum after Protease VII Digestion of Liver Microsomes from Rats Treated with Safrole for 8 weeks

<table>
<thead>
<tr>
<th></th>
<th>Cytochrome P450</th>
<th>Cytochrome b,</th>
<th>455nm Absorption Maximum</th>
<th>Cytochrome b, in supernatant</th>
<th>Cytochrome b, in pellet</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>Pellet*</td>
<td>Supernatant*</td>
<td>Total+ Recovery%</td>
<td>Pellet*</td>
<td>Supernatant*</td>
</tr>
<tr>
<td>CONTROL</td>
<td>4.0</td>
<td>0.0</td>
<td>30.6</td>
<td>2.8</td>
<td>9.1</td>
</tr>
<tr>
<td>SAFROLE</td>
<td>12.9</td>
<td>0.0</td>
<td>44.0</td>
<td>12.2</td>
<td>38.0</td>
</tr>
</tbody>
</table>

* Mean values, expressed as nmol/gram liver. 4 rats per group.
† Recovery calculated as % of values for undigested microsomes
§ Mean value expressed in absorbance units
Table 4.7 shows that after digestion the 455nm absorption maximum was quantitatively recovered in the 105,000g pellet, despite loss of up to 60% of the cytochrome P450 content. However, the ratio cytochrome b<sub>5</sub> in supernatant cytochrome b<sub>5</sub> in pellet was virtually identical for microsomes both from control and safrole-treated rats, indicating that the contribution of the metabolite complex to the b<sub>5</sub> spectrum was negligible.

h) Enzyme Histochemical Changes in the Liver During Chronic Administration of Safrole

After administration of safrole for 1 week the only histochemical change observed was a slight centrilobular depression of glucose-6-phosphatase. After 8 weeks, this depression was more marked (Fig. 4.5) and there was a slight, generalised loss of aniline hydroxylase. Succinate dehydrogenase appeared normal but there were striking lysosomal changes. These were an increase in size, a loss of the pericanalicular distribution of the lysosomes and the presence of enlarged lysosomes and autophagic vacuoles randomly distributed within many centrilobular hepatocytes (Fig. 4.6). Changes after 25 weeks were similar, but there was an increase in the number of autophagic vacuoles. Although there was a generalised loss of aniline hydroxylase, some individual hepatocytes showed a striking stimulation of aniline hydroxylase activity (Fig. 4.7). The non-nodular tissue of rats killed after 80 weeks did not differ greatly from that seen at 25 weeks, although there was a decrease in succinate dehydrogenase and an irregular distribution of acid phosphatase, some cells showing almost no activity whilst in other areas enlarged lysosomes and autophagic vacuoles were randomly distributed within the cells. Nodular tissue was characterised by great variability histochemically – as it was biochemically. Large areas of cells did
FIG. 4.5  Glucose-6-phosphatase (x 72).

A. Untreated control liver - even distribution

B. Safrole week 8 - centrilobular loss.
FIG. 4.6 Acid phosphatase (x 450).

A. Untreated control liver (cf. Fig. 3.3A).
B. Safrole week 8 - clumping, enlargement and scattering of lysosomes.
FIG. 4.7 Aniline hydroxylase (x 180).

A. Untreated control liver - even distribution.
B. Safrole week 25 - generalised loss with individual cell stimulation.
FIG. 4.8  Saffrole week 80. Loss of glucose-6-phosphatase in hyperplastic nodule (x 72).

FIG. 4.9  Saffrole week 80. Acid phosphatase - autophagic vacuoles (arrowed) in nodule (x 600).
FIG. 4.10 Safrole week 80, acid phosphatase (x 450)

A. Increased activity and scattering of lysosomes in nodule
B. Reduced activity in nodule
not stain for glucose-6-phosphatase whilst some cells appeared normal (Fig. 4.8): succinate dehydrogenase was generally very low but again, some cells appeared normal. Aniline hydroxylase activity was low—quite comparable with the surrounding, non-nodular tissue. In some areas lysosomal distribution was relatively normal, in other areas their numbers were greatly reduced (Fig. 4.10) whereas in still other areas, there were increased numbers of lysosomes and autophagic vacuoles were abundant (Fig. 4.9) — in short, a grossly disturbed pattern.

j) Macroscopic Changes in the Liver

Up to and including week 42, the livers of the treated rats were slightly enlarged but otherwise, grossly normal. Gross changes were evident in the livers of treated rats killed at weeks 75 and 85 (Fig. 4.11). In some rats, there was no recognisable normal liver tissue, the whole being replaced by irregular, single or multiple nodules of up to 3 cm in diameter, whilst in other rats, nodules were present only in one or two lobes of the liver and some of these might be only 1-2 mm in diameter. Some nodules, up to 1.5 cm in diameter were similar in colour and texture to the adjacent parenchyma whilst others were associated with large amounts of connective tissue and areas of necrosis. Some nodules were cystic and filled with blood or clear fluid. No invasion of neighbouring organs was noted. In one rat killed at 75 weeks, ascitic fluid was present although the liver of this rat was subsequently shown to contain levels of drug metabolising enzymes higher than those of the other treated rats. No abnormalities were observed in control rats.
FIG. 4.11 Safrole week 80 - gross appearance of nodule-bearing livers.
k) Histological Changes in the Liver

Centrilobular cell enlargement was evident at all stages of safrole treatment, this enlargement being particularly marked at 80 weeks (Fig. 4.12). Individual cell necrosis was observed in the livers of some treated rats killed at 8 weeks and at 25 weeks several necrotic hepatocytes were present in almost every centrilobular area (Fig. 4.13): careful examination revealed no such changes in liver from control rats. No other changes were apparent up to and including week 42, although small cytoplasmic vacuoles frequently seen in control tissue, were not seen in tissue from treated rats.

It was not possible to determine whether coadministration of phenobarbitone exerted any protective effect against the necrogenic effect of safrole since in this experiment, individual cell necrosis was observed in both the treated and untreated rats. However, mild inflammatory cell infiltration was also evident and electron microscopy revealed the presence of virus particles in hepatocytes of both treated and untreated rats. Although these findings were restricted to this particular experiment, it is clear that the significance of individual liver cell necrosis in the strain of rats used and under the conditions of the present experiments must be assessed with caution.

The principal histological features of the non-nodular liver tissue obtained from treated rats killed at weeks 80 and 85 included varying degrees of fatty change, intracellular oedema, hydropic degeneration, bile duct proliferation, vascular dilatation and liver cell necrosis (Fig. 4.14). Small areas of hyperplasia surrounded by a rim of compressed hepatocytes were seen quite frequently in this tissue. The nodular lesions subjected to biochemical analysis (4.3c) were all
FIG. 4.12 Centrilobular cell enlargement
A. Safrole week 1 (Haematoxylin and eosin (H & E) x 450).
B. Safrole week 80 ("""""""" x 180).
FIG. 4.13 Safrole week 25 - individual cell necrosis (arrowed) around central vein (H & E x 450)
FIG. 4.14 Safrole week 80 - histological features of non-nodular liver

A. Cell enlargement and hydropic degeneration (H & E x 180)

B. Higher power of part of A - several pyknotic nuclei are arrowed (H & E x 770)
FIG. 4.14 (continued)

C. Bile duct hyperplasia (H & E x 180)
D. Fatty change (H & E x 180)
FIG. 4.15 Safrole week 80 - histological features of nodule

A. Nodule (on right) containing a portal tract and compressing adjacent parenchyma (H & E x 72).

B. Arrangement of liver cords within the nodule (H & E x 180).
diagnosed as hyperplastic nodules. Within these nodules, there was dilatation of the sinusoids and no recognisable lobular arrangement. However, portal tracts were present and the trabecular arrangement was preserved with liver cell cords no more than two cells thick: the cells were fairly regular and not greatly enlarged. The nodules compressed the surrounding parenchyma but they were not associated with extensive fibrosis, nor did they show any tendency to invade the surrounding parenchyma (Fig. 4.15). No nuclear abnormalities were apparent in either the nodule or the adjacent non-nodular liver tissue.

1) Ultrastructural Changes in the Liver

After administration of safrole for 1 week, free ribosomes were abundant and there was a moderate increase in smooth endoplasmic reticulum (Fig. 4.19). After 8 weeks, proliferation of the smooth endoplasmic reticulum was marked, the rough endoplasmic reticulum appeared dilated and occasional autophagic vacuoles were evident. An increased number of free ribosomes was no longer apparent. By week 25 most of the extra-nuclear space was occupied by smooth endoplasmic reticulum (Fig. 4.17): in some cells this appeared as dilated tubules while in others it was finer and more densely packed. Autophagic vacuoles were abundant and frequently they appeared to have enclosed areas of smooth endoplasmic reticulum. Some of these vacuoles contained tightly-packed whorls of smooth membrane (Fig. 4.18). The rough endoplasmic reticulum was dilated and had completely lost its normal, parallel array formation. Similar changes were observed after 80 weeks in the non-nodular liver, whilst in the nodular tissue there appeared to be less smooth, and slightly more - grossly disorganised - rough endoplasmic reticulum (Fig. 4.20). Apart from this, there was great variability in the number and size of mitochondria, in the number of
FIG. 4.16  Untreated control rat liver - typical appearance of centrilobular hepatocytes.  N-nucleus; SER-smooth endoplasmic reticulum; RER-rough endoplasmic reticulum; M-mitochondria; L-lysosome; P-peroxisome; LD-lipid droplet; BC-bile canalicus (X 7,900).
FIG. 4.17  Safrole week 25. The greater part of the cytoplasm is occupied by smooth endoplasmic reticulum (x 7,900)
FIG. 4.18  Safrole week 25 - extensive proliferation and local dilatation of smooth endoplasmic reticulum and two autophagic vacuoles (arrowed) (x 27,500).

FIG. 4.19  Safrole week 1. Apart from some increase in the content of smooth endoplasmic reticulum and free ribosomes, the tissue appears normal (x 20,500).
FIG. 4.20A Safrole week 80 - non-nodular liver. There is an abundance of smooth endoplasmic reticulum - some of it grossly dilated - and several autophagic vacuoles (arrowed) are present (x 13,700).

FIG. 4.20B Safrole week 80 - nodule. Portions of two cells are shown divided by a longitudinally sectioned bile canaliculus (BC). Note the variable appearance of the rough endoplasmic reticulum (arrowed) and the dilated tubules of smooth endoplasmic reticulum (x 20,500).
autophagic vacuoles and in the size and vesiculation of Golgi apparatus. At no stage were any nuclear or nucleolar changes evident.
4.4 Discussion

A response of liver enlargement and drug metabolising enzyme induction was seen (Chapter 3) to be sustained during prolonged administration of BHT. Similar results have been obtained with phenobarbitone (see 3.4). These findings contrast with the effects of compounds carcinogenic or acutely toxic to the liver, many of which produce a progressive depression of drug metabolising enzyme activity (Meyer and Barber, 1973; Vorne and Arvela, 1971). The hepatic effects of safrole were biphasic and cannot easily be ascribed to either of these categories of response. Initial enzyme induction was associated with an enlarged, but morphologically normal liver - indicative according to Golberg (1966) of a physiological liver response - whereas subsequent reversal of this induction correlated with the appearance of histochemical and, ultimately, histological changes indicative of hepatotoxicity (Feuer et al., 1965; Grasso et al., 1974). Reversal of drug metabolising enzyme induction during chronic administration of safrole must be distinguished from the depressive effects of hepatotoxins and carcinogens referred to above. The former comprised a return of some, but not all, activities of the enzyme system to control level, the latter comprised a non-specific fall from control level.

Effects of Safrole on Drug Metabolising Enzyme Activity

Despite its known hepatocarcinogenicity, short term administration of safrole elicited a marked induction of drug metabolising enzyme activity resembling that produced by BHT or phenobarbitone. However, safrole cannot be simply classified as a phenobarbitone-type inducer (see 1.4) in view of its action in preferentially stimulating the
2-hydroxylation of biphenyl - an effect typical of carcinogenic polycyclic hydrocarbons (Creaven and Parke, 1966) - and in inducing the appearance of the absorption maximum at 455nm. In further contrast to the action of phenobarbitone, the present results have shown that the high levels of enzyme activity initially induced are not maintained on prolonged administration of safrole.

This decline in hydroxylating enzyme activity cannot readily be attributed to non-specific effects such as decreased bioavailability, increased metabolism via an alternative pathway or a direct toxic action of safrole on the liver, since certain components of the drug hydroxylase system remained greatly elevated even after the appearance of gross pathological changes in the liver. Rather, the persistence of elevated levels of microsomal protein and cytochrome c reductase suggests that a state of induction is maintained, but that the expression of this as increased enzyme activity is in some way impaired. The rate of reduction of cytochrome P450, rather than its actual concentration, is regarded as the rate-limiting step in drug metabolism (Gigon et al., 1969). Whilst the activity of NADPH-cytochrome P450 reductase may not be directly reflected in the activity measured as NADPH-cytochrome c reductase (Davies et al., 1969), it seems probable, in view of the maintained induction of the latter, that the decrease in enzyme activity derives from changes in the terminal oxygenase, cytochrome P450, such that its concentration does become rate-limiting. In support of this view, it is known that the interaction of safrole, or a metabolite thereof, with liver microsomes in vitro results in a non-competitive inhibition of ethylmorphine metabolism (Franklin, 1972a) and an inhibition of up to 50% in the binding of CO to cytochrome P450 (Philpot and Hodgson, 1971), these effects paralleling the appearance of the absorption maximum at 455nm. If safrole interacts in a similar manner in vivo, it follows
that the true level of cytochrome P450 in microsomes from safrole-treated rats is some 40-50% greater than the apparent level, i.e. the induction of cytochrome P450 is effectively 'masked'. Experimental evidence to support this contention is presented in Chapter 7. However, since the extent of this 'masking', as judged by the level of the 455nm absorption maximum, was similar at 1 week, when there was enzyme induction, and at 16 and 25 weeks when enzyme activity was no more than control levels, it cannot directly account for the fall in hydroxylating enzyme activity. By the same argument, the apparent decrease in the level of cytochrome P450 between 1 and 16 weeks cannot be attributed to increased inhibition of CO-binding, but must represent a genuine decrease. Thus, the decline in enzyme activity appears to result from a fall in the true cytochrome P450 content to a level at which 'masking' by metabolite binding, reduces the amount of active cytochrome P450 to around control level. For the substrates ethylmorphine and aniline, this conclusion is supported by calculation of the ratio

\[
\frac{\text{enzyme activity}}{\text{cytochrome P450 concentration}}
\]

between 1 and 25 weeks. In the case of biphenyl, more complex changes appear to be involved since the decreased rate of its 4-hydroxylation was associated with a halving of this ratio.

An explanation for this differential effect on the rate of metabolism of the two type I substrates, ethylmorphine and biphenyl, may lie in the finding that under certain conditions, biphenyl, but not ethylmorphine, can displace the bound safrole metabolite from cytochrome P450. This is discussed in Chapter 7. In a similar vein, it is notable that ethoxyquin induces the metabolism of ethylmorphine but not of biphenyl (Parke et al., 1972) and that piperonyl butoxide, a
methylenedioxyphenyl derivative related to safrole, when administered to rats 1 hr prior to killing, inhibits the metabolism of aminopyrine competitively but inhibits the metabolism of biphenyl non-competitively (Friedman et al., 1972).

The view that the effects of safrole on drug metabolising enzyme activity are dependent on the balance between induction of cytochrome P450 and its inactivation by metabolite binding, is supported by the finding that coadministration of phenobarbitone with safrole prevented the fall in enzyme activity produced by safrole alone. Phenobarbitone probably acts not only by increasing the total concentration of cytochrome P450, but also by accelerating the metabolism and excretion of safrole. This might involve induction of metabolism not leading to formation of the metabolite complex, or an increased rate of further metabolism of the bound metabolite - possibly via induction of glucuronyl transferase (Halac and Sicignano, 1969). The latter seems plausible since piperonyl butoxide is known to induce hepatic glucuronyl transferase activity (Goldstein et al., 1973).

The decrease in cytochrome P450 content, unaccompanied by any fall in cytochrome c reductase activity, suggests that safrole itself, or formation of the metabolite complex, may exert a specific influence on the balance between synthesis and degradation of cytochrome P450. An investigation of these rates (see Greim et al., 1970), and the rate of turnover of the metabolite complex in vivo should be fruitful. It seems improbable that the allyl group, which in certain other molecules causes destruction of microsomal haem and an impairment of haem biosynthesis (Abritti and De Matteis, 1971/72; De Matteis, 1971, 1973), exerts any such effect in the case of safrole in view of the potent induction of cytochrome b5 elicited by safrole. This finding is perplexing, for
powerful inducers such as phenobarbitone or 3-methylcholanthrene produce only modest increases in cytochrome b₅ concentration (Estabrook et al., 1971). It has been proposed that electron transfer via cytochrome b₅ is responsible for the stimulatory effects of NADH on drug metabolism (Hildebrandt and Estabrook, 1971). More recent work has shown that cytochrome b₅ can donate the second of the two electrons required for mixed function oxidation (Baron et al., 1973) but that NADPH, via NADPH-cytochrome c reductase, is the obligatory source of the first electron (Sasame et al., 1973). Since, in the present study, the activity of NADPH-cytochrome c reductase remained induced, and since this flavoprotein cannot accept electrons from NADH-reduced cytochrome b₅ (Sasame et al., 1973), it is difficult to envisage a function for the massive amounts of cytochrome b₅ induced on prolonged administration of safrole. However, since piperonyl butoxide (Goldstein et al., 1973) and isosafrole (Lake, 1974) also appear to induce the formation of large amounts of cytochrome b₅, the possibility that it functions in formation of the 455nm absorbing species produced by these methylenedioxyphenyl compounds cannot be excluded.

Although the extent of the induction of cytochrome c reductase and formation of the 455nm absorption maximum was very similar, the very slow apparent rate of the latter reaction argues against a direct, quantitative relationship between these two activities. The results are more consistent with the view that the activity of cytochrome c reductase reflects the effective maintenance of enzyme induction, as discussed above, and that the observed parallel increase in formation of the 455nm absorption maximum results from the slow rate of this reaction such that, in this case, the concentration of cytochrome P450 is not rate limiting. Thus, it seems improbable that these findings represent a specific
induction of safrole metabolism, although the unknown nature of the reaction - particularly its relationship to the further metabolism and excretion of safrole - hinders further interpretation.

Interrelationships between the biochemical and the morphological changes produced by safrole

In considering the possible significance of the changes in drug metabolising enzyme activity observed in vitro, account must be taken of the influence of increased liver weight on the total drug metabolic capacity of the intact treated rats. When expressed per total amount of liver per 100g body weight (i.e. in relation to relative liver weight), only the activity of biphenyl 4-hydroxylase decreased to below control levels although metabolic activity toward all three substrates examined decreased markedly from the initially induced levels. In fact, the measurements of hexobarbitone sleeping time indicate that the trends observed in vitro may reflect quite closely the situation obtaining in vivo and that prolonged administration of safrole does lead to an impairment of drug metabolism.

If the initial enzyme induction is viewed as a response to the metabolic load posed by ingestion of safrole, the demise of this response might be envisaged as being disadvantageous to the animal. A biphasic effect on drug metabolising enzyme activity rather similar to that produced by safrole was observed by Hutterer et al. (1969) during the chronic administration of dieldrin to rats. The decline in enzyme activity occurred while the liver remained histologically and ultrastructurally normal but was followed by the appearance of mitochondrial injury. This sequence of events led the authors to conclude that loss of drug metabolising capacity resulted in the intracellular accumulation of levels of dieldrin toxic to the
mitochondria. No such precise chronology of biochemical and morphological events can be discerned from the present results with safrole. It can only be said that during the initial phase of enzyme induction the liver showed no abnormalities whereas the phase of decline was associated with the appearance of histochemical and ultrastructural evidence of injury. This situation lends itself equally well to the view that cell injury was the cause of the decline as it does to the converse, that cell injury was in some way a consequence of the failure or breakdown of an adaptive response. However, the finding discussed earlier that certain components of the drug metabolising enzyme system are maintained at high levels, would tend to favour the second of these two possibilities.

Changes in hepatotoxic effects following alterations in drug metabolising enzyme activity depend largely on the relative toxicities of the parent compound and its metabolites. Little information of this type is available in the case of safrole. Borchert et al. (1973a, b) have shown that 1'-hydroxysafrole is a much more potent hepatocarcinogen than safrole itself and that production of this metabolite is increased some 10-fold in rats pretreated with phenobarbitone or 3-methylcholanthrene. However, the practical significance of these findings may not be great, since safrole did not induce this pathway of its own metabolism nor was tumour incidence or mortality altered after 14 months in rats treated simultaneously with safrole and phenobarbitone (Borchert et al., 1973b). A role for the intact methylenedioxy ring in the hepatotoxicity of safrole is suggested by observations that on chronic administration, methylenedioxybenzene but not allylbenzene or eugenol (4-allyl-1-hydroxy 2-methoxybenzene) produced liver changes similar to, although less marked than those produced by safrole (Hagen et al., 1965; Taylor et al.,
These morphological findings correlate with biochemical observations that both safrole and methylenedioxybenzene can combine with and inactivate cytochrome P450 - as complexes absorbing at 455nm - whereas allylbenzene and eugenol cannot (Franklin 1971; Chapter 7). The present results do not permit interpretation of the biphasic effect of safrole on drug metabolising enzyme activity in terms of increased or decreased tissue levels of safrole or its metabolites. Nevertheless it seems probable that an early result of the demise of the initial enzyme induction would be a disturbance of the equilibrium between intake and excretion of safrole. It is possible therefore, that the cell injury produced by safrole might be initiated by an increase in its intracellular concentration. Such a hypothesis was advanced by Hutterer et al. (1969) to explain their results with dieldrin. Whilst it is almost certainly too simplistic, pharmacokinetic studies to determine whether changes in enzyme activity could be correlated with changes in tissue levels of safrole would seem to be justified. The availability of suitably radio-labelled safrole would enable accurate collection of such data by pulse-labelling during the chronic administration of unlabelled safrole.

The degree of proliferation of the smooth endoplasmic reticulum increased progressively up to week 25 of treatment but it was unaccompanied by any similar increase in drug metabolising enzyme activity or microsomal protein content. Normally, increases in these two parameters are closely related (Fouts, 1971). It is noteworthy however, that a dissociation of smooth membrane proliferation from enhanced drug metabolising enzyme activity, as seen with safrole, was also a feature of early dieldrin intoxication (Hutterer et al., 1968; 1969). The abnormal nature of the excessive smooth membrane proliferation induced by safrole is also indicated by the ultrastructural
finding that the autophagy observed histochemically appeared to be associated with these membranes. The histological findings in the present study were quite consistent with those reported in previous studies with safrole (see 4.1). Results obtained with safrole-induced liver nodules are discussed in Chapter 8 in the context of the nodules induced by Ponceau MX and 2-acetylaminofluorene.
CHAPTER FIVE

BIOCHEMICAL AND MORPHOLOGICAL CHANGES IN THE LIVER DURING CHRONIC DIETARY ADMINISTRATION OF PONCEAU MX TO RATS
5.1 Introduction

The water soluble, red food colour, Ponceau MX (Ponceau R; Ponceau 2R; Colour Index (1956) No 16150) was defined by the British Standards Institute (1963) as the disodium salt of 1-(2,4- or mixed xylylazo)-2-naphthol-3,6-disulphonic acid. It was removed from the list of colours permitted in foods in the U.K. in 1971 on the grounds of its hepatotoxicity and possible hepatocarcinogenicity.

Commercial Ponceau MX consisted of a mixture of closely related dyes with 2-naphthol-3,6-disulphonic acid as the common constituent. Variations derived from the amine component of the molecule which could be either monomethylated, polymethylated or ethylated. According to Pla Delfina (1968), dyes formed from up to 11 different amines might commonly be present, although in practice derivatives of the dimethylanilines, 2,4-(45-60%) 2,5-(15-25%) and 2,6-xylidine(12-20%) were the principal components. The composition of different samples of Ponceau MX varied according to the composition of the initial coal-tar distillate subjected to the nitrating and reducing process. In the closely related Ponceau 3R (C.I. 16155), dyes formed from trimethylanilines - principally 2,4,5-trimethylaniline - accounted for up to 50% of the total dye (Lindstrom, 1961; Pla Delfina, 1968; Manell, 1964).

Early studies failed to demonstrate clearly any toxic or carcinogenic effects of Ponceau MX. Cook et al. (1940) found no tumours related to the administration to mice of 2-3 mg per day of Ponceau MX for up to 25 months. Similarly, Waterman and Lignac (1958) found no significant increase in tumour incidence in mice fed 1 mg per day of Ponceau MX for up to 24 months. Bonser et al. (1958) treated
mice with 0.05% Ponceau MX in the drinking water and after a further observation period of 9 months found benign caecal polyps, but no other tumours, in 4 out of 6 surviving animals. No tumours were found by Willheim and Ivy (1953) in rats ingesting for 19 months a diet containing 4% Ponceau MX.

In considering their negative results, Cook et al. (1940) subscribed to a view that "... rapid elimination of these water-soluble sodium sulphonates would tend to prevent their exerting a carcinogenic influence". Such a view overlooked the possible consequences of reductive cleavage of the azo-link. This process, first demonstrated by Sisley and Porcher (1911) in dogs fed Orange 1, is effected principally by the gut microflora and is now a well recognised facet of the metabolism of azo compounds (Radomski and Mellinger, 1962; Walker, 1970). In the case of Ponceau MX, azo-reduction yields a water soluble aminosulphonic acid (1-amino-2-naphthol-3,6-disulphonic acid) and a mixture of water-insoluble aromatic amines (Daniel, 1962). Since, by analogy with the results of Radomski and Mellinger (1962), the absorption of "intact" Ponceau MX is unlikely to exceed 5% and since the aminosulphonic acid is rapidly excreted, unchanged, in the urine (Daniel, 1962), the amines are likely to be the prime determinants of the biological activity of Ponceau MX.

The potential toxicity of these amines was indicated by Treon et al. (1949, 1950), although their studies were conducted with commercial 'xylidine' which contained a mixture of isomeric xylidines. More specifically, Lindstrom et al. (1963) showed that 2,4-xylidine was hepatotoxic, chronic dietary administration to rats producing cholangiofibrosis, bile duct proliferation, hepatic cell
necrosis and foci of hepatic cell hyperplasia: no such changes were produced by 2,6-xylidine. Similar results were obtained by Pla Delfina (1968) who fed rats diets containing 3% of Ponceau MX synthesised from pure 2,4- or 2,6-xylidine. However, in dogs, not only were these xylidines more acutely toxic but 2,6-xylidine produced more severe liver damage than 2,4-xylidine (Magnusson et al., 1971). 3,4-Xylidine, present to the extent of 2-6% in Ponceau MX can give rise to pituitary tumours in rats (Boyland and Sims, 1959) and 2,4,6-trimethylaniline present in small amounts (0.05-3%) in Ponceau 3R is weakly hepatocarcinogenic (Morris et al., 1964).

The chronic hepatotoxicity of 2,4-xylidine (Lindstrom et al., 1963) and the weak hepatocarcinogenicity of Ponceau 3R (Grice et al., 1961; Hansen et al., 1963), suggested that similar effects might be exerted by Ponceau MX and more recent studies support this view. Thus, liver enlargement with fatty change and cloudy swelling was observed by Hall et al. (1966) in rats fed diets containing 0.5-2.0% of Ponceau MX for 90 days. Ikeda et al. (1966) found that rats given dietary levels of 0.2-5.0% of Ponceau MX containing over 85% of the 2,4-xylazo derivative for 15 months developed lesions diagnosed as liver cell adenomas. In a subsequent study with mice (Ikeda et al., 1968) similar findings were reported, although some lesions were classified as hepatocellular carcinoma. Rats given dietary levels of 0.125-1.0% Ponceau MX(mixed xylylazo derivative) for 2 years developed liver lesions which were interpreted by Grasso et al. (1969) as nodular hyperplasia following hepatocellular necrosis. This interpretation has been a matter of some controversy (see Bonser and Roe, 1970).
The disposition in the rat of $^{35}$S-labelled Ponceau MX was studied by Urakubo (1967) who reported that after oral administration it was excreted mainly in the faeces and after intravenous administration, mainly in the urine - unchanged. However, these observations are probably misleading since, after cleavage of the azo-link, they are informative only of the naphthol portion of Ponceau MX, an entity known to be poorly absorbed from the gastrointestinal tract (Radomski and Mellinger, 1962) and also to be rapidly excreted, unchanged, in the urine (Daniel, 1962). The metabolism of the xylidine components of Ponceau MX was investigated by Lindstrom (1961) and Lindstrom et al. (1963). They found that the hepatotoxic 2,4-xylidine was metabolised by oxidation of the 4-methyl group to give 3-methyl-4-aminobenzoic acid, whereas the relatively non-toxic 2,5- and 2,6- xylidines were metabolised almost exclusively to the corresponding 4-aminophenols. The extent to which cytochrome P450 is involved in these reactions is uncertain. Tonkopii and Fateev (1967) reported that 2,4-xylidine given intraperitoneally one hour before hexobarbitone increased the duration of anaesthesia, suggesting an interaction of this xylidine with cytochrome P450. Grasso et al. (1969) cite unpublished experiments showing stimulation of drug metabolising enzyme activity when weanling rats were given a diet containing 2% Ponceau MX for 14 days.

In this chapter, experiments have been performed, as in Chapter 4, to determine whether any correlations exist between the effects of Ponceau MX on drug metabolising enzyme activity and the development of pathological changes in the liver. Liver nodules from the treated animals have been studied, as have some effects of the constituent xylidines of Ponceau MX.
5.2 Materials and Methods

For one experiment liver and liver nodular tissue was obtained from Wistar albino rats maintained for 56 weeks on a diet containing 2% Ponceau MX in the animal unit of the British Industrial Biological Research Association, Carshalton, Surrey.

All other materials were as described in 2.1.

Methods

a) Chronic Administration of Ponceau MX

Female rats were maintained on a diet containing 1.0% (w/w) Ponceau MX as described in 2.2. This dose level was selected with reference to the study of Grasso et al. (1969) as being sufficient to produce a high incidence of liver nodules without significantly increasing mortality.

At weeks 1, 16, 25, 42 and 80, 4 treated and 4 control rats were killed and the livers used for the assays described in 2.3. In addition, these assays were carried out on tissue obtained from liver nodules at weeks 66, 70 and 80. The procedures and precautions applied in obtaining this tissue were described in 4.2a.

Enzyme histochemistry (2.3) was carried out on liver samples from rats killed at weeks 1, 8, 16 and 25 and on nodular, and adjacent, non-nodular liver from rats killed at 56 and 80 weeks. Liver samples from the same rats were examined by light microscopy and, in some cases, by electron microscopy (2.3).

b) Administration of 2,4-, 2,5- and 2,6-Xyolidine

Three experiments were performed. The first involved the
administration of each isomer at a level of 0.25% (w/w) in the diet for 7 days to groups of 4 female rats, a fourth group receiving control diet only. In the second experiment the isomers were administered by oral intubation of 5% (w/v) solutions in groundnut oil at a dose level of 150 mg/kg/day for 7 days. Controls received groundnut oil alone. Finally rats were fed the 0.25% 2,4-xylididine diet for 1 and 8 weeks.

c) Interaction of 2,4-, 2,5- and 2,6-Xylidine with Cytochrome P450

The kinetic constants of $K_s$ and $\Delta OD_{max}$ were determined as described in 2.3. Xylidines were added as solutions 500 mM in 50% (v/v) ethanol to produce the following, final cuvette concentrations (mM):-

0.2, 0.3, 0.4, 0.5, 0.6, 0.8, 1.2, 2.0.
5.3 Results

a) Animal Body Weights and Food Intake

Growth curves for the control and Ponceau MX-fed rats are shown in Fig. 5.1. The growth rate of the treated rats was slightly, but significantly (after 4 weeks), depressed. Initial diet rejection was followed by a rapid stabilisation of food intake at a level slightly below that of the control group (Table 5.2). Over the first 12 weeks, the depression of body weight of the treated rats correlated well with the depression of food intake: subsequently the relative decrease in body weight was not accompanied by any further reduction of food intake (Table 5.1). However, measurements of body weight after about week 40 were influenced by the development of severe respiratory disease in many of the rats. Whilst this outbreak affected roughly equal numbers of control and treated rats, there were many more deaths amongst the treated rats, severely reducing the numbers available for experimentation.

The effects of Ponceau MX on body weight and food intake are summarised in Table 5.1 and the approximate daily intake of Ponceau MX, in mg/kg body weight, calculated from the food intake data, is shown in Table 5.2.

b) Biochemical Changes During Chronic Administration of Ponceau MX

These data are presented in the form treated as % of control values, in Fig. 5.2 and Table 5.3. The full data, with statistical treatment, are included in the Appendix (Table A3).

The induction of drug metabolising enzyme activity elicited by Ponceau MX was much less marked than that produced by either BHT or safrole. After 1 week, the treated rats showed increases - per gram
FIG. 5.1
Growth Curve for Rats Maintained on Control (●) or 1.0% Ponceau MX (▲) Diet.

Points represent mean values. Significant differences between means are shown:

b = p<0.01
c = p<0.001
TABLE 5.1
Mean Body Weight and Food Intake of Ponceau MX-Treated Rats as % of Control

<table>
<thead>
<tr>
<th></th>
<th>WEEKS ON DIET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
</tr>
<tr>
<td>Body Weight</td>
<td>91</td>
</tr>
<tr>
<td>Food Intake*</td>
<td>.93</td>
</tr>
</tbody>
</table>

* Total food intake during weeks 1-12, 13-26 and 27-50 as % of control

TABLE 5.2
Mean Food Intake (g/rat/day) for Control and Ponceau MX-treated Rats and Approximate Daily Ponceau MX Intake (mg/kg body wt)

<table>
<thead>
<tr>
<th></th>
<th>WEEKS ON DIET</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>CONTROL</td>
<td>13.9</td>
</tr>
<tr>
<td>1.0% PONCEAU MX</td>
<td>10.9</td>
</tr>
<tr>
<td>Approx.Ponceau MX</td>
<td>767</td>
</tr>
</tbody>
</table>
liver - of 25-75% in the activities of ethylmorphine N-demethylase, biphenyl 4-hydroxylase and NADPH-cytochrome c reductase and in the contents of cytochrome P450 and b5. In contrast, the activity of aniline 4-hydroxylase was significantly depressed, being only 64% of the control value. There were small increases in the content of microsomal (17%) and total liver (8%) protein and in the activity of glucose-6-phosphate dehydrogenase (12%), whilst the activity of succinate dehydrogenase showed a decrease of 20% which was not statistically significant. Liver weight was significantly increased (35%). In common with the effect of safrole, Ponceau MX produced a preferential increase in the 2-hydroxylation of biphenyl.

After administration of Ponceau MX for 8 weeks there was no marked decrease in the induced levels of drug metabolising enzyme activity such as was seen with safrole. The activity of ethylmorphine N-demethylase decreased slightly but that of biphenyl 4-hydroxylase increased from its 1 week level and, following its early inhibition, the activity of aniline 4-hydroxylase showed a significant increase. These increases were not paralleled by any further increase in the content of cytochrome P450 or the activity of NADPH-cytochrome c reductase. The large increase in liver weight resulted in greatly elevated levels of all parameters when expressed per total liver per 100g body weight and this was true for all subsequent sampling intervals.

Continued administration of Ponceau MX brought about a gradual decrease in drug metabolising enzyme activity such that at the time of appearance of grossly recognisable liver nodules (about 60 weeks) the activity of all parameters - expressed per gram liver - had returned to, or fallen below, control levels. For the reasons outlined in 4.3b, values expressed per total liver per 100g body weight when there was
Measurements of Some Drug Metabolising Enzymes and Related Parameters at Intervals During Chronic Dietary Administration of Ponceau MX to Female Rats.

Mean values for the treated rats are expressed as % of control values. (4 rats per group). Significant differences are shown (Units per g live only): a = p<0.05, b = p<0.01, c = p<0.001.

- Units per g liver
- Units per mg microsomal protein
- Units per total liver per 100g body weight
FIG. 5.2

NADPH-CYTOCHROME C REDUCTASE

Percent Control vs. weeks

CYTOCHROME P450

CYTOCHROME b5

MICROSOMAL PROTEIN
TABLE 5.3

Measurements of Some Parameters Additional to those shown in Fig. 5.2*

<table>
<thead>
<tr>
<th></th>
<th>WEEKS</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>245</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>80</td>
</tr>
<tr>
<td>Glucose-6-Phosphate dehydrogenase</td>
<td>112</td>
</tr>
<tr>
<td>Total liver protein</td>
<td>108</td>
</tr>
</tbody>
</table>

* Results expressed as % of control in units per g liver.
c) Biochemical Investigations of Ponceau MX-Induced Liver Nodules

Histologically, an area of hyperplasia was observed in the liver of one rat as early as 25 weeks; however, sufficient nodular tissue for biochemical analysis was not normally present before 60-70 weeks. Tables 5.4 - 5.6 show the results obtained after 66, 70 and 80 weeks in the form nodular and non-nodular treated liver as % of control, and nodular as % of non-nodular treated liver. The basic data are given in the appendix (Tables A7 - A9).

As in the case of the safrole-induced nodules, the results were characterised by considerable variability such that large differences between mean values for the nodules and either the control or non-nodular treated liver were not always statistically significant. However, a consistent feature of the nodules was a content of microsomal protein significantly lower than that of liver from control rats or the non-nodular part of livers from treated rats. Consequently, when values were expressed per mg microsomal protein, increases in the nodule were accentuated and decreases were less marked relative to the control and non-nodular treated liver. In general, most parameters of microsomal enzyme activity were lower in the nodule than in the control or the non-nodular treated liver, although these decreases were much less marked, and in some instances no longer apparent, when values were expressed per mg microsomal protein. Fairly consistently the activities of biphenyl and aniline 4-hydroxylase were higher in the nodular tissue, even when expressed per gram of tissue. In contrast, the activities of ethylmorphine N-demethylase and NADPH-cytochrome c
Measurements of Some Drug Metabolising Enzymes and Related Parameters in Non-Nodular and Nodular Liver Tissue from Rats Given a Diet Containing 1% Ponceau MX for 66 Weeks

Values are means from 5 control and 5 treated rats and nodular tissue from 4 of the treated rats

<table>
<thead>
<tr>
<th></th>
<th>Units per g liver</th>
<th>Units per mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NON-NODULAR*</td>
<td>NODULAR*</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>95</td>
<td>95</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>59c</td>
<td>71</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>85</td>
<td>118</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>200</td>
<td>226</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>89</td>
<td>60a</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>102</td>
<td>49b</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>123</td>
<td>59b</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>84a</td>
<td>50c</td>
</tr>
<tr>
<td>Total protein</td>
<td>93c</td>
<td>80c</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>93</td>
<td>63c</td>
</tr>
</tbody>
</table>

* Results expressed as % of, and compared statistically to values for untreated control rats
† Results compared statistically to values for non-nodular liver

a = p<0.05  b = p<0.01  c = p<0.001
### TABLE 5.5

Measurements of Some Drug Metabolising Enzymes and Related Parameters in Non-Nodular and Nodular Liver Tissue from Rats Given a Diet Containing 1% Ponceau MX for 70 Weeks

Values are means from 4 control and 6 treated rats and nodular tissue from each of the treated rats

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units per g liver</th>
<th>Units per mg microsomal protein</th>
<th>NODULAR AS % OF NON-NODULAR</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NON-NODULAR*</td>
<td>NODULAR*</td>
<td>NODULAR AS % OF NON-NODULAR*</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>76</td>
<td>50b</td>
<td>65a</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>58b</td>
<td>84</td>
<td>142a</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>87</td>
<td>130</td>
<td>150b</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>243b</td>
<td>182a</td>
<td>76</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>80</td>
<td>51c</td>
<td>63c</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>96</td>
<td>50c</td>
<td>53c</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>129</td>
<td>72a</td>
<td>56b</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>90</td>
<td>73c</td>
<td>81b</td>
</tr>
<tr>
<td>Total protein</td>
<td>111a</td>
<td>112b</td>
<td>101</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>88</td>
<td>59c</td>
<td>67b</td>
</tr>
</tbody>
</table>

* Results expressed as % of, and compared statistically to values for untreated control rats
† Results compared statistically to values for non-nodular liver

a = p<0.05  b = p<0.01  c = p<0.001
TABLE 5.6

Measurements of Some Drug Metabolising Enzymes and Related Parameters in Non-Nodular and Nodular Liver Tissue from Rats Given a Diet Containing 1% Ponceau MX for 80 Weeks

Values calculated from means for 4 control and 6 treated rats and nodular liver tissue from 4 of the treated rats

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Units per g liver</th>
<th>Units per mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NON-NODULAR*</td>
<td>NODULAR*</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>88</td>
<td>59a</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>68c</td>
<td>118</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>71b</td>
<td>135</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>171a</td>
<td>114</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>76a</td>
<td>46c</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>102</td>
<td>71a</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>104</td>
<td>74a</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>92</td>
<td>68b</td>
</tr>
<tr>
<td>Total protein</td>
<td>88a</td>
<td>90</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>85a</td>
<td>59c</td>
</tr>
</tbody>
</table>

* Results expressed as % and compared statistically to, values for untreated control rats
† Results compared statistically to values for non-nodular liver

a = p<0.05    b = p<0.01    c = p<0.001
reductase and the content of cytochrome P450 were usually lower in the nodule expressed either per gram of tissue or per mg microsomal protein. The activity of the mitochondrial marker enzyme, succinate dehydrogenase, was similarly depressed in the nodule. Total protein content of the nodules was either the same or slightly less than that of the control and the non-nodular treated liver, in contrast to the findings for microsomal protein. A similar pattern of results was observed in all three experiments and there was no obvious correlation between enzyme activity and size of the nodules. Similar results were also obtained in a preliminary experiment with tissue from rats fed 2% Ponceau MX for 56 weeks. In this experiment there was a striking inverse relationship between activities in the nodule and the non-nodular treated liver within any one animal.

d) Effects of 2,4-, 2,5- and 2,6-Xylidine on Drug Metabolising Enzyme Activity

Results of the administration of xylidine isomers in the diet and by oral intubation are shown in Figs. 5.3 and 5.4 respectively, expressed as % of control values. Results of the dietary administration of 2,4-xylidine for 1 and 8 weeks are shown in Fig. 5.5 expressed in the same manner.

When xylidines were given in the diet the 2,4- isomer produced marked increases in all the parameters studied whilst 2,6-xylidine had little effect. The effect of 2,5-xylidine was intermediate between these two. Only 2,4-xylidine produced a significant increase in liver weight, whilst both 2,4- and 2,5- but not 2,6-xylidine elicited very marked increases in biphenyl 2-hydroxylase - approximately twice the respective increases in biphenyl 4-hydroxylase.
Effect of Dietary Administration for 1 Week of 2,4-, 2,5- and 2,6-xylidine on Some Drug Metabolising Enzymes and Related Parameters

Mean values for the treated rats are expressed as % of control values (4 rats per group) in units per g liver.

Treatments are shown as: 24, 25 and 26 = 2,4-, 2,5- and 2,6-xylidine respectively (see 5.2b)
Effect of Administration by Oral Intubation for 1 Week of 2,4-, 2,5- and 2,6-xylidine on Some Drug Metabolising Enzymes and Related Parameters

Mean values for the treated rats are expressed as % of control values (4 rats per group) in units per g liver.

Treatments are shown as: 24, 25 and 26 = 2,4-, 2,5- and 2,6-xylidine respectively (see 5.2b)

- Ethylmorphine N-demethylase
- Aniline 4-hydroxylase
- Biphenyl 4-hydroxylase
- Cytochrome P450
- Cytochrome b5
- Microsomal Protein
- Relative liver weight
- Biphenyl 2-hydroxylase
Rather different results were obtained when the xylidines were given by oral intubation. The stimulatory effects of 2,4- and 2,5-xylidine were much less pronounced, with the exception of their effects on biphenyl 2-hydroxylase which was unchanged and the effect of 2,4-xylidine on relative liver weight which was increased. Although remaining slight, the stimulatory effect of 2,6-xylidine appeared to be greater when given by oral intubation.

It should be noted that 2,4-xylidine, when given in the diet, depressed food consumption to a much greater extent than 2,5- or 2,6-xylidine. Consequently, over the 7 days, the mean dose level for 2,4-xylidine was approximately 150 mg/kg as opposed to approximately 250 mg/kg for the other two isomers. The dose level given by intubation was selected to be comparable with that for 2,4-xylidine given in the diet: therefore the dose level of 2,5- and 2,6-xylidine given by intubation was lower than that given dietarily. None of the isomers had any effect on food consumption or rate of body weight gain when given by intubation.

The increases in drug metabolising enzyme activity produced by dietary administration of 2,4-xylidine for 1 week were much less pronounced when administration was continued for 8 weeks. An exception was biphenyl 2-hydroxylase, the activity of which increased slightly relative to the control value. The relative liver weight increased from 150 to 200% of the control value after 8 weeks.

No histological changes in the liver were seen in any of these experiments.
Mean values for the treated rats are expressed as % of control values (4 rats per group). Significant differences are shown (units per g liver only):

- a = p < 0.05, b = p < 0.01, c = p < 0.001.

- Units per g liver
- Units per mg microsomal protein

**ETHYLmorphine N-DEMETHYLASE**

**BIPHENYL 2-HYDROXYLASE**

**ANILINE 4-HYDROXYLASE**

**BIPHENYL 4-HYDROXYLASE**

**CYTOCHROME b5**

**MICROSOMAL PROTEIN**

**CYTOCHROME P450**

**ABSOLUTE LIVER WT**

**RELATIVE LIVER WT**
e) Interaction of Xylidine Isomers with Cytochrome P450

2,4- and 2,5-xylidine produced typical type II interaction spectra with liver microsomes from untreated female rats. 2,6-xylidine, in concentrations up to 5mM, produced no spectral change at all. The kinetic constants determined were as follows:

<table>
<thead>
<tr>
<th></th>
<th>$K_s$ (M x 10^{-4})</th>
<th>$\Delta A_{max}$</th>
<th>No. of determinations</th>
</tr>
</thead>
<tbody>
<tr>
<td>2,4-Xylidine</td>
<td>10.0 ± 0.2</td>
<td>0.053 ± 0.005</td>
<td>3</td>
</tr>
<tr>
<td>2,5-Xylidine</td>
<td>9.2 ± 0.3</td>
<td>0.037 ± 0.002</td>
<td>3</td>
</tr>
</tbody>
</table>

f) Enzyme Histochemical Changes in the Liver During Chronic Administration of Ponceau MX

After administration of Ponceau MX for 1 week, histochemistry revealed a centrilobular depression of glucose-6-phosphatase (as Fig. 4.5) and a slight, generalised loss of succinate dehydrogenase. Despite the depression observed biochemically, there was no histochemical change in aniline hydroxylase activity. Liver sections stained by the Gomori acid phosphatase technique showed a decreased number of lysosomes in the centrilobular cells with some cells containing enlarged, randomly-distributed lysosomes (Fig. 5.6). Marked centrilobular depression of glucose-6-phosphatase was consistently noted at subsequent intervals. Succinate dehydrogenase activity appeared variable but although no consistent pattern was evident the overall impression was of decreased activity - in agreement with the biochemical findings. At week 8 and subsequently a slight centrilobular depression of aniline hydroxylase was observed although at later intervals (week 25 onwards) some individual cells appeared to contain high levels of aniline hydroxylase (as Fig. 4.7).
FIG. 5.6  Ponceau MX week 1. Acid phosphatase.

A. Reduced activity in centrilobular areas (x 72).

B. High power of centrilobular area showing enlarged, randomly distributed lysosomes (x 720).
The lysosomal changes which were confined to centrilobular areas at week 1 progressed subsequently and by week 25, involved the greater part of the liver lobule. Autophagic vacuoles were seen in some areas at week 8 and subsequently, but these were never as frequent as with safrole (4.3h). The nodules induced by Ponceau MX showed a virtually complete loss of histochemically demonstrable glucose-6-phosphatase (as Fig. 4.8). In some nodules or in some areas within nodules the activity of succinate dehydrogenase appeared considerably reduced. Aniline hydroxylase activity in the nodules was fairly evenly distributed and appeared comparable to or greater than that in periportal areas of the non-nodular liver (Fig. 5.7). Lysosomal distribution was quite similar to that observed in the safrole-induced nodules, i.e. a wide variation in numbers, size and distribution of the lysosomes. However, autophagic vacuoles were less frequently present.

9) Macroscopic Changes in the Liver

Apart from enlargement, no changes were observed before week 42 when the liver of 1/4 rats killed contained a single small nodule (approx. 0.2 cm diameter), the colour and consistency of which was similar to that of the adjacent parenchyma. Between weeks 52 and 58, 13 of the remaining 30 treated rats died - apparently as a result of respiratory infection - and in 8 of these rats the liver contained similar nodules ranging from 0.2 - 0.6 cm in diameter. Of the rats killed at weeks 66, 70 and 80, the livers of 4/5, 6/6 and 4/6 respectively contained nodules. These might be single, non-necrotic nodules as described above, but up to 1.5 cm in diameter, and there might be several such nodules in any one liver. In other cases multiple nodules up to 3 cm in diameter might be present, these
FIG. 5.7 Ponceau MX week 80 - nodule showing aniline hydroxylase activity equal to or greater than in adjacent, non-nodular liver (X 72).

FIG. 5.8 Ponceau MX week 25 - isolated area of liver cell hyperplasia (H & E x 180)
comprising areas of necrosis, blood or clear fluid-filled cysts and other relatively "normal" areas. These multiple nodules usually accounted for more than 75% of the total "liver" weight and were often associated with large amounts of connective tissue. The macroscopic appearance of the nodules induced by Ponceau MX was essentially identical to that of the safrole-induced nodules shown in Fig. 4.11. Ascites fluid was not observed, even in animals whose liver was grossly abnormal and no invasion of neighbouring organs was noted.

h) Histological Changes in the Liver

Centrilobular cell enlargement was evident in the livers of 3/4 rats treated with Ponceau MX for 1 week but only occasionally at subsequent sampling intervals. Individual centrilobular cell necrosis (as Fig. 4.13) was noted in 2/4 treated rats killed at week 1 and with a similar, or increased frequency at subsequent intervals up to week 42: this lesion was not observed in control rats at any stage (cf. 4.3k). A small area of liver cell hyperplasia was noted in 1 rat killed at week 25 (Fig. 5.8) and at week 42 the liver of 3/4 rats contained several such areas.

Non-nodular liver tissue sampled at week 80 showed slight fatty change, small areas of hydropic degeneration and liver cell necrosis and occasional areas of hyperplasia (Fig. 5.9). Nodular lesions compressed the surrounding hepatocytes but, as in the case of safrole, all the nodules examined biochemically were diagnosed as hyperplastic. Within these nodules the normal liver architecture was absent and the cells were irregularly enlarged but occasional portal tracts were present
(Fig. 5.10). The histological findings in the present study were thus in accord with those described by Grasso et al. (1969).

j) Ultrastructural Changes in the Liver

At week 1 clusters of normally arranged rough endoplasmic reticulum were prominent and there appeared to be an increased number of free ribosomes. There was no unequivocal proliferation of smooth endoplasmic reticulum and the other cell components appeared normal. An abundance of rough endoplasmic reticulum was also observed at week 25. In some areas this appeared somewhat dilated and the normal parallel array formation was absent. Proliferation and local dilatation of the smooth endoplasmic reticulum was pronounced in some cells (Fig. 5.11) but in the majority there was only a marginal increase in this component. Extensive stacks of dilated rough endoplasmic reticulum were prominent in the non-nodular liver at week 80 (Fig. 5.12). In some cells the smooth endoplasmic reticulum and Golgi apparatus were grossly dilated whilst in other cells these appeared relatively normal. This variable appearance was also a feature of the nodular tissue as is evident from Figs.5.13A and B. Some of the swollen rough endoplasmic reticular elements in both nodule and non-nodular liver appeared to be undergoing a loss of bound ribosomes. In many respects the appearance of both nodule and non-nodular liver was similar although areas of relatively normal cells were more frequently encountered in the nodule. In neither tissue were mitochondrial, nuclear or nucleolar abnormalities observed.
FIG. 5.9  Ponceau MX week 80 - histological features of non-nodular liver.

A. Hydropic degeneration (H & E x 180).

B. Fatty change and liver cell necrosis (H & E x 180).
A. Part of a large nodule with compression of adjacent parenchyma: note the uniform structure of the nodule (H & E x 72)

B. Higher power view of part of a nodule showing the arrangement of the cell cords: relatively normal liver occupies the far right of the field (H & E x 180)
FIG. 5.11  Ponceau MX week 25 - abundant rough endoplasmic reticulum and mild proliferation, together with local dilatation, of the smooth endoplasmic reticulum (x 20,500)

FIG. 5.12  Ponceau MX week 80 - non-nodular liver. Proliferation and gross dilatation of both rough and smooth endoplasmic reticulum (x 20,500)
FIG. 5.13A  Ponceau MX week 80 - nodule. Numerous free ribosomes and clusters of dilated rough endoplasmic reticulum are present (x 20,500).

FIG. 5.13B  Ponceau MX week 80 - different cell from the same nodule as in Fig. 5.13A, showing gross dilatation of the smooth endoplasmic reticulum and numerous free ribosomes (x 27,500).
5.4 Discussion

Hepatic nodular hyperplasia following the chronic ingestion of Ponceau MX has been previously reported (Grasso et al., 1969), the lesions produced resembling both grossly and histologically those seen in the present study. Such results, however, contrast with those of earlier studies (see 5.1) in which dietary levels higher than that used here were without long-term effect in rats. An explanation for these discrepancies lies most probably in the variable composition of different dye samples and, since the systemic effects of Ponceau MX are probably dependent on its intestinal azo-reduction, in differences in the composition of the intestinal microflora. Certainly it is known that this flora can vary quite markedly between rats from different colonies as judged, for example, by differences in rates of reduction of azo-compounds (Walker, 1970) and in the extent of conversion of cyclamate to cyclohexylamine (Hill, 1970).

These same considerations, particularly the chemical heterogeneity of Ponceau MX, impose difficulties in interpretation of the biochemical changes observed. Thus, the early inhibition of aniline 4-hydroxylase in rats fed the intact dye contrasted with the stimulatory effect of each of the three principal (Pla Delfina, 1968) constituent xylidines when these were fed individually. This suggests that the changes elicited by Ponceau MX may represent no more than the balance between the simultaneous action of stimulatory and inhibitory constituent compounds. Evaluation of this possibility is hindered by ignorance of the precise amine composition of the dye sample utilised. However, apart from the result with aniline 4-hydroxylase, the changes produced by Ponceau MX appeared compatible with those produced by 2,4-, 2,5- and 2,6-xylidine.
Although Ponceau MX elicited a relatively weak induction of drug metabolising enzymes it caused a marked increase in liver weight. Accordingly, the calculated 'total liver activity' values for these enzymes were greatly in excess of control values and remained so in contrast to the changes produced by safrole. A somewhat similar situation produced in mice was described by Beckett et al.,(1972). They found that the hypolipidaemic drug Nafenopin evoked little increase in drug metabolising enzyme activity, as measured in vitro on a per g liver basis, but produced an approximate doubling in liver weight. Despite this, a corresponding doubling of 'total liver' drug metabolising capacity was not observed in vivo, the hexobarbitone sleeping time of the treated mice being only slightly decreased. This would suggest that values for 'total activity' predicted from measurements of liver weight and enzyme activity in vitro, may bear little relation to the drug metabolising capacity of the intact animal. Certainly there is no evidence to suggest that liver enlargement in itself represents an adaptive response to foreign compound administration. Indeed, as pointed out in 1.7, liver enlargement unaccompanied by increased drug metabolising enzyme activity may be indicative of incipient injury.

The observation as early as 1 week of histochemical changes involving glucose-6-phosphatase and lysosomal acid phosphatase provides some support for this view. Such changes were never observed with BHT, and in the case of safrole, only after the demise of the initial enzyme inductive response. The situation after administration of Ponceau MX for 1 week might therefore be equated with that prevailing after 8 weeks in the case of safrole. Accordingly, less significance can be attached to the biphasic nature of the drug metabolising enzyme response to Ponceau MX and the gradual, uniform (c.f. safrole) downward progression of the graphs in Fig. 5.2 is probably a non-specific reflection of progressing hepatocellular injury.
Such progression is also indicated by the gradual extension of the lysosomal changes, which initially were confined to centrilobular areas, to involve the greater part of the lobule. At week 25 and at subsequent intervals, scattered, individual centrilobular cells showed marked stimulation of aniline hydroxylase activity, indicating that the hepatocytes in this zone may be heterogeneous in their response to Ponceau MX (and/or its metabolites). A similar effect was noted after administration of 2,4-xylidine for only 7 days (Grasso et al., 1974). This observation was correlated with electron microscopic observations that individual centrilobular cells showed proliferation of the smooth endoplasmic reticulum whilst the majority did not. A similar effect on the smooth endoplasmic reticulum was apparent in the present study with Ponceau MX. The relationship of these various hepatic changes produced by Ponceau MX to the subsequent development of liver nodules is discussed in Chapter 8.

The divergence between effects on drug metabolising enzyme activity and liver weight seen with Ponceau MX was also notable with 2,4-xylidine - particularly when given by oral intubation - but not with 2,5- or 2,6-xylidine. 2,4-xylidine is known to be hepatotoxic, chronic administration leading to changes similar to those produced by Ponceau MX (Lindstrom et al., 1963). In the study of Grasso et al. (1974), 2,4-xylidine given by oral intubation evoked little or no increase in aminopyrine demethylase activity or cytochrome P450 concentration and gave rise to lysosomal abnormalities within 7 days. Fairly similar biochemical results were obtained in the present study. In contrast, when given in the diet for 7 days, 2,4-xylidine elicited a marked stimulation of drug metabolising enzyme activity. It would be of interest, therefore, to determine whether lysosomal changes occur also in this case or whether their first appearance can be correlated with the subsequent (8 weeks)
It is known that the isomeric dimethylanilines differ in their biological activity (see 5.1) and the three isomers studied here differed in their respective effects on liver weight and drug metabolising enzyme activity. Whilst 2,4-xylidine was 5-6 times more potent in producing liver enlargement than 2,5 or 2,6-xylidine, the relative efficacy of these isomers as enzyme inducers followed more evenly the sequence 2,4->2,5->2,6-xylidine. This sequence appeared to correlate with the extent to which each compound was able to elicit a type II cytochrome P450 interaction spectrum. These differences in ability to undergo spectrally apparent interaction with cytochrome P450 may be due to the increasing steric hinderance (Gorrod et al., 1972) and/or hydrophobicity around the nitrogen atom with the shift of the 4-methyl group in 2,4-xylidine through position 5- to position 6- as in 2,6-xylidine. These factors would favour a shift towards the production of a type I spectrum (Gorrod et al., 1972) and it might be that the apparent failure of 2,6-xylidine to give rise to any spectral change results from an equal generation of type I and type II spectral components. This seems improbable however, since injection of 2,6-xylidine into rats 60 minutes prior to killing the animals was without effect on the metabolism of either type I or type II substrates. In contrast, 2,4- and 2,5-xylidine under the same conditions produced a marked inhibition of both reactions. It appears, therefore that 2,6-xylidine does not interact appreciably with cytochrome P450 and this presumably accounts for its minimal influence on drug metabolising enzyme activity. The factors contributing to the greater stimulatory effect of 2,4- compared with 2,5-xylidine on both drug metabolising enzyme activity and liver weight are not clear. The apparent affinity ($K_s$) of each compound for cytochrome P450 was similar and the differences
in $\Delta OD_{\text{max}}$ are unlikely to be important in this context since spectrally apparent interaction appears to bear little relation to metabolism in the case of type II substrates (Jefcoate et al., 1969; Mannering, 1971). The differences in metabolism between 2,4- and 2,5-xylidine (Lindstrom, 1961; Lindstrom et al., 1963) are probably of more direct relevance to their differing effects on drug metabolising enzyme activity.

From a metabolic viewpoint, it is perhaps not surprising that 2,4-xylidine appears to be more hepatotoxic than 2,5- or 2,6-xylidine (Lindstrom et al., 1963). The substituted ring 4- but unsubstituted ring 2- position would tend to favour 2-hydroxylation, a reaction frequently associated with increased biological activity (Clayson, 1962). Similarly, by analogy with the hepatocarcinogenic 2,4,6-trimethylaniline, it is possible that N-hydroxylation is important in the toxicity of 2,4-xylidine (Weisburger and Weisburger, 1973). Either of these reactions might be favoured by the demise of the initial enzyme induction produced by 2,4-xylidine.

Differences in the effects of 2,5- and 2,6-xylidine on liver weight and enzyme activity according to their mode of administration were not so pronounced as with 2,4-xylidine, particularly when consideration is given to the possible influence of the lower dose level of these two compounds when given by oral intubation. Similar differences in effect between intubation and dietary administration have been observed with the antioxidant, butylated hydroxyanisole (P. Grasso, personal communication). Since with both of these modes of administration access to the systemic circulation must be via the gastrointestinal tract, the different effects produced must presumably relate to differences in the kinetics of absorption and distribution of the compound. Oral intubation
usually involves administration of the entire daily dose at a single time, moreover, a time at which the gastrointestinal tract is likely to be relatively empty. Accordingly, the maximum plasma level achieved is likely to be considerably higher than when the same dose is ingested in the diet over a period of 12 hours or more. In the case of 2,4-xylidine, this raises the possibility of acute toxic damage as a cause both of the decreased enzyme response and of the relatively increased liver weight in the intubation experiment. It would be of interest to compare the course of development of pathological changes in the liver produced by 2,4-xylidine given in the diet or by intubation. A further possibility is that large differences in plasma level/time relationships may lead to qualitative alterations in routes of metabolism and excretion. This could account for the apparently greater effect of 2,6-xylidine on drug metabolising enzyme activity when given by oral intubation despite the lower overall dose administered. Some of these factors have recently been discussed by Chasseaud and Taylor (1974). Such differences may not result with all enzyme inducers, however, since Hashimoto et al. (1972) found that the degree of induction of ethylmorphine N-demethylase, aniline 4-hydroxylase and cytochrome P450 was very similar when cedrene was administered to rats either intraperitoneally, by oral intubation or by inhalation.
CHAPTER SIX

BIOCHEMICAL AND MORPHOLOGICAL CHANGES IN THE LIVER DURING CHRONIC DIETARY ADMINISTRATION OF 2-ACETYLMINOFLUORENE TO RATS
6.1 Introduction

The carcinogenicity of 2-acetylaminofluorene (AAF; N-2-fluorenyl-acetamide) was discovered after the compound had been patented by the United States Department of Agriculture as a potential insecticide. Toxicity studies revealed that 3-11 months after commencement of AAF administration, rats developed tumours in a number of tissues including liver, kidney, lung, urinary bladder, pancreas, ear duct, mammary gland and gastrointestinal tract (Wilson et al., 1941; Cox et al., 1947). Subsequent work showed AAF to be carcinogenic in most species of experimental animal— with the exception of the guinea pig— although the site and incidence of tumour development varied markedly according to the species, strain and sex of animal: these studies have been comprehensively reviewed by Weisburger and Weisburger (1958). Of the species examined, the rat has proved to be the most susceptible to the carcinogenic action of AAF and in the male rat, the principal target organ is the liver. The almost exclusively hepatocellular derivation of the liver tumours in this animal and their precedence by the early development of hyperplastic nodules (Epstein et al., 1967; Skoryna and Webster, 1951; Teebor and Seidman, 1970), has led to the extensive utilisation of AAF as a model hepatocarcinogen.

In practice, the development of hepatocellular carcinoma is considerably influenced by the dosing schedule employed, chiefly because of the chronic hepatotoxicity of AAF. At dose levels leading to a high tumour incidence, the continuous administration of AAF produces severe cirrhosis and consequent early mortality may preclude the development of frank carcinoma (Reuber, 1965). Epstein et al. (1967) avoided this difficulty by treating their rats with alternate cycles of AAF and control diet: under these conditions the hepatotoxic effects of AAF were minimised.
and an incidence of hepatocellular carcinoma approaching 90% was obtained after 8-10 months.

The hyperplastic liver nodule has been implicated by a number of authors as a precursor to the development of hepatocellular carcinoma (Epstein et al., 1967; Farber, 1968, 1973a,b, 1974; Merkow et al., 1971; Stewart and Snell, 1959) and as such, nodules induced by AAF have received considerable attention. Areas of hyperplasia appear at an early stage in the livers of male rats given AAF, discrete, macroscopically visible nodules being evident after 6-15 weeks (Kitigawa and Sugano, 1973; Teebor and Seidman, 1970). The morphology of these nodules, of the subsequently appearing carcinomas and of the liver tissue adjacent to each of these lesions has been described in detail by Epstein et al. (1967), Farber (1973b), Kitigawa (1971), Merkow et al. (1967, 1969) and Reuber (1965). Earlier studies have been reviewed by Stewart and Snell (1959) whilst Flaks (1970, 1971, 1972, 1973) has provided sequential ultrastructural studies of the changes produced in rat liver by both AAF and its non-carcinogenic isomer, 4-acetylaminofluorene. Histologically the nodules comprised fairly uniform, enlarged hepatocytes but the normal lobular hepatic architecture was lacking and portal tracts were scant or absent. Carcinoma tissue was characterised by a cell population much more variable in size, shape and staining affinity. Ultrastructurally both nodule and carcinoma contained prominent clusters of smooth endoplasmic reticulum but the rough endoplasmic reticulum was reduced in quantity and had lost its normal parallel array formation. Both tissues contained annulate lamellae but additionally, there were nuclear and mitochondrial abnormalities and more frequent autophagic vacuoles in the carcinoma. In comparison, liver adjacent to the nodules and carcinomas resembled untreated, control liver although histologically, areas of cholangiofibrosis and diffuse hyperplasia were evident.
Results of the morphological studies referred to above are compatible with the view of the hyperplastic nodule as a forerunner of the hepatocellular carcinoma but unequivocal evidence to this effect is lacking. Perhaps the most direct evidence for such a relationship comes from the studies of Epstein et al. (1968) and Teebor and Becker (1971). The former showed that after administration to rats of $^{14}$C-AAF, DNA- and glycogen-bound label persisted for months in the cells of the nodules and subsequently in the carcinomas but not in the adjacent liver cells. The latter found that if hyperplastic nodules either did not develop, or were allowed, before a certain critical stage, to regress, then the subsequent incidence of hepatocellular carcinoma was less than 5% (1/25). However, Peraino et al. (1973) observed a tumour incidence of 21% (22/106) after 9 months in male rats given AAF at a dietary level of 0.02% for 18 days, a treatment unlikely to give rise to early hyperplastic nodules. This would suggest that the early development of hyperplastic nodules may not be mandatory for the subsequent production of tumours. A number of reports on the biochemical characteristics of the nodules are available and these are discussed in Chapter 8.

The female rat is much less susceptible than the male to the hepatocarcinogenicity of AAF (Sidransky et al., 1961; Skoryna and Webster, 1951; Teebor and Seidman, 1970) although females of different strains may differ considerably in sensitivity (Weisburger and Weisburger, 1958). Females are, however, more susceptible than males to the development of ear duct and mammary gland carcinoma (Miller et al., 1958) and prolongation of lifespan by surgical removal of mammary tumours increased the incidence of liver tumours (Sidransky et al., 1961). Hyperplastic nodules were found only infrequently in these animals and the liver tumours produced were more highly differentiated than in male rats. It is noteworthy that the early liver cell proliferation produced by AAF in
the male rat does not occur in the female (Farber 1956; Jackson and Irving, 1972). Moreover, induction of cell division by partial heptectomy of AAF-fed female rats did not lead to the development of hepatocellular carcinoma despite the irregular, nodular liver regeneration observed (Jackson and Irving, 1973). However, gonadectomised female rats given testosterone did develop liver tumours in response to AAF and, conversely, hypophysectomised or thyroidectomised male rats were markedly more resistant than their controls (De Baun et al., 1970a). The effect of these procedures with regard to the early development of hyperplastic nodules was not stated.

Most chemical carcinogens require metabolism to a reactive species for the exertion of their carcinogenic activity (Miller, 1970) and AAF is no exception in this respect. Essentially two major pathways for its metabolism are known, namely ring- and N-hydroxylation, although the quantitative importance of each differs in different animal species (Weisburger and Weisburger, 1958). In the rat, the fluorene nucleus undergoes hydroxylation principally at the 3-, 5- and 7- positions (Cramer et al., 1960a) but trace amounts of the 1-, 6- and 8-hydroxy derivatives have also been identified (Weisburger and Weisburger, 1958). These ring hydroxylated metabolites are inactive and are excreted chiefly in the urine as glucuronides. N-hydroxylation of AAF was first demonstrated by Cramer et al. (1960b). The product, N-hydroxy-2-acetylaminofluorene (N-OHAAF), was shown to be a more potent carcinogen than AAF, giving rise to higher levels of macromolecule bound fluorene derivatives in the liver (De Baun et al., 1970a; Irving and Veazy, 1969) and producing tumours at sites of its administration (Miller et al., 1961) as well as in species in which AAF is inactive, e.g. the guinea pig (Miller et al., 1964). Moreover, synthetic N-hydroxylation converted the inactive 7-hydroxyAAF into a potent carcinogen (Gutmann et al., 1967).
However, the observation that N-OHAAF was formed in equal amounts from AAF by the susceptible male and the resistant female rat (Weisburger et al., 1964) and its low reactivity with cellular macromolecules under physiological conditions in vitro (Miller et al., 1968; Miller, 1970) suggested a requirement for further metabolic activation. Recent evidence points to the sulphate ester of N-OHAAF as a key ultimate carcinogenic metabolite of AAF in rat liver, the formation of this highly electrophilic reactant being mediated by a soluble sulphotransferase (De Baun et al., 1970a; De Baun et al., 1970b; King and Phillips, 1968; Weisburger et al., 1972). In support of this view, Gutmann et al. (1972) correlated AAF hepatocarcinogenicity with metabolic activation using two strains of rat differing in their ability to N-hydroxylate AAF and to sulphate-esterify N-OHAAF: other species, strain and sex differences in susceptibility to AAF hepatocarcinogenesis also correlated well with differences in hepatic sulphotransferase activity (De Baun et al., 1968; De Baun et al., 1970a; Jackson and Irving, 1972). However, the existence of other activation mechanisms seems probable since other target tissues of AAF, e.g. ear duct and mammary gland, do not possess detectable N-OHAAF-sulphotransferase activity (see Bartsch et al., 1973). The metabolic activation of AAF and other carcinogenic aromatic amines and amides has been the subject of a number of recent reviews (Irving, 1972; Miller, 1970; Miller and Miller, 1969; Weisburger and Weisburger, 1973).

The ring hydroxylation of AAF appears to be a typical, cytochrome P450-mediated mixed function oxidation (Cramer et al., 1960a) but the nature of the N-hydroxylation reaction has been a matter of some controversy. In apparent confirmation of the studies of Kampffmeyer and Kiese (1965) on nitrogen oxidation, Matsushima et al. (1972) and Matsushima and Weisburger (1972) reported that the N-hydroxylation of
AAF was not necessarily cytochrome P450-dependent. In a rather more thorough approach, however, Thorgiersson et al. (1973) produced seemingly unequivocal evidence for the involvement of P450 in this reaction. These considerations are important with regard to the influence of effectors of cytochrome P450 on AAF carcinogenesis. Thus, coadministration of 3-methylcholanthrene with AAF markedly reduced tumour incidence at all sites in the rat (Marugami et al., 1967; Miller et al., 1958), a result, according to Lotlikar et al. (1967), of marked induction of the ring hydroxylation (detoxication) of AAF. Coadministration of butylated hydroxytoluene (BHT) (Grantham et al., 1973) or phenobarbitone (Peraino et al., 1971, 1973) also reduces the hepatocarcinogenicity of AAF in the rat although apparently via a different mechanism to 3-methylcholanthrene. Thus, BHT stimulates the N-hydroxylation of AAF to a considerably greater extent than the ring-hydroxylation (Grantham et al., 1973) and the key effect here appears to lie in acceleration of the excretion of N-OHAAF as a result of induction of glucuronyl transferase (Weisburger and Weisburger, 1973).

Despite these considerations, little is known of the effects that AAF itself exerts on the activity of the drug metabolising enzymes. In male rats there is evidence that AAF causes a depression of drug metabolic activity which may or may not be preceded by a transitory enzyme induction (Trams et al., 1961). No comparable measurements appear to have been made in the female rat.

In the experiments described in this chapter, AAF has been administered to both female and male rats in order that the results obtained in the previous chapters could be compared with measurements of the same parameters in rats treated with a known hepatocarcinogen. Male rats were utilised since it was not possible to obtain adequate...
nodular liver tissue from the female rats to permit biochemical analysis. Whilst the higher basal levels of drug metabolising enzyme activity in male rats (Gigon and Bickel, 1971) precluded direct comparisons with the liver nodules induced in female rats by safrole and Ponceau MX, it was considered that comparisons on a 'percent of control' basis were valid.
6.2 Materials and Methods

a) Chronic Administration of AAF to Male and Female Rats

Male and female rats were maintained on a diet containing 0.06% (w/w) AAF. This dietary level has been shown to produce a high incidence of hepatic nodular hyperplasia, and, subsequently, hepatocellular carcinoma (Teebor and Becker, 1971; Teebor and Seidman, 1970). Female rats received the AAF diet continuously. For comparability with the experiments using female rats, both in this and in previous chapters, it was clearly desirable that the male rats should also receive the AAF diet continuously. The difficulties associated with this were mentioned in 6.1 and were reinforced by the death of 8/12 male rats after ingestion of the AAF diet for 3-4 weeks in a preliminary experiment. As a compromise therefore, control diet was substituted for the AAF diet during weeks 3 and 4, week 9 and weeks 13 and 14 and 18 and 19.

At weeks 1, 8, 16 and 24 (females) and 1 and 8 (males), 4 treated and 4 control rats were killed and the livers used for the assays described in 2.3. At week 16, 6 treated male rats were killed on the assumption that these would have developed liver nodules (cf. Teebor and Seidman, 1970): all had but there was insufficient nodular tissue to permit biochemical analysis. The remaining 8 male rats were killed at week 22 and from the livers of 6 of these, nodular tissue was obtained. In each case, multiple, small (2-5 mm diameter) smooth surfaced nodules were present and these were carefully freed from the surrounding parenchyma and pooled to give one sample of nodular tissue for each treated rat.

Enzyme histochemistry, histology and electron microscopy (2.3)
were carried out on liver samples from a number of both male and female rats.

b) Interaction of AAF with cytochrome P450

For these investigations, AAF was prepared as a solution 100 mM in a mixture of absolute ethanol and acetone (75:25 v/v).
6.3 Results

a) Animal Body Weights and Food Intake

Growth curves for female and male rats are shown in Figs. 6.1 and 6.2 respectively. In both sexes, but particularly in the males, ingestion of the AAF diet resulted in severe depression of growth rate such that from week 1 onwards, the mean body weight of the treated rats was very significantly ($p < 0.001$) less than that of the controls. 'Catch-up' growth during the periods when male rats received control in place of AAF diet was only minimal. The mean body weight of the male rats reached a maximum at week 14 and thereafter was decreased gradually in parallel with the deteriorating condition of the animals. Mean body weight for the female rats continued to increase up to week 24. Although the food intake of both treated and control rats was similar on day 1, the intake of the treated rats of each sex subsequently decreased and remained markedly depressed throughout the experiment. The extent of this depression correlated with the depression of growth rate in the male rats, but less closely in the females, whose growth rate was less severely affected. The effects of AAF on body weight and food intake are summarised in Table 6.1 and the approximate daily intake of AAF, in mg/kg body weight, calculated from the food intake data is shown in Table 6.2.

b) Condition of Rats During AAF Feeding

Apart from the decreased rate of body weight gain, the female rats showed no obvious signs of ill health prior to week 21. At this stage, several of the remaining 16 rats developed outgrowths from the side of the head which appeared to originate from the ear duct. Since this is a known target organ of AAF (Miller et al., 1958; Weisburger and
FIG. 6.1

Growth Curve for Female Rats Maintained on Control (●●●) or 0.06% AAF (▲▲▲) Diet

Points represent mean values. Significant differences between means are shown: $c = p<0.001$
Fig. 6.2
Growth Curve for Male Rats Maintained on Control (•—•) or 0.06% AAF (△—△) Diet

Points represent mean values. Significant differences between means are shown: a = p<0.05  b = p<0.01  c = p<0.001
### TABLE 6.1

Mean Body Weight and Food Intake of 2-Acetylaminofluorene-Treated Rats as % of Control

<table>
<thead>
<tr>
<th>WEEKS ON DIET</th>
<th>FEMALE</th>
<th>MALE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>12</td>
<td>26</td>
</tr>
<tr>
<td>Body Weight</td>
<td>74</td>
<td>69</td>
</tr>
<tr>
<td>Food Intake*</td>
<td>61</td>
<td>63</td>
</tr>
</tbody>
</table>

* Total food intake during weeks 1-12 and 13-26 (female) and 1-12 (male) as % of control

### TABLE 6.2

Mean Food Intake (g/rat/day) for Control and 2-Acetylaminofluorene-Treated Rats and Approximate Daily 2-Acetylaminofluorene Intake (mg/kg/body)

<table>
<thead>
<tr>
<th>WEEKS ON DIET</th>
<th>FEMALE</th>
<th>MALE</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>1</td>
<td>2</td>
</tr>
<tr>
<td>CONTROL</td>
<td>15.5</td>
<td>14.8</td>
</tr>
<tr>
<td>0.06% 2-Acetylaminofluorene</td>
<td>8.1</td>
<td>8.7</td>
</tr>
<tr>
<td>Approx.AAF Intake</td>
<td>38</td>
<td>37</td>
</tr>
<tr>
<td></td>
<td>43</td>
<td>40</td>
</tr>
</tbody>
</table>

* Rats receiving control diet (see 6.)
Weisburger, 1958), no further investigations were made. It was noted, however, that in 9/10 rats that developed these lesions, only the right ear was involved: the 10th rat had tumours in both ears. From week 23 onwards the condition of the remaining rats deteriorated rapidly with severe loss of weight in some animals. In 2 rats, killed in extremis, there was evidence of jaundice. At week 30 the remaining 3 rats were given control diet in place of AAF diet. The size of the ear tumours necessitated killing these rats after a further 16 weeks and although in each case the liver was grossly abnormal, no tumours were observed in this organ.

The male rats appeared to be much more susceptible to the toxic effects of AAF. Of the 8 rats dying within 4 weeks of commencement of AAF feeding, all showed ascitic fluid on opening the abdomen and in 4 animals there was evidence of pronounced jaundice. Ear tumours were not observed in rats surviving up to week 24.

c) Biochemical Changes During Chronic Administration of AAF

Data for female and male rats, in the form treated as % of control values, are presented in Figs. 6.3 and 6.4 respectively and in Table 6.3. The full data with statistical treatment are included in the Appendix (Tables A4 and A5). Since the changes produced in each sex were quite dissimilar, it is convenient to consider the experiments with male and female rats separately.

**Female:** When administered to female rats AAF proved to be a moderately powerful inducer of drug metabolising enzyme activity. After 1 week there were increases - per gram of liver - of 60-140% in the activities of ethylmorphine N-demethylase, aniline 4-hydroxylase and biphenyl 4-hydroxylase. The activity of NADPH-cytochrome c reductase
Measurements of Some Drug Metabolising Enzymes and Related Parameters at Intervals During Chronic Dietary Administration of 2-Acetylaminofluorene to Female Rats

Mean values for the treated rats are expressed as % of control values (4 rats per group). Significant differences are shown (units per g liver only):

- $a = p < 0.05$
- $b = p < 0.01$
- $c = p < 0.001$

**Units per g liver**

- **ETHYL MORPHINE N-DEMETHYLASE**
- **ANILINE 4-HYDROXYLASE**
- **BIPHENYL 4-HYDROXYLASE**
- **NADPH-CYTOCHROME C REDUCTASE**

**Cytochrome P450**

**Cytochrome b5**

**Microsomal Protein**

**Absolute Liver Weight**

**Relative Liver Weight**
Measurements of Some Drug Metabolising Enzymes and Related Parameters at Intervals During Chronic Dietary Administration of 2-Acetylaminofluorene to Male Rats

Mean values for the treated rats are expressed as % of control values (4 rats per group). Significant differences are shown (units per g liver only):

\[ a = p<0.05 \quad b = p<0.01 \quad c = p<0.001 \]

- • Units per g liver
- △△△ Units per mg microsomal protein

**FIG. 6.4**

- **ETHYLMORPHINE N-DEMETHYLASE**
- **CYTOCHROME P450**
- **ANILINE 4-HYDROXYLASE**
- **CYTOCHROME b_{5}**
- **BIPHENYL 4-HYDROXYLASE**
- **MICROSOMAL PROTEIN**
- **ABSOLUTE LIVER WEIGHT (---•---)**
- **RELATIVE LIVER WEIGHT (0---0)**
<table>
<thead>
<tr>
<th>WEEKS ON DIET</th>
<th>MALE</th>
<th>FEMALE</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>142</td>
<td>89</td>
</tr>
<tr>
<td>8</td>
<td>587c</td>
<td>90</td>
</tr>
<tr>
<td>16</td>
<td>184b</td>
<td>90 b</td>
</tr>
<tr>
<td>22</td>
<td>128</td>
<td>85 b</td>
</tr>
</tbody>
</table>

- Biphenyl 2-hydroxylase
- Succinate dehydrogenase
- Total liver protein

*Results expressed as % of control in units per g liver.*

*Some parameters additional to those shown in Figs. 6.2 and 6.3.*
was raised by 35% and the content of cytochromes P450 and b5 by 53 and 76% respectively. In contrast there was only a marginal increase in microsomal protein content (12%) and there was no change in the total liver protein content. The increase in biphenyl 2-hydroxylase was less than that for the 4-hydroxylase and, at this early stage, there was no depression of succinate dehydrogenase. Despite the enzyme induction there was no increase in relative liver weight and the absolute liver weight was significantly decreased - in parallel with the body weight.

Continued administration of AAF brought about a sharp fall in the levels of NADPH-cytochrome c reductase and cytochromes P450 and b5 back to control values or below. Although lower than their 1 week levels, the rates of metabolism of ethylmorphine, aniline and biphenyl remained significantly elevated at week 8 and that of biphenyl remained so even at week 24 when liver damage was grossly apparent. Succinate dehydrogenase activity decreased steadily to reach 69% of control at week 24 whilst the activity of biphenyl 2-hydroxylase increased to 3 times the control level at 16 weeks. Microsomal protein content decreased gradually but progressively whereas no clear trend could be discerned in the values for total protein content. The relative liver weight increased steadily being more than double that of the controls at week 24. One of the rats killed at 24 weeks appeared jaundiced: levels of drug metabolising enzymes in the liver of this animal were virtually undetectable and the activity of succinate dehydrogenase was less than 20% of control. Subsequent histological examination revealed that much of the hepatic parenchyma had been replaced by an extensive lymphosarcoma.
Male: The administration of AAF to male rats resulted in a fairly uniform depression of drug metabolising enzyme activity which, with the exception of the small increases in biphenyl 4- and 2-hydroxylases and cytochrome b5, was evident at week 1 and became progressively more marked on continued administration. Thus, at week 22 the activities of ethylmorphine N-demethylase, aniline 4-hydroxylase, biphenyl 4-hydroxylase and NADPH-cytochrome c reductase and the levels of cytochromes P450 and b5 were only 24-58% of control levels. The depression of microsomal protein content from week 1 onwards, but particularly at week 22, resulted in somewhat higher values when these parameters were expressed on a specific activity basis. Succinate dehydrogenase activity and total protein content fell to 63 and 80% of control respectively at week 22. Relative liver weight was maximal after 16 weeks at 270% of control.

d) Biochemical Investigations of AAF-Induced Liver Nodules

These investigations were confined to tissue obtained from the male rats. Results are presented in Table 6.4 in the form nodular and non-nodular liver as % of control, and nodular as % of non-nodular liver. The basic data are given in the Appendix (Table A10).

It was evident that, with respect to the parameters measured, the nodular tissue resembled closely the surrounding parenchyma: in both tissues drug metabolising enzyme activity was very significantly lower than control levels.
<table>
<thead>
<tr>
<th></th>
<th>Units per g liver</th>
<th>Units per mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>NON-NODULAR*</td>
<td>NODULAR AS % OF NON-NODULAR*</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>27c</td>
<td>38c</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>24c</td>
<td>16c</td>
</tr>
<tr>
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<td>55c</td>
<td>51c</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
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<td>128</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>58b</td>
<td>60b</td>
</tr>
<tr>
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<td>28c</td>
<td>24c</td>
</tr>
<tr>
<td>Cytochrome b₅</td>
<td>53b</td>
<td>52b</td>
</tr>
<tr>
<td>Microsomal protein</td>
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<td>66c</td>
</tr>
<tr>
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<td>81c</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>63c</td>
<td>65c</td>
</tr>
</tbody>
</table>

* Results expressed as % of, and compared statistically to, values for untreated control rats
† Results compared statistically to values for non-nodular liver
a = p<0.05   b = p<0.01   c = p<0.001
e) Interaction of AAF with Cytochrome P450

No recognisable substrate interaction spectrum was obtained when AAF was added to liver microsomes from untreated male or female rats at concentrations up to 1mM.

f) Enzyme Histochemical Changes in the Liver During Chronic Administration of AAF

Female rats: After 1 week there was a slight but definite centrallobular depression of glucose-6-phosphatase and stimulation of aniline hydroxylase. There were no clear cut changes in acid phosphatase distribution although in some centrallobular and mid-zonal cells "clumping" of lysosomes with loss of the normal pericanalicular distribution was evident. At week 8, the centrallobular depression of glucose-6-phosphatase was pronounced and stimulation of aniline hydroxylase was no longer apparent. There was a slight, overall loss of succinate dehydrogenase. Lysosomal changes comprised a slightly decreased centrallobular activity of acid phosphatase with aggregation of lysosomes in the centre of some cells, as at week 1. Enlarged individual lysosomes were present in some scattered groups of cells but there were very few autophagic vacuoles. At week 16, changes were similar to those seen at week 8. In the livers of 2 rats, however, microscopic nodules were present and their constituent cells showed levels of enzyme activity lower than in the surrounding parenchyma. This loss of enzyme activity was most marked in the case of glucose-6-phosphatase, activity of which was virtually undetectable in the hyperplastic nodules (as Fig. 6.5).
Male rats: Administration of the AAF diet for 1 week resulted in decreased activity of glucose-6-phosphatase throughout the liver lobule but particularly in the centrilobular areas. No changes were detected in either the activity or distribution of succinate dehydrogenase or aniline hydroxylase. Lysosomal changes were similar to those seen in the female rats at week 1. After 8 weeks the normal lobular architecture of the liver was disrupted by the presence of numerous areas of hyperplasia. These micronodules were virtually devoid of glucose-6-phosphatase activity although the surrounding parenchyma stained quite heavily (Fig. 6.5). The activity of succinate dehydrogenase was variable throughout the "normal" liver but in the nodules it was markedly reduced (Fig. 6.5). There was an overall decrease in aniline hydroxylase activity with the nodules staining only slightly more weakly than the remainder of the liver. Sections stained for acid phosphatase presented a very damaged picture: in the nodules there was an overall loss of activity whilst in the surrounding liver enlarged lysosomes and autophagic vacuoles were abundant.

Enzyme histochemistry was not carried out on liver from male rats killed at weeks 16 and 24 or from female rats killed at week 26.

g) Macroscopic Changes in the Liver

Female Rats: No changes in the gross appearance of the liver at autopsy were noted until week 16 when in 2 out of 4 rats killed the liver had a fairly granular surface and a mottled appearance. At week 26 the liver in all 4 rats was markedly enlarged and gross pathological changes were evident. The surface of the liver was irregular with a rather pale, mottled appearance and in 2 rats several small (1-3 mm diameter),
FIG. 6.5 AAF (male) week 8 (x 72)

A. Loss of succinate dehydrogenase in hyperplastic nodule

B. Loss of glucose-6-phosphatase in hyperplastic nodule
smooth surfaced, tan-coloured nodules were present.

Male Rats: Abnormalities were first seen at week 8, when in all 4 rats killed the surface of the liver was finely granular and rather pale, almost yellowish in colour. By week 16, livers were greatly increased in size and numerous smooth-surfaced, pale tan-coloured nodules (1-3 mm diameter) protruded from the surface of the major lobes. Similar changes were evident at week 24. At this stage, larger smooth-surfaced nodules were present (2-6 mm diameter) and these were sharply demarcated from the rough, irregular surface of the intervening liver tissue. Ascitic fluid or evidence of jaundice was not seen. The gross appearance of the liver nodules induced by AAF has been illustrated in several publications (Epstein et al., 1967; Farber, 1973b; Reuber, 1965) Fig. 6.6 shows the liver of a male rat treated for 16 weeks with AAF.

h) Histological Changes in the Liver

Female Rats: No changes were observed prior to week 16. In 2 rats killed at this stage there were several small areas of liver cell hyperplasia and occasional small foci of hepatocytes in the early stages of hydropic degeneration. The liver of 2 other rats showed no abnormalities. Histopathological changes were present in the liver of all 4 rats killed at week 26. In one, the hepatic parenchyma had been extensively replaced by a lymphosarcoma. Patchy hydropic degeneration, which did not appear to follow a lobular distribution, was a feature of each of the other 3 livers and in 2 of these, there was also slight fatty change. Numerous areas of hyperplasia were present. These were usually composed of fairly uniform enlarged cells and the larger hyperplastic nodules compressed the surrounding parenchyma. Occasional portal tracts
FIG. 6.6 AAF (male) week 16 - gross appearance of liver. Numerous, small (1-3 mm diameter) pale nodules are present in all lobes of the liver. (This liver was preserved in buffered formalin prior to photography).
were present within the nodules but in 1 rat an area of adenoid and papilliform architecture and dilated sinusoids within a large, structureless nodule indicated the presence of an early hepatocellular carcinoma. There was little fibrosis and no evidence of nuclear changes or cell necrosis in the livers examined.

**Male Rats:** Histological changes were first observed at week 8, when in each of the 4 rats killed the presence of numerous small areas of hyperplasia imparted a micronodular appearance to the liver. The cells in these hyperplastic nodules were generally larger than those of the adjacent parenchyma and their nuclei were frequently enlarged and vesicular with prominent nucleoli (Fig. 6.7). Mitotic figures were seen quite frequently in the nodules but not in the adjacent liver (Fig. 6.9). The latter contained areas of fatty change and early hydropic degeneration. Bile duct hyperplasia and early fibrosis were also noted (Fig. 6.8). A similar picture was seen at 16 weeks although the nodules were larger and associated with more extensive fibrosis. There was no evidence of liver cell necrosis at this stage. By week 24 there was little recognisable liver tissue and the overall picture was one of micronodular cirrhosis (Fig. 6.10A). Within some of the larger nodules there was fatty change, hydropic degeneration and, occasionally, individual cell necrosis. A majority of cells contained enlarged, vesicular nuclei (Fig. 6.10B). Occasional portal tracts were present within most of the larger nodules.

No histological abnormalities were observed in the livers of any of the male or female control rats.
FIG. 6.7 AAF (male) week 8

A. Nuclear enlargement and vesiculation in hyperplastic nodule (on right) (H & E x 180).

B. Higher power of hyperplastic nodule in A (H & E x 450).
FIG. 6.9 AAF (male) week 8 - bile duct hyperplasia and early fibrosis (H & E x 180).

FIG. 6.9 AAF (male) week 16 - mitotic figures (arrowed) within nodule (H & E x 450).
FIG. 6.10 AAF (male) week 24

A. Micronodular cirrhosis (H & E x 72).

B. Enlarged nuclei within a nodule (H & E x 450).
j) Ultrastructural Changes in the Liver

Electron microscopy was carried out only on liver tissue from male and female rats given the AAF diet for 1 week. In the females, the hepatocytes appeared to contain an increased amount of smooth, but a slightly reduced amount of rough endoplasmic reticulum (Fig. 6.11). The smooth endoplasmic reticulum in the male rat, whilst somewhat increased in amount, was quite markedly dilated. There was a distinctly reduced amount of rough endoplasmic reticulum. This too appeared somewhat dilated and assumed an unusual configuration encircling the mitochondria (Fig. 6.12). There were frequent areas of apparently "unoccupied" intracellular space. Although the rats were starved prior to killing, it is possible (cf. Epstein et al., 1967) that some of this space represents glycogen. Although apparently normal in structure, the mitochondria in the male rat appeared to be swollen as is evident from a comparison of Figs. 6.11 and 6.12, both of which were taken at the same magnification. Measurements of succinate dehydrogenase both biochemically and histochemically revealed no differences between the males and females at this stage or subsequently.
FIG. 6.11 AAF week 1 (female). Apart from an increased amount and some dilatation of the smooth endoplasmic reticulum, the tissue appears relatively normal (x 20,500).

FIG. 6.12 AAF week 1 (male). Note swollen appearance of the mitochondria (cf Fig. 6.11), reduced amount and unusual arrangement of the rough endoplasmic reticulum, dilated smooth endoplasmic reticulum and areas of "empty" intracytoplasmic space. (x 20,500).
The biological activity of AAF has been extensively investigated at both the morphological and biochemical levels and forms the subject of a very considerable literature. In the present experiments the observed changes in body weight, liver weight and in the gross and microscopic appearance of the liver were substantially in agreement with those reported for other studies using comparable dietary levels of AAF (Albert et al., 1972; Epstein et al., 1967; Flaks, 1970; Jackson and Irving, 1972; Sidransky et al., 1961; Teebor and Seidman, 1970; Trams et al., 1961). Although overall consumption of the AAF diet was markedly reduced in both sexes, intake was essentially normal on day 1 of feeding. This situation, the converse of that seen with safrole and Ponceau MX, suggests that toxic anorexia, rather than unpalatability, was a key factor in the reduced consumption of AAF diet. In the female rats there was an apparent increase in food conversion ratio. Numerous factors could give rise to such an effect, making interpretation difficult without additional information (Sharratt, 1972).

The uniform depression of drug metabolising enzyme activity caused by AAF in the male rats is consistent with the reported effects of a number of hepatotoxins on this enzyme system (Donelli et al., 1970; Smuckler et al., 1967). Trams et al. (1961) found that hepatic N-demethylase and aromatic hydroxylase activities fell to 20-40% of control over a 24 week period of administration of a diet containing 0.025% AAF to male rats. The effects of AAF on other microsomal enzymes appear to be variable since Trams et al. (1961) reported a significant elevation of glucuronyl transferase activity whilst in the present study, the activity of glucose-6-phosphatase
could be shown histochemically to be markedly depressed.

The action of AAF on drug metabolising enzyme activity in the female rat contrasted sharply with its observed effects in the male. Although this sex difference might be considered to reflect the fact that AAF is more potently hepatocarcinogenic in the male rat, the available evidence suggests that it is more probably the result of a greater sensitivity of the male rat to the hepatotoxic effects of AAF. Thus, in the present study, early fatalities occurred only in the males, an observation made also by Wilson et al. (1941), and histopathological changes in the liver were seen much earlier in this group than in the females. Furthermore, depression of drug metabolising enzyme activity in the male occurred at a very early stage and therefore well before the liver cells are reported to show any features of neoplastic growth (Slifkin et al., 1972; Teebor and Becker, 1971; Teebor and Seidman, 1970). Many carcinogens and/or their metabolites cause inhibition of protein synthesis (e.g. Hurtin and Arrhenius, 1965). A sex difference in the rate or extent of production of such a metabolite of AAF - as has been shown for the production of AAF N-sulphate (Jackson and Irving, 1972) - could therefore account for the early depression in the level of microsomal protein observed in the male rats. On the other hand, Teebor and Seidman (1970) reported that the activities of glucose-6-phosphatase, tryptophan pyrrolase and tyrosine transaminase were depressed to a similar extent in both male and female rats fed a diet containing 0.06% AAF for up to 12 weeks. The present results confirmed this effect on glucose-6-phosphatase and showed that the activity of succinate dehydrogenase was also depressed to a similar extent in both sexes. Thus it appears that relatively specific effects on the synthesis of particular microsomal enzyme proteins may be involved in
the observed sex difference in drug metabolising enzyme response to AAF. These effects might be related to the reported differences in the extent to which AAF becomes bound to t- and r-RNA fractions in male and female rat livers (Irving and Veazy, 1971).

The apparent failure of AAF to give rise to a recognisable substrate interaction spectrum with cytochrome P450 in liver microsomes from either sex of untreated rat contrasts with results obtained with hamster and mouse liver microsomes. In these preparations, AAF elicits a typical type I interaction spectrum (F.J. McPherson, personal communication). Although metabolism may not always correlate closely with spectrally apparent binding to cytochrome P450, it is noteworthy that the basal levels of AAF-ring hydroxylases are much lower in the rat than in the hamster (Cramer et al., 1960a). Moreover, the observed decline in aromatic hydroxylase activity in the male rats may result in a shift in the relative proportions of ring- and N-hydroxylated metabolites formed from AAF. This might account for the observation of Miller et al. (1960) that the proportion of N-OHAAF excreted in the urine increased from an initial 1-2% to some 10-14% after 14 weeks. Nevertheless, it seems unlikely that these factors are of primary importance in predisposing the rat to the hepatocarcinogenicity of AAF since among species capable of N-hydroxylation AAF, differences in hepatocarcinogenicity correlate closely with differences in N-OHAAF sulphotransferase activity (De Baun et al., 1970a).

The biochemical results obtained with AAF - at least in the male rat - contrast sharply with those obtained with safrole and Ponceau MX although, as mentioned above, it is doubtful whether this contrast is
directly related to the hepatocarcinogenicity of AAF. Since the differing response of male and female rats to AAF appears to correlate with their differing levels of sulphotransferase activity, it is difficult to argue that the induction of drug metabolising enzyme activity in the female rat plays an important part in the relative resistance of this animal to the effects of AAF. The converse of this situation is more probably correct. Once established however, the enzyme induction may exert a protective effect in view of the known ameliorating effects of drug metabolising enzyme inducers on the toxic and carcinogenic action of AAF in the rat (Peraino et al., 1971, 1973; Ulland et al., 1973). That AAF is a potential inducer even in the male rat is suggested by its proliferative influence on hepatocyte smooth endoplasmic reticulum (Flaks, 1970). Meldolesi (1967) has pointed out that smooth membrane proliferation may still occur in response to chemicals which are themselves, or whose metabolites are inhibitors of protein synthesis.

The sequence of morphological changes produced by AAF was quite different from that produced by either safrole or Ponceau MX. In particular the hyperplastic nodules which developed in the livers of both male and female rats were not preceded by cell necrosis. This absence of early necrosis, which is in agreement with published findings (Farber, 1956; Flaks, 1970; Sidransky et al., 1961; Teebor and Seidman, 1970), imparts on the AAF-induced nodules a significance rather different from that of the nodules induced by safrole and Ponceau MX. The latter not only developed at a much later stage but were invariably preceded by cell necrosis. The significance of this difference is discussed in Chapter 8. Despite the absence of necrosis, mild fibrosis was evident in the livers of male rats at week 8. It is
possible that this early response, also noted by Flaks (1970), is related to the action of N-OHAAF. This substance, when administered in relatively large doses, is known to cause massive periportal necrosis (De Baun et al., 1970b). The extensive fibrosis subsequently seen surrounding the nodules of hyperplasia in both male and female rats may reflect a pressure effect of the rapidly growing hyperplastic tissue on the surrounding parenchyma. The nuclear changes observed in the male, but not the female rats from week 8 onwards were very similar to those described by Teebor and Seidman (1970). These authors also noted the confinement of such changes to male rats. In contrast to these findings, Flaks (1970) observed no nuclear abnormalities in the livers of male rats fed 0.05% AAF for 8-12 weeks. This may reflect strain differences in susceptibility to the hepatotoxic or carcinogenic effects of AAF since there was also little early nodule formation in these livers.

Despite the evident hepatotoxicity of AAF, lysosomal changes of the type produced at an early stage by safrole and Ponceau MX were not prominent prior to the appearance of histological abnormalities in the livers of AAF-treated rats. The histochemical changes in acid phosphatase and glucose-6-phosphatase associated with the development of early nodules in the male rats were in agreement with the findings of Kitigawa and Sugano (1973).

The early mortality of all the rats used in this series of experiments could most probably have been averted by avoiding continuous administration of the AAF diet and by withdrawing this completely after an appropriate period of time (see Farber, 1973b; Teebor and Becker, 1971). However, for comparison of the biochemical
results with those obtained with safrole and Ponceau MX continuous administration was desirable. Since the compromise of withdrawing the AAF diet for brief intervals was not successful, it would seem preferable for future studies to adopt the procedure of Teebor and Becker (1971). Those hyperplastic nodules that did not regress on withdrawal of the diet and the subsequently developing hepatocellular carcinomas could then be compared with nodules persisting in safrole- and Ponceau MX-treated rats after cessation of treatment.
CHAPTER SEVEN

STUDIES ON THE INTERACTION OF SAFROLE WITH
HEPATIC MICROSONAL CYTOCHROME P450
7.1 Introduction

Evidence that a metabolite of safrole binds tenaciously to cytochrome P450, producing a spectral species absorbing maximally at 455nm, was outlined in section 4.1. It was subsequently suggested that inactivation of cytochrome P450 in this manner was largely responsible for the reduced ability to metabolise foreign compounds, shown both by in vitro and in vivo techniques, to result during chronic administration of safrole to rats. Experiments were therefore carried out to investigate more closely the nature of this metabolite - cytochrome P450 interaction and in particular, to determine whether or not it was reversible.

Studies in vitro suggest that it is from the methylenedioxy moiety of safrole that the species absorbing at 455nm is derived. Thus, of the compounds methylenedioxybenzene, allylbenzene and eugenol (4-allyl-1-hydroxy-2-methoxybenzene), only methylenedioxybenzene interacts with cytochrome P450 to produce an absorption maximum at 455nm (Franklin, 1971). In addition to an intact methylenedioxy ring, a suitably lipophilic character appears to be necessary since related, but more polar compounds e.g. methylenedioxybenzoic acid (piperonylic acid) do not undergo this interaction (Lake and Parke, 1972). According to Casida et al. (1966), extensive cleavage of the methylenedioxy ring occurs both in vitro and in vivo after oral administration of safrole to mice. This reaction requires NADPH and oxygen and is thought to involve hydroxylation of the methylene group followed by loss of formaldehyde to produce catechol derivatives. None of these possible derivatives, when added to liver microsomes either in the presence or absence of NADPH, produce any increase in absorption at 455nm (unpublished results of B.G. Lake, 1972). Therefore it appears that the species responsible for the 455nm absorption maximum is an intermediate produced during oxidative metabolism of the methylene group.
In considering their efficacy as pesticide synergists (see reviews by Casida, 1970, 1973), Hennessey (1965) proposed that methylenedioxyphenyl compounds could interact irreversibly with the drug metabolising enzyme system. He postulated that loss of a hydride ion from the methylene ring gave rise to the electrophilic benzodioxolium ion which could then bind covalently to, and inactivate, the enzyme protein. An alternative view (Hansch, 1968), held that an anionic free radical species was produced by homolytic cleavage of a proton from the methylene group. Based on more recent theory, Ullrich and Schnabel (1973) have proposed the metabolic formation of a carbanion by abstraction of a methylene proton by the strongly nucleophilic active oxygen species formed on donation of the second electron to the reduced cytochrome P450-oxygen-substrate complex. Formation of the absorption maximum at 455nm is explained by the ligand binding of this carbanion to cytochrome P450. This mechanism is based on observations with the fluorene anion and with the compounds 2-nitropropane and carbon tetrachloride both of which can give rise, non-enzymically, to anionic species.

The 455nm absorbing species derived from safrole possesses considerable stability as judged by its presence in microsomes isolated from rats treated in vivo with safrole (Lake and Parke, 1972; Parke and Rahman, 1971). A carbanion formed as proposed by Ullrich and Schnabel (1973) could possess such stability in the relatively aprotic environment of the lipophilic substrate binding site of cytochrome P450. Factors influencing, and the mechanism of the decay of the 455nm absorbing species are unknown. Franklin (1972b) reported that addition of uridine diphosphate-glucuronic acid, s-adenosylmethionine, cysteamine, hexobarbital or metyrapone to microsomes containing excess NADPH but limiting amounts of piperonyl butoxide, did not influence the rate of decay of the 455nm absorption maximum.

Stable interaction of a metabolite either with the type I substrate
binding site or involving ligand binding to cytochrome P450 haem would result in inhibition of drug metabolism. Accordingly, Franklin (1972a) has observed a direct relationship between the degree of inhibition of ethylmorphine demethylation and the amount of 455nm absorbing species formed in vitro from different, limiting amounts of piperonyl butoxide. In the same vein, an intact methylenedioxy moiety, with both methylene hydrogens unsubstituted, was shown to be essential for high synergistic activity in the structure-activity correlations of Metcalf et al. (1966) and Wilkinson et al. (1966).

Results presented in this chapter show that under certain conditions the metabolite-cytochrome P450 complex can be dissociated and suggest that the novel interactions of safrole may provide a useful tool for studying substrate-cytochrome P450 interactions.
7.2 Methods

a) Animal pretreatment and preparation of microsomes

Female rats, initial body weight 120-150g, were given a diet containing 0.25%(w/w)safrole (see 2.2) for 7-14 days, or were injected intraperitoneally with safrole, phenobarbitone or 3-methylcholanthrene as described in 2.2. Washed liver microsomes, prepared as described in 2.3, were suspended in 0.1M sodium phosphate buffer pH 7.6 to a concentration of 2 mg protein per ml, measured by the method of Lowry (2.3). For experiments involving the use of glycerol, microsomes were suspended in 0.1M sodium phosphate buffer pH 7.6 containing 20%(v/v)glycerol.

b) Determinations of $K_s$ and $\Delta \text{OD}_{\text{max}}$

These were made as described in 2.3. Biphenyl was added as solutions 50 and 250 mM, safrole 100 mM and aniline 500 mM, all in absolute ethanol. The following final cuvette concentrations (mM) were utilised:-

Biphenyl: 0.04, 0.05, 0.06, 0.08, 0.10, 0.14, 0.20 (added as 50 mM solution)
  0.30, 0.40, 0.50, 0.60, 0.80, 1.00 (added as 250 mM solution)
Safrole: 0.04, 0.06, 0.08, 0.10, 0.12, 0.16, 0.20, 0.28, 0.40, 0.80
Aniline: 0.20, 0.30, 0.40, 0.60, 0.80, 1.20, 2.00.

c) Measurement of Enhanced Interaction Spectra Associated with Loss of 455 nm Absorption in Microsomes from Safrole-Pretreated Rats

All measurements were carried out using a Pye-Unicam SP 1800 dual beam spectrophotometer at ambient temperature after allowing at least 60 minutes for instrument warm-up. Oxidised microsomal suspension was added into each of two glass cuvettes and substrate solution added to the sample cuvette. The resultant interaction spectrum was recorded at intervals - cuvettes being removed from the light path between recordings - until no further increase
occurred (15-120 minutes). Any residual 455nm absorption was then recorded, firstly after addition of NADH and then after addition of sodium dithionite to the sample cuvette. Dithionite was then added also to the reference cuvette, the cuvette positions in the spectrophotometer reversed, and the reduced difference spectrum recorded. The resultant type III spectrum (Philpot and Hodgson, 1971) provided a measure of the 455nm absorption of the original reference suspension relative to that of the substrate-treated suspension. The process of spectral enhancement could be halted at any stage, and measurements of residual 455nm absorption made, by addition of NADH to the sample cuvette.

With the following exceptions, all substrate solutions were prepared in absolute ethanol:- SKF 525A, ethylmorphine hydrochloride, aminopyrine and nicotinamide were dissolved in distilled water, 2,4-xylidine in 50% (v/v) aqueous ethanol and 2-acetylaminofluorene in ethanol/acetone (75:25 v/v).

All substrate concentrations quoted in the text are final cuvette concentrations in m.mols/litre.
7.3. Results
7.3.1. Some Properties of the 455nm Absorbing Species

a) Spectral Characteristics

The NADH-reduced minus oxidised difference spectrum of liver microsomes prepared from rats pretreated with safrole (safrole microsomes*) showed an absorption maximum at 455nm in addition to the spectrum of reduced cytochrome b₅. The magnitude of this 455nm maximum was not further increased by addition of NADPH and only slightly so (< 5%) by addition of Na₂S₂O₄.

Incubation of control microsomes* with safrole and NADPH, but not NADH, led to the progressive appearance of an absorption maximum at 455nm. This maximum disappeared when residual NADPH was oxidised by bubbling oxygen through the cuvette contents, but was fully and immediately regenerated by addition of NADH. A 455nm absorption maximum generated by similar incubation of control microsomes with piperonyl butoxide could only be re-reduced with NADPH or Na₂S₂O₄, and not NADH.

The difference spectrum between NADPH-reduced control microsomes in the presence and absence of safrole, had maxima at 427 and 455nm. This spectrum, generated under similar conditions from piperonyl butoxide, was termed type III by Philpot and Hodgson (1971). A "type III" spectrum was also resolved when NADH-reduced safrole microsomes were compared with NADH-reduced control microsomes although, in contrast to the spectrum described above, the 427nm maximum was greater than the 455nm maximum, probably due to the increased cytochrome b₅ content of the safrole microsomes. These observations are summarised in Fig. 7.1.

* Liver microsomes prepared from rats treated with safrole are referred to throughout as safrole microsomes and those from untreated rats, as control microsomes.
### Cuvette Contents

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</tbody>
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### Spectral Change

- **Wavelength (nm)**: 400-420-440-460-480
- **Minutes**: 2-4-15
- **ΔOD 400-455 nm**

- **Type III spectrum**
  - **ΔOD 400-455 nm**
  - **Wavelength (nm)**: 400-420-440-460-480
b) Effect of Deoxycholate Treatment and Isooctane Extraction on the 455nm Absorption Maximum

Conversion of cytochrome P450 to P420 by solution of sodium deoxycholate (final concentration approx. 1%) in safrole microsomes, resulted in a loss of 455nm redox absorption. Sequential measurements, in the presence of 20% glycerol to retard the breakdown of cytochrome P450, showed that loss of 455nm redox absorption paralleled the conversion of cytochrome P450 to P420. Conversion of cytochrome P450 to P420 did not appear to be slower in safrole than in control microsomes.

Extraction of safrole microsomes with isoctane (2.3) resulted in complete loss of the ability to generate an interaction spectrum on addition of a type I substrate. However, the 455nm absorption maximum was not diminished after extraction, recovery approaching 100% when expressed per unit of microsomal protein recovered (Table 7.1). In the safrole, but not the control microsomes, 10-20% of the cytochrome P450 was recovered as P420.

c) Stability of the 455nm Absorbing Species During Storage of Safrole Microsomes

The relative rates of decay of the 455nm absorption maximum and cytochromes P450 and b5 determined during storage of safrole microsomes at approximately 20°C are shown in Fig. 7.2. Under these conditions, the 455nm absorbing species appeared to be rather less stable than either cytochrome P450 or b5.
<table>
<thead>
<tr>
<th>Experiment No.</th>
<th>Microsomes</th>
<th>Cytochrome P450</th>
<th>Cytochrome b5</th>
<th>455nm Absorption Maximum</th>
<th>Microsomal Protein</th>
</tr>
</thead>
<tbody>
<tr>
<td>1</td>
<td>Control</td>
<td>76§</td>
<td>69</td>
<td>-</td>
<td>70</td>
</tr>
<tr>
<td></td>
<td>Safrole</td>
<td>43</td>
<td>58</td>
<td>61</td>
<td>59</td>
</tr>
<tr>
<td>2</td>
<td>Control</td>
<td>119</td>
<td>116</td>
<td>-</td>
<td>105</td>
</tr>
<tr>
<td></td>
<td>Safrole</td>
<td>76</td>
<td>94</td>
<td>98</td>
<td>99</td>
</tr>
</tbody>
</table>

* Values expressed as % of those for phosphate buffer-extracted microsomes (see 2.3 o)

§ Mean values. Expt. 1, 2, rats/group, Expt. 2, 3 rats/group. Rats treated dietarily with safrole (see 7.2a)
Decay of Cytochromes P450 and $b_5$ and the 455nm Absorption Maximum During Storage of Safrole Microsomes at $20^\circ$C.
7.3.2. Substrate-Cytochrome P450 Interactions after Pretreatment of Rats with Safrole

a) Determination of Kinetic Constants at Intervals During Chronic Administration of Safrole

After administration of safrole for 1, 8, 16 and 25 weeks, the kinetic constants $K_s$ and $\Delta \text{OD}_{\text{max}}$ were determined for the spectrally apparent interactions with cytochrome P450 of biphenyl, safrole and aniline. For the determinations of these values (Table 7.2) the cytochrome P450 content of the control and safrole microsomes was equalised after measurement of their CO spectra. However, subsequent findings (see 7.3.3a) showed that as a result of inhibition of CO binding, the actual cytochrome P450 content of the safrole microsomes was some 40% greater than that determined by the CO spectrum. Consequently, the kinetic constants determined are of limited absolute value; they are included since they are of interpretative value.

The spectral minimum of the aniline (type II) interaction with safrole microsomes was blue-shifted to around 395nm, producing a spectrum resembling that given by n-octylamine in microsomes from 3-methylcholanthrene pretreated rats (Jefcoate et al. 1969).

b) Displacement of the 455nm Absorption Maximum

If the observed effects of safrole pretreatment on substrate metabolism and interaction were attributed to the purported safrole metabolite bound to cytochrome P450, it seemed possible that high concentrations of alternative substrates of cytochrome P450 might displace the bound metabolite. Support for this contention was obtained.

Addition of up to 4mM of the type I substrates biphenyl or ethylbenzene to oxidised safrole microsomes, followed by reduction with NADH, did not alter the intensity of the 455nm absorption maximum either
Table 7.2
Kinetic Constants for Substrate Interaction with Cytochrome P450 after Pretreatment of Rats with Safrole

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Pretreatment Time (Weeks)</th>
<th>1</th>
<th>8</th>
<th>16</th>
<th>25</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td></td>
<td>Control</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>BIPHENYL</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>(High Affinity)</td>
<td>$K_s$</td>
<td>0.54 ± 0.03</td>
<td>0.59 ± 0.07</td>
<td>0.53 ± 0.03</td>
<td>0.50 ± 0.05</td>
</tr>
<tr>
<td></td>
<td>$\Delta \text{OD}_{\text{max}}$</td>
<td>0.047± 0.003</td>
<td>0.053± 0.003</td>
<td>0.063± 0.002</td>
<td>0.049± 0.004</td>
</tr>
<tr>
<td>Treated</td>
<td>$K_s$</td>
<td>0.49 ± 0.04</td>
<td>0.81 ± 0.08</td>
<td>0.75 ± 0.05$^a$$^2$</td>
<td>0.60 ± 0.09</td>
</tr>
<tr>
<td></td>
<td>$\Delta \text{OD}_{\text{max}}$</td>
<td>0.058± 0.03</td>
<td>0.050± 0.002</td>
<td>0.062± 0.001</td>
<td>0.050± 0.003</td>
</tr>
<tr>
<td>BIPHENYL</td>
<td>(Low Affinity)</td>
<td>$K_s$</td>
<td>1.48 ± 0.25</td>
<td>1.67 ± 0.31</td>
<td>1.57 ± 0.29</td>
</tr>
<tr>
<td></td>
<td>$\Delta \text{OD}_{\text{max}}$</td>
<td>0.060± 0.002</td>
<td>0.069± 0.001</td>
<td>0.083± 0.007</td>
<td>0.061± 0.004</td>
</tr>
<tr>
<td>Treated</td>
<td>$K_s$</td>
<td>1.48 ± 0.007</td>
<td>2.53 ± 0.10</td>
<td>2.73 ± 0.46</td>
<td>2.15 ± 0.41</td>
</tr>
<tr>
<td></td>
<td>$\Delta \text{OD}_{\text{max}}$</td>
<td>0.075± 0.006</td>
<td>0.074± 0.004</td>
<td>0.094± 0.007</td>
<td>0.077± 0.005</td>
</tr>
<tr>
<td>ANILINE</td>
<td>$K_s$</td>
<td>4.67 ± 0.60</td>
<td>6.04 ± 0.19</td>
<td>5.63 ± 0.31</td>
<td>5.40 ± 0.42</td>
</tr>
<tr>
<td></td>
<td>$\Delta \text{OD}_{\text{max}}$</td>
<td>0.028± 0.001</td>
<td>0.029± 0.001</td>
<td>0.028± 0.003</td>
<td>0.025± 0.002</td>
</tr>
<tr>
<td>Treated</td>
<td>$K_s$</td>
<td>4.30 ± 0.61</td>
<td>3.58 ± 0.16$^b$</td>
<td>3.34 ± 0.40$^a$</td>
<td>3.80 ± 0.19$^a$</td>
</tr>
<tr>
<td></td>
<td>$\Delta \text{OD}_{\text{max}}$</td>
<td>0.041± 0.002$^b$</td>
<td>0.043± 0.002$^b$</td>
<td>0.047± 0.001$^b$</td>
<td>0.033± 0.001$^a$</td>
</tr>
<tr>
<td>SAFROLE</td>
<td>$K_s$</td>
<td>0.71 ± 0.05</td>
<td>0.77 ± 0.06</td>
<td>0.75 ± 0.06</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>$\Delta \text{OD}_{\text{max}}$</td>
<td>0.072± 0.002</td>
<td>0.060± 0.001</td>
<td>0.70 ± 0.002</td>
<td>–</td>
</tr>
<tr>
<td>Treated</td>
<td>$K_s$</td>
<td>1.01 ± 0.09</td>
<td>1.13 ± 0.17</td>
<td>1.01 ± 0.03$^a$</td>
<td>–</td>
</tr>
<tr>
<td></td>
<td>$\Delta \text{OD}_{\text{max}}$</td>
<td>0.107± 0.008$^b$</td>
<td>0.088± 0.005$^b$</td>
<td>0.097± 0.005$^b$</td>
<td>–</td>
</tr>
</tbody>
</table>

1. Values are means (3 rats per group) ± SEM expressed in M x 10^{-4}.
2. Means significantly different from control. $a = p < 0.05$  $b = p < 0.01$
immediately, or on standing for up to 30 minutes. However, if NADH was not added until 30 minutes after addition of substrate (1mM ethylbenzene), a marked decrease in absorption at 455nm was seen. By increasing the time interval between addition of substrate and reduction with NADH, complete loss of 455nm absorption could be achieved.

c) Enhancement of Substrate Interaction Spectra Accompanying Loss of 455nm Absorption

When the oxidised difference spectrum was recorded at intervals after addition of 1mM ethylbenzene to safrole microsomes, a progressive enhancement of the type I spectrum was observed, resulting in a final spectral change 2-3 times the kinetically determined \( \Delta O D_{\text{max}} \). No such effect could be reproduced in control microsomes. The time required for maximal enhancement of the type I spectrum was very similar to that required to produce complete loss of 455nm absorption (section (a) above) and the close, inverse relationship between these two events is illustrated in Fig. 7.3. Further, in 12 different preparations of safrole microsomes the degree of spectral enhancement appeared to be directly related to the initial magnitude of the 455nm absorption maximum (Fig. 7.4). Finally, the dithionite reduced difference spectrum between safrole microsomes (sample cuvette) and the same microsomes plus 1mM ethylbenzene (reference cuvette) showed the progressive appearance of a type III spectrum, clearly indicating the decreased amount of 455nm absorbing species in the ethylbenzene-treated microsomes.

The time taken to effect complete displacement of the 455nm absorption maximum varied between about 15 and 120 minutes dependent on a number of factors which are considered in section 7.3.2f. The process of displacement could be halted at any stage by reduction of the microsomes with NADH.
Fig. 7.3

Inverse Relationship between Enhancement of Type I Spectral Change and Loss of 455nm Absorption on Addition of Ethylbenzene (1.0 mM) to Safrole Microsomes

Types 1 spectral change; 455nm absorption

For details of procedure see 7.2c
Correlation of Initial Absorbance at 455nm with Degree of Spectral Enhancement on Addition of Type I Substrate to Safrole Microsomes

For details of procedure see 7.2c
d) Characteristics of Enhanced Interaction Spectra

The enhanced substrate interaction spectrum associated with displacement of the 455nm absorption maximum did not differ, other than in magnitude, from the type I spectrum produced on first addition of the substrate. Wavelengths of the Soret maximum and minimum were not altered and the visible minimum at around 575nm - characteristic of type I interactions - intensified in parallel with enhancement of the Soret spectrum. When the initial type I component of an enhanced spectrum was cancelled out by addition of an equal amount of substrate to the reference cuvette, the residual "spectrum due to enhancement" again appeared identical to a type I spectrum.

Type I spectra produced by high (> 1mM) concentrations of certain substrates, e.g. biphenyl, safrole, show an increase in absorption towards 350nm which is not attributable to the absorption spectrum of the substrate. Enhancement of such spectra was accompanied by a progressive return to baseline absorption at 350nm. The "spectrum due to enhancement" similarly showed baseline absorption at 350nm.

Some spectra, typical of those described in sections b) c) and d) above are shown in Fig. 7.5.

e) Correlation of Physicochemical Properties with Activity in Displacing the 455nm Absorption Maximum

A range of substrates of cytochrome P450 was utilised to determine whether ability to displace the 455nm absorption maximum was a property common to all such substrates and if it were not, what features were peculiar to those substrates possessing this ability.

The substrates examined are shown in Table 7.3 classified as active, partially active and inactive in displacing the 455nm absorption maximum. Where available, Hansch values (log partition coefficient between n-octanol
FIG. 7.5  Some Spectra of Safrole Microsomes

BEFORE DISPLACEMENT

NADPH-reduced minus oxidised difference spectrum

Wavelength nm

AFTER DISPLACEMENT

Type 1 substrate-cytochrome P450 interaction spectrum

Ethyl isocyanide spectrum

nm
Table 7.3  Substrates Active and Inactive in Displacing the 455 nm Absorption Maximum

<table>
<thead>
<tr>
<th>Active</th>
<th>Partially Active</th>
<th>Inactive</th>
</tr>
</thead>
<tbody>
<tr>
<td>Butylbenzene</td>
<td>4.13†</td>
<td>Hexylbenzene</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>4.09</td>
<td>Imipramine</td>
</tr>
<tr>
<td>Isopropylbenzene</td>
<td>3.66</td>
<td>Carbon tetrachloride</td>
</tr>
<tr>
<td>BHA</td>
<td>≈3.6</td>
<td>Benzene</td>
</tr>
<tr>
<td>Octyl carbamate</td>
<td>3.51</td>
<td>Hexobarbitone</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>3.37</td>
<td>Piperonal</td>
</tr>
<tr>
<td>Allylbenzene</td>
<td>3.23</td>
<td>4-methoxyphenol</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>3.13</td>
<td></td>
</tr>
<tr>
<td>Eugenol</td>
<td>2.99</td>
<td></td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>2.73</td>
<td></td>
</tr>
<tr>
<td>Toluene</td>
<td>2.69</td>
<td></td>
</tr>
<tr>
<td>Safrole</td>
<td>2.28*</td>
<td></td>
</tr>
<tr>
<td>Hexane</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>NN-dimethylaniline</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4,4'-dimethylbiphenyl</td>
<td>-</td>
<td></td>
</tr>
<tr>
<td>4-phenylanisole</td>
<td>-</td>
<td></td>
</tr>
</tbody>
</table>

† Hansch values taken from Leo et al., (1971) except those marked * which were kindly supplied by Dr. M.S. Tute of Pfizer Ltd., Sandwich, Kent.
### TABLE 7.4

Structural Formulae of Some Substrates Active or Inactive in Displacing the 455nm Absorption Maximum

<table>
<thead>
<tr>
<th>ACTIVE</th>
<th>INACTIVE</th>
</tr>
</thead>
<tbody>
<tr>
<td>Safrole</td>
<td>Hexobarbital</td>
</tr>
<tr>
<td>Ethylbenzene</td>
<td>Ethylmorphine</td>
</tr>
<tr>
<td>Allylbenzene</td>
<td>SKF 525A</td>
</tr>
<tr>
<td>Cyclohexane</td>
<td>2-Acetylaminofluorene</td>
</tr>
<tr>
<td>Naphthalene</td>
<td>Aminopyrine</td>
</tr>
<tr>
<td>Biphenyl</td>
<td>Butylated Hydroxytoluene</td>
</tr>
</tbody>
</table>

**Type II Substrates**
and water) are included as an index of lipophilicity. Substrates classified as active displaced all the NADH-reducible 455nm absorption and left no more than 10% reducible by Na₂S₂O₄. Partially active substrates might displace up to 50% of NADH-reducible 455nm absorption but might leave as much as 90% reducible by Na₂S₂O₄ (see section 7.3.2f).

Clearly, many substrates were inactive and these remained so, irrespective of the substrate concentration used. Such substrates tended to be less, although some were much more, lipophilic than those that were active, all of which were highly lipophilic (Table 7.3). A distinction between active and inactive substrates emerged more clearly when structural formulae were compared (Table 7.4). Thus, inactive substrates were either large or sterically "bulky" molecules - or were type II substrates - active substrates being comparatively "simple" molecules. In an attempt to assess further the relative importance of lipophilicity and steric factors, experiments were performed with a series of alkylbenzenes in which lipophilicity increased with increasing alkyl chain length. Results of these experiments are summarised in Fig. 7.6. Comparison of the highly lipophilic but sterically very "bulky" 2,6-di-tert-butyl-4-methylphenol (BHT) with the less lipophilic but less "bulky" 2-tert-butyl-4-methoxyphenol (BHA) showed the former to be inactive but the latter to be active.

Atypical results were obtained with carbon tetrachloride, 4-methoxyphenol and piperonal. The type I spectrum of carbon tetrachloride underwent enhancement but there was no concomitant loss of Na₂S₂O₄-reducible 455nm absorption, probably because carbon tetrachloride itself gives rise to an absorption maximum at 454nm in Na₂S₂O₄-reduced microsomes (Reiner and Uehleke, 1971). 4-Methoxyphenol gave no detectable interaction spectrum with control microsomes and little or no immediate spectrum with safrole microsomes. With phenobarbitone microsomes it gave a reverse type I (Schenkman et al., 1972b) spectrum whilst the enhanced spectrum with safrole microsomes resembled a
FIG. 7.6

Spectral Enhancement Produced by Benzene and some Alkylbenzenes

Safrole microsomes were suspended in phosphate/glycerol buffer as per 7.2a. The final cuvette concentration of each substrate was 1.0 mM. Benzene 0—0; Toluene △—△; Ethylbenzene •——•; n-Butylbenzene ■—■; n-Hexylbenzene ▲—▲.
normal type I spectrum. The spectrum produced by piperonal in phenobarbitone microsomes and both its initial and enhanced spectrum with safrole microsomes resembled a type I spectrum but the wavelength of the maximum was increased to about 408nm. Franklin (1971) reported that piperonal did not bind to phenobarbitone microsomes. All of the type II substrates utilised were inactive in displacing the 455nm absorption maximum.

f) Some Factors Affecting the Rate of Displacement of the 455nm Absorption Maximum

A remarkable feature of the process of displacement was the very slow rate at which it occurred. The effect on this rate, of displacing substrate concentration, glycerol, iso-octane extraction and the presence of non-displacing substrate was therefore examined. Displacement was monitored by following the progressive enhancement of the substrate (ethylbenzene) interaction spectrum.

Substrate Concentration and Glycerol Spectral enhancement proceeded more rapidly with increasing substrate concentration (Fig. 7.7) although when spectral change (ΔOD) was plotted as % of initial ΔOD, the differences in rate at different concentrations were much less marked. When measurements were made in the presence of glycerol the rate of enhancement was decreased: at substrate concentrations of 0.04, 0.40 and 2.00 mM the rate was 4.2, 3.1 and 2.1 times respectively, slower in the presence than in the absence of glycerol when measurements of ΔOD over the first 5 minutes were compared. Analysis of values for "initial velocity" of enhancement at different displacing substrate concentrations in the form of double reciprocal plots (Lineweaver and Burk, 1934), yielded well-fitting straight lines and indicated that glycerol was acting as a "non-competitive inhibitor" of the displacement process (Fig. 7.8).

Under the conditions of these experiments, the minimum concentration of ethylbenzene required to effect complete displacement was between 0.1 and 0.2 mM
Fig. 7.7

Rate of Spectral Enhancement at Various Concentrations of Ethylbenzene Added to Safrole Microsomes

For details of procedure see 7.2c. Microsomes were suspended in phosphate/glycerol buffer as per 7.2a.
Lineweaver-Burk Plot Showing Effect of Glycerol on the Initial Rate of Spectral Enhancement Associated with Loss of 455nm Absorption

Glycerol present (see 7.2a) $V_{max} = 0.080\text{A}/10\text{ min.}$

Glycerol absent $V_{max} = 0.200\text{A}/10\text{ min.}$

$K_m = 6.7 \times 10^{-4}\text{M}$
in the absence, but around 1.0mM in the presence of glycerol. These concentrations appeared to hold for preparations of safrole microsomes with quite widely varying apparent levels of the 455nm absorption maximum.

When displacement was incomplete, addition of \( \text{Na}_2\text{S}_2\text{O}_4 \) to the NADH-reduced sample might elicit an increase of 10-50% in the residual 455nm absorption. This contrasts with the minimal additional effect of \( \text{Na}_2\text{S}_2\text{O}_4 \) on "intact" NADH-reduced safrole microsomes (see 7.3.1a).

Isooctane Extraction: Although no initial type I spectral change was generated on addition of up to 2mM of ethylbenzene to isooctane-extracted safrole microsomes, extraction did not prevent the time-dependent appearance of a "type I" spectrum nor did it affect the rate at which this spectrum underwent enhancement. In both extracted and unextracted microsomes complete displacement of the 455nm absorption maximum was effected and the \( \Delta \text{OD}_{\text{max}} \) in the extracted was smaller than that in the unextracted microsomes by an amount equal to the magnitude of the \( \Delta \text{OD}_{\text{initial}} \) in the unextracted microsomes. Substrates inactive as displacers remained so after isooctane extraction.

Presence of Non-Displacing Substrate: SKF 525A was added to safrole microsomes at a concentration in excess of that required to elicit the maximum type I spectral change in these microsomes. This treatment reduced by 70% the type I spectrum produced on addition of 1 mM ethylbenzene but did not prevent, or retard, the subsequent enhancement of this spectrum and complete displacement of the 455nm absorption maximum. No type I spectrum was generated on addition of 1 mM ethylbenzene to SKF 525A-saturated control microsomes.
7.3.3. **Some Consequences of Formation and Displacement of the 455nm Absorption Maximum**

a) **Effects on Cytochrome P450-CO, and Type II substrate Interaction Spectra**

Formation in vitro of the 455nm absorption maximum from piperonyl butoxide was paralleled by inhibition of CO-binding to cytochrome P450 (Philpot and Hodgson, 1971). To determine whether a similar inhibition resulted from the interaction of safrole with cytochrome P450 in vivo, the CO-spectrum of safrole microsomes was measured before and after displacement.

Ethylbenzene (1mM) was added to safrole microsomes and the suspension immediately reduced with Na$_2$S$_2$O$_4$. The cytochrome P450-CO spectrum of this suspension was compared with that of the same suspension but which had been incubated at room temperature with ethylbenzene (1mM) for 90 minutes prior to reduction with Na$_2$S$_2$O$_4$. In 5 preparations of safrole microsomes displacement of the 455nm absorption maximum resulted in increases of between 41 and 48% in the cytochrome P450-CO spectrum.

The displacing substrate must be present in both cuvettes to show this effect fully. If it is present only in the sample cuvette, a type III spectrum will be generated in the reference cuvette on reduction with Na$_2$S$_2$O$_4$, the 455nm maximum of which will subtract from the 450nm maximum of the cytochrome P450-CO spectrum in the sample cuvette.

The type II interaction spectrum of aniline in safrole microsomes did not undergo enhancement (see 7.3.2e), but did so when ethylbenzene (1mM) was present in the microsomal suspension. The spectrum increased by 60-70% as compared with 41-48% in the case of the CO- spectrum. This additional increase probably derives from the known stimulatory effect of type I substrates on the magnitude of type II spectra (Leibman et al., 1969), an effect which could be elicited to an equal extent in both safrole and control.
microsomes with both displacing and non-displacing type 1 substrates.

b) Effects on the Cytochrome P450-Ethylisocyanide Spectrum

As an approach to determining whether qualitative changes in cytochrome P450, other than binding of the metabolite, occurred after safrole pretreatment, the ethylisocyanide spectrum of safrole microsomes was examined before and after displacement of the 455nm absorption maximum.

In control microsomes at pH 7.6 the ratio between the two maxima of the ethylisocyanide spectrum 430nm/455nm was 0.66 (mean of 3 observations). Dietary administration of safrole for 1 week brought this ratio to a value of 1.21. After administration of safrole for 25 weeks, control and treated values were 0.73 and 1.40 respectively. This alteration of the ethylisocyanide spectrum could be consistently demonstrated after dietary administration of safrole but not when safrole was given by intraperitoneal injection (150 mg/kg/day for 4 days). In this case, the level of the 455nm absorption maximum relative to the (apparent) level of cytochrome P450 was less than when safrole was given in the diet. However, if microsomes from rats treated by injection were incubated in vitro with safrole and NADPH, the resultant increase in the 455nm absorption maximum was paralleled by an increase in the ratio 430nm/455nm for the ethylisocyanide spectrum from 0.8-0.9 to 1.0-1.1.

After displacement of the 455nm absorption maximum, the 430nm/455nm ratio reverted from 1.21 to 0.76 (mean of 3 observations), a value close to that observed for control microsomes. This reversion was achieved purely by an increase in the 455nm maximum of the ethylisocyanide spectrum. The total spectrum (430nm + 455nm) was increased by 31% after displacement. The effect of displacement of the 455nm absorption maximum on the ethylisocyanide spectrum of safrole microsomes is illustrated in Fig. 7.5.
For the reasons outlined in 7.3.3a, it is essential that the displacing substrate is present in both sample and reference cuvettes for the demonstration of these effects on the ethylisocyanide spectrum.

c) Effects on the Apparent Level of Cytochrome b₅

After displacement of the 455nm absorption maximum the cytochrome b₅ spectrum was decreased by 5-15% dependent on the amount of 455nm absorption displaced. These findings support the conclusion reached in 4.3g that the very high apparent levels of cytochrome b₅ induced during chronic administration of safrole do not result simply from a contribution to the b₅ spectrum of the 427nm maximum of the type III spectrum.
7.3.4 Formation in vitro of the 455nm Absorption Maximum

The formation in vitro of a 455nm absorption maximum from piperonyl butoxide has been described in some detail by Franklin (1971, 1972a,b). Most of Franklin's findings could be confirmed using safrole and only a few additional observations are presented here.

a) Effects of Different Pretreatments in vivo on the Rate of Formation in vitro of the 455nm Absorption Maximum

Rates of formation of the 455nm absorption maximum (measured as described in 2.3) in microsomes from untreated rats and rats pretreated with phenobarbitone, safrole or 3-methylcholanthrene are given in Table 7.5B. Two observations were of particular interest. Firstly, the specific increases in initial rate induced by each pretreatment exceeded by several fold the respective increases in the specific content of cytochrome P450 and secondly, whilst the extent of the reaction (i.e. total ΔOD 455-490nm per incubation) was normally quite closely related to the initial rate, 3-methylcholanthrene pretreatment increased the extent more than the initial rate by a factor of approximately 2.5 (Table 7.5B).

In one experiment, a small quantity of a preparation of human liver microsomes was available. Upon incubation with safrole and NADPH an increase in absorption at 455nm was observed and on subsequent reduction with Na₂S₂O₄, a type III spectrum could be generated. Although quantitative measurements could not be undertaken, the rate of reaction appeared to be very low - some 25-50% of that in untreated rat liver microsomes.
### TABLE 7.5

**In Vitro Formation of the 455nm Absorption Maximum:** A. From Compounds Related to Safrole: B. Effect of In Vivo Pretreatments

**A. Microsomes**

<table>
<thead>
<tr>
<th>Substrate (Assay concentration 0.5 mM)</th>
<th>Initial Rate $\left(\Delta\text{OD}_{455-490}\text{nm/unit P450/hr}\right)$</th>
<th>Extent $\Delta\text{OD}_{455-490}\text{nm/total unit P450}$</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Safrole</td>
<td>0.10</td>
<td>0.0270</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>0.13 (130)*</td>
<td>0.0410 (148)</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Safrole</td>
<td>0.26</td>
<td>0.1010</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>0.39 (150)</td>
<td>0.1380 (137)</td>
</tr>
<tr>
<td>Piperonal</td>
<td>0.00</td>
<td>0.0000</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.00</td>
<td>0.0000</td>
</tr>
<tr>
<td>Allylbenzene</td>
<td>0.00</td>
<td>0.0000</td>
</tr>
<tr>
<td>Safrole</td>
<td>0.26</td>
<td>0.0099</td>
</tr>
<tr>
<td>Piperonyl butoxide</td>
<td>0.37 (142)</td>
<td>0.1210 (122)</td>
</tr>
<tr>
<td>Piperonal</td>
<td>0.11</td>
<td>0.0045 (45)</td>
</tr>
<tr>
<td>Eugenol</td>
<td>0.00</td>
<td>0.0000</td>
</tr>
<tr>
<td>Allylbenzene</td>
<td>0.00</td>
<td>0.0000</td>
</tr>
</tbody>
</table>

**B. Microsomes**

<table>
<thead>
<tr>
<th>Substrate</th>
<th>Initial Rate $\left(\Delta\text{OD}_{455-490}\text{nm/hr}\right)$</th>
<th>Extent $\Delta\text{OD}_{455-490}\text{nm/mg microsomal protein}$</th>
<th>Extent/rate (per unit P450) X 100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Control</td>
<td>0.15</td>
<td>0.28</td>
<td>0.0017</td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>0.37 (247)†</td>
<td>1.51 (540)</td>
<td>0.0041 (241)</td>
</tr>
<tr>
<td>Safrole</td>
<td>0.43 (286)</td>
<td>0.72 (257)</td>
<td>0.0054 (317)</td>
</tr>
<tr>
<td>3-Methylcholanthrene</td>
<td>0.51 (340)</td>
<td>1.21 (432)</td>
<td>0.0153 (900)</td>
</tr>
</tbody>
</table>

† For details of pretreatments see 2.2c
§ Values represent means from 2 experiments
* Figures in parentheses are values expressed as % of value for safrole
† † † † † † † † † † control
b) Other Methyleneedioxyphenyl and Related Compounds as Substrates for \textit{in vitro} Formation of the 455nm Absorption Maximum

The results of this experiment are summarised in Table 7.5A. Neither of the compounds without an intact methylenedioxy ring - eugenol and allylbenzene - give rise to any increase in absorption at 455nm. Piperonyl butoxide supported a faster rate of reaction than did safrole whilst piperonal gave rise to an increase in absorption at 455nm only in safrole microsomes and not in phenobarbitone microsomes.

c) Effect of Isooctane Extraction on \textit{in vitro} Formation of the 455nm Absorption Maximum

Abolition of the ability to generate a type I spectrum by isooctane extraction, resulted in loss of the ability of phenobarbitone microsomes either to form, or to bind, the species responsible for the 455nm absorption maximum. In safrole microsomes this ability was reduced but not abolished, the rate being 50% and the extent 30% of that in unextracted microsomes (Table 7.6). However, when safrole was added to the reaction mixture 30 minutes prior to the addition of NADPH - i.e. producing conditions under which displacement could occur - the reaction rate was increased to 75% and the extent to 85% of that of the unextracted microsomes. When the displacing substrate naphthalene was added to the extracted safrole microsomes 30 minutes prior to the addition of safrole and NADPH, the rate of reaction remained at 75%, but the extent was increased to 121% of that in the unextracted safrole microsomes.
### TABLE 7-6

**Effect of Isooctane Extraction on the *In Vitro* Formation of the 455nm Absorption Maximum**

<table>
<thead>
<tr>
<th>Microsomes *</th>
<th>Substrate Additions</th>
<th>Initial Rate †</th>
<th>Extent</th>
<th>Extent/ RateX100</th>
</tr>
</thead>
<tbody>
<tr>
<td>Phenobarbitone</td>
<td>Safrole 0.8 mM</td>
<td>0.20</td>
<td>0.0026</td>
<td>1.30</td>
</tr>
<tr>
<td>Safrole</td>
<td>&quot; &quot;</td>
<td>0.22</td>
<td>0.0032</td>
<td>1.45</td>
</tr>
<tr>
<td>Isooctane Extracted</td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Phenobarbitone</td>
<td>Safrole 0.8 mM</td>
<td>0.00</td>
<td>0.0000</td>
<td>-</td>
</tr>
<tr>
<td>Safrole</td>
<td>&quot; &quot;</td>
<td>0.11</td>
<td>0.0010</td>
<td>0.91</td>
</tr>
<tr>
<td>&quot;</td>
<td>Safrole 0.8 mM 30 min prior to addition of NADPH</td>
<td>0.17</td>
<td>0.0027</td>
<td>1.59</td>
</tr>
<tr>
<td>&quot;</td>
<td>Safrole 0.8 mM:Naphthalene 0.5 mM added 30 min prior to safrone &amp; NADPH</td>
<td>0.17</td>
<td>0.0040</td>
<td>2.35</td>
</tr>
</tbody>
</table>

* For details of pretreatment see 2.2c
† For definition see Table 7.5.
‡ Mean values: incubations performed in triplicate on pooled microsomal preparations (2 rats per treatment group)
7.4 Discussion

A number of substrates of cytochrome P450 are now known to give rise to species absorbing maximally at 455nm (Franklin 1971, 1974; Schenkman et al., 1972a; Werringloer and Estabrook, 1973) and a potent inhibition of drug metabolism in vitro accompanies the formation of such a species from piperonyl butoxide (Franklin, 1972a). A similar effect in vivo was shown in Chapter 4 with safrole and, in consequence, some in vitro studies have been performed to explore the nature of the interaction of safrole with cytochrome P450.

The addition of relatively low concentrations of certain substrates to microsomes from rats pretreated with safrole brought about a gradual disappearance of the 455nm absorption band which characterises the redox difference spectrum of these microsomes. Changes in magnitude of the 455nm absorption maximum correlated closely with quantitative changes in the cytochrome P450-interaction spectrum of the added substrate. This, together with the appearance of a type III spectrum (Philpot and Hodgson, 1971) when untreated safrole microsomes were compared with the same microsomes after substrate-induced abolition of the 455nm absorption maximum, supports the view that the 455nm maximum derives from a species bound to cytochrome P450 and suggests that the observed spectral changes represent the displacement of this bound species. In support of this conclusion, abolition of the 455nm maximum resulted in an increase of up to 50% in the magnitude of the CO-spectrum of cytochrome P450, inhibition of CO binding being known to accompany formation in vitro of the 455nm absorbing species from piperonyl butoxide (Philpot and Hodgson, 1971).

Nature of Metabolite Binding

The changes in the ethylisocyanide spectrum of safrole microsomes indicate an effect of the bound metabolite rather than the synthesis of a modified form of cytochrome P450 (Shoeman et al., 1969; Sladek and
Mannering, 1969) since they were reversible on displacement of the metabolite and since they could be produced \textit{in vitro}. Piperonyl butoxide elicits changes in the ethylisocyanide spectrum similar to those produced by safrole (Philpot and Hodgson, 1971/72; Skrinjaric-Spoljar et al., 1971). Displacement increased only the 455nm maximum of the ethylisocyanide spectrum. This is interesting since 3-methylcholanthrene pretreatment, which preferentially increases the 455nm ethylisocyanide maximum (Sladek and Mannering, 1969) and which apparently induces a high-spin form of cytochrome P450 (Waterman and Mason, 1972), resulted in the binding of a considerably greater amount, per unit of cytochrome P450, of the species responsible for the (safrole) 455nm absorption maximum. Possibly therefore, the safrole metabolite binds preferentially to a high-spin form of cytochrome P450. Since characteristic changes in the spin state of cytochrome P450 accompany the binding of ligands (Cammer et al., 1966; Jefcoate et al., 1969; Jefcoate and Gaylor, 1969), electron spin resonance spectroscopy should be valuable in investigating further the nature of the binding and displacement of the safrole metabolite.

Increased binding of the two ligands, CO and ethylisocyanide, attendant on abolition of the 455nm absorption maximum, implies that binding of the safrole metabolite involves a direct interaction with the haem iron of cytochrome P450. The concomitant increase in binding of the type II substrate aniline supports the same conclusion, the greater extent of this increase probably being attributable to the presence of the displacing type I substrate (Leibman et al., 1969). This implied inhibition of aniline binding in safrole microsomes need not be contradicted by the observed increases in both the total binding ($\Delta OD_{\text{max}}$) and the affinity ($K_s$) for aniline after safrole pretreatment. These results, calculated on a basis of equal CO-cytochrome P450 concentrations in both the
control and safrole microsomes (see 7.3.2a), may be regarded as informative only of changes in that proportion of the cytochrome P450 in the safrole microsomes not involved in metabolite binding. In piperonyl butoxide treated microsomes, inhibition of the type II binding of pyridine paralleled the inhibition of CO binding (Philpot and Hodgson, 1972).

Studies with biphenyl indicate that binding of the safrole metabolite also involves - or at least affects - the type I substrate binding site. Applying the argument put forward above, recalculation of the results to a basis of equal total cytochrome P450 concentrations, assuming a P450 concentration in the safrole microsomes 40% greater than that determined by the CO spectrum, shows that after administration of safrole for 8 weeks the ΔODmax of both the high and the low affinity biphenyl interaction is greatly reduced, whilst the Ks does not differ greatly from the control. This effect, analogous to non-competitive inhibition, is consistent with an "occupation" of the biphenyl (type I) binding site in a proportion of the cytochrome P450 population and accords with the observed inhibition of biphenyl 4-hydroxylase (Chapter 4) and the reported non-competitive inhibition of type I substrate metabolism produced by piperonyl butoxide (Franklin, 1972a; Friedman et al., 1972).

For the formation in vitro of the 455nm absorbing species from safrole, a requirement for spectrally apparent type I interaction with cytochrome P450 appeared absolute since isooctane extraction of phenobarbitone microsomes completely eradicated this activity. Normal type I substrate metabolism, in contrast, is only slightly decreased by isooctane extraction (Leibman and Estabrook, 1971). Once formed, however, as in safrole microsomes, the 455nm absorption maximum was unaffected by isooctane extraction, suggesting that stable binding of the metabolite is not dependent on that fraction of the microsomal phospholipid associated with
the production of type I spectra: this stability presumably derives from interaction with cytochrome P450 haem. Since iso-octane extraction did not prevent the development, or decrease the magnitude of the displacement-associated type I spectrum, the bound metabolite may stabilise a specific site which, on removal of the metabolite, is capable of interacting with certain type I substrates to produce an enhanced interaction spectrum. The lack of effect of high concentrations of the non-displacing substrate, SKF 525A, on displacement and the associated spectral changes, suggests that such a site differs from the "normal" type I site. However, any such difference(s) must be subtle to allow the enhanced interaction spectrum to resemble so closely a "normal" type I spectrum and to account for an observation that in safrole microsomes the binding of displacing and non-displacing substrates was inhibited to a similar degree.

It may be concluded that generation of the 455nm absorption maximum requires a spectrally detectable type I interaction of safrole with cytochrome P450 and that subsequent metabolite binding involves both a direct interaction with the haem and the type I substrate binding site. Since most type I substrates are chemically unsuitable for direct haem liganding (Imai and Sato, 1967) the requirement for NADPH and O2 must be in the generation of a species sufficiently nucleophilic to undergo such interaction. Measurement of the absolute spectrum of cytochrome P450, which exhibits characteristic changes on interaction of substrates and ligands (Remmer et al., 1969) might provide further evidence as to the nature of the metabolite interaction.

Factors Affecting Displacement

The most striking difference between substrates active and inactive in displacing the bound metabolite was in chemical structure. Whilst all the active substrates were highly lipophilic, several substrates equally,
or more lipophilic were inactive. However, without exception all the (type I) inactive substrates were structurally more complex and sterically "bulky" than the active substrates. The view that, given a certain degree of lipophilicity, activity in displacing the bound metabolite is governed by molecular "size" is supported by observations with the series benzene-hexylbenzene and with the pair of compounds BHA and BHT. In the latter, the presence of an additional tert-butyl group in BHT - despite the attendant increase in lipophilicity - results in complete loss of activity. The steric dimensions of a tert-butyl group are greater than those of an unsubstituted benzene ring. Activity in displacing the bound metabolite appears to be unrelated to the affinity (Ks) of the substrate for cytochrome P450. Thus, the inactive SKF 525A has an affinity some 100-fold greater than that of most displacers and in the alkylbenzene series, affinity increases with increasing alkyl chain length (unpublished observations of K. Al-Galainy, 1973). However, it may be significant that benzene, which was an unexpectedly poor displacer, has an affinity some 10-fold less than that of methyl- or ethylbenzene.

Although none of the type II substrates examined was particularly lipophilic, their inactivity as displacers results more probably from their fundamentally different interaction with cytochrome P450. This is interesting since the bound metabolite appears to interact with the type II (haem) binding site and since certain amphetamine derivatives which give type II interaction spectra can themselves give rise to 455nm absorbing species (Franklin, 1974). A common mechanism for the generation of 455nm absorbing species from both type I and type II compounds is possible since many type II substrates probably interact also with the type I binding site (Gorrod and Temple, 1973; Schenkman, 1970).
Activity or inactivity in displacing the bound metabolite thus appears to be unrelated to ability or inability to give rise to a 455nm absorbing species. Of the active substrates only safrole can do so, whereas amongst the inactive substrates both piperonyl butoxide and SKF 525A can give rise to 455nm absorbing species. Clearly, as implied by the isooctane studies, different interactions are involved in formation and displacement of these species. Some insight into the nature of the binding site involved in displacement might be gained from dimensional analysis of molecular models of active and inactive substrates. Within the present results, differences in rate of displacement between different substrates give no clear indication as to what constitutes a "best fit" in this respect.

Mechanistic Considerations

The molecular events underlying the substrate-induced displacement of the 455nm absorption maximum and the accompanying spectral changes are unknown. The process cannot represent a simple replacement of the bound species by the displacing substrate since type I substrates could not "replace" an interaction with haem, since the accompanying spectral changes were type I in nature and since displacement resulted in reversal of the inhibition of CO and ethylisocyanide binding to cytochrome P450. The increase in CO binding never exceeded 50% and cannot directly account for the 200-350% increase in type I spectral change. Conceivably, all of the cytochrome P450 "unmasked" by displacement might be capable of forming the type I spectral complex as opposed to only the 12% (Ullrich, 1969) able to do so in normal microsomes. That CO itself had no effect on the magnitude of the 455nm absorption maximum suggests a high affinity for the metabolite-haem interaction. However, when the relationship between displacing substrate concentration and rate of displacement was analysed as a double reciprocal plot, the intercept on the x axis, which might be regarded as "the concentration of substrate required to produce half-
maximal rate of displacement", gave a value similar to that of the $K_s$ for many type I substrates - an affinity some 100 times less than that for CO. This reinforces the view that displacing substrates act indirectly rather than by competition for a common binding site. Glycerol decreased the "$V_{\text{max}}"$ without affecting the "$K_m"$ for displacement which suggests that it does not exert a direct effect on the metabolite interaction: possibly its viscosity hinders access of the displacer to the metabolite interaction site. Any such effect of viscosity could be examined with an "inert" agent such as polyvinylpyrrolidine.

If, as proposed by Ullrich and Schnabel (1973), the 455nm absorption maximum reflects the ligand interaction of a carbanion with cytochrome P450, disappearance of the maximum could represent the reprotonation of this species. Such a suggestion is energetically acceptable and could explain the length of time required for displacement since the hydrophobic environment of the bound metabolite would not be conducive to rapid reprotonation. Implicit in this proposal however, is an action of the displacing substrate in precipitating, or greatly accelerating reprotonation, an action difficult to envisage other than as a substrate-induced conformational change resulting in increased hydrophilicity around the bound metabolite. This proposal could be tested by the use of an appropriate fluorescent probe.

**Significance of Displacement**

The displacement process described here has shown that safrole does not interact "irreversibly" with cytochrome P450. Storage of safrole microsomes indicates that even without such external manipulation breakdown of the metabolite-cytochrome P450 complex precedes the destruction of cytochrome P450 itself. The apparent storage stability of cytochrome P450 in safrole microsomes may thus be attributed to a restoration of CO
binding attendant upon dissociation of the metabolite complex. The stability of the metabolite complex in vivo is more difficult to assess since it is unknown whether or not its formation represents a "dead end" in terms of metabolism. The 455nm absorbing species in safrole microsomes appeared to be relatively stable in the presence of NADPH and O₂ suggesting that further mixed function oxidation does not contribute to its breakdown: reprotonation of a carbanion would regenerate the parent compound. It will be necessary to determine the turnover rate of the metabolite complex in vivo before the potential in vivo significance of the displacement process can be assessed.

The relationship between the displacement process and rates of drug metabolism is unclear. An approach to this problem was made by measuring the in vitro metabolism of biphenyl before and after biphenyl-mediated displacement of the bound metabolite. In this experiment an increase of more than 100% in the type I spectrum of biphenyl was accompanied by an increase of only some 10% in the rate of 4-hydroxylation of biphenyl. Thus, substrate-induced displacement may not lead to a restoration of metabolic activity. However, even if this is so, the behaviour of substrates either as displacers or non-displacers appears to be of some importance in view of the effects noted in Chapter 4 where, on chronic administration of safrole, the metabolism of biphenyl was impaired to a much greater extent than that of the non-displacing substrates, ethylmorphine and aniline. It is unlikely, on structural grounds, that the endogenous, physiological substrates of cytochrome P450 would be active as displacers although this possibility merits investigation.

The observations reported in this chapter have been concerned with the interaction of safrole with cytochrome P450. However, the properties of the 455nm absorbing species generated from safrole differ in some
respects from those reported for a number of other compounds. In particular, the ability of NADH, as opposed to NADP, to reduce the safrole-derived 455nm absorbing species might imply that binding of the safrole metabolite produces rather more fundamental changes in cytochrome P450. This might account for the considerable stability of the safrole metabolite complex as compared with that formed, for example from piperonyl butoxide (Lake and Parke, 1972) but it suggests that the phenomena described here may not be entirely reproducible with 455nm absorbing species formed from other compounds.

The mechanism proposed by Ullrich and Schnabel (1973) suggests that many substrates of cytochrome P450 are potentially capable of giving rise to metabolite-cytochrome P450 complexes of the type apparently formed from safrole and recent reports indicate that this is so (Buening and Franklin 1974; Franklin 1974 a,b). Adverse effects might ensue in at least two different ways from an interaction of this nature with cytochrome P450. Firstly, the impairment of drug metabolism resulting from the formation of relatively stable metabolite complexes may have important repercussions both for the metabolism of the parent compound and of other substrates of the drug metabolising enzyme system. The possible significance of this situation in influencing the hepatotoxicity of safrole was considered in Chapter 4. These considerations could also be relevant in the case of a drug, chemically suitable for complex formation and administered in high doses for prolonged periods - particularly in patients receiving multiple drug therapy. Secondly, as pointed out by Ullrich and Schnabel (1973), carbanions formed from some compounds are unstable in the presence of oxygen: these might readily form peroxides which could lead to the
destruction of autooxidisable cell components. Clearly, therefore, further investigations of the nature of metabolite-cytochrome P450 complexes and the factors influencing their formation and breakdown in vivo will be important.
CHAPTER EIGHT

GENERAL DISCUSSION
General Discussion

Despite the frequency with which increased liver weight and drug metabolising enzyme activity are encountered following the exposure of experimental animals to foreign compounds, the interrelationships between these phenomena and the production of liver injury are incompletely understood. The present series of investigations suggests that a key factor influencing the production of both cytopathological and gross pathological changes by compounds causing liver enlargement is the extent and the duration of their effects on drug metabolising enzyme activity.

Significance of Enzyme Induction

Although the precise physiological role of the drug metabolising enzyme system remains uncertain, the importance of its function in the metabolism of lipid-soluble foreign compounds is generally accepted (Brodie 1962; Gelboin et al., 1972) and, indeed, forms the basis for the interpretation of liver enlargement accompanied by increased activity of these enzymes as representing an adaptive response. From the present results, support for this interpretation derives not only from the apparently innocuous character of BHT, which produced persistent induction of the drug metabolising enzymes, but also from the converse situation, namely the ultimate development of pathological changes within the enlarged liver when this was accompanied by only a transitory or weak induction of the drug metabolising enzymes, as in the case of safrole and Ponceau MX.

However, the concept of enzymic adaptation to chemical fluctuations in the environment has a broader basis than the changes elicited by foreign compounds in the rat liver. Thus, the maintenance
of homeostasis in a non-constant environment necessitates, both at cellular and tissue level, the continual regulation and adjustment of the myriad of metabolic processes occurring in the body. Such control operates ultimately via changes in enzyme activity. From early studies with bacteria it is known that an important regulatory mechanism involves the induction or repression of the synthesis of specific enzyme proteins mediated by changes in concentration of either their substrate or reaction product in catabolic and anabolic sequences respectively (Davis, 1961; Jacob and Monod, 1961). A well-known example is the induction of β-galactosidase that follows the transfer of _E. coli_ grown in a lactose-free medium to one containing this sugar as the sole carbon source. This response enables continued survival under otherwise hostile conditions (Jacob and Monod, 1961). Comparable examples are, of necessity, less easy to identify in multicellular organisms but several examples of substrate-mediated enzyme induction are well documented. A case in point is the induction of tryptophan pyrrolase which follows a tryptophan load in rats (Knox, 1962), whilst the transfer of rats from a mixed diet to one high in carbohydrate or protein results in the induction of carbohydrate (Fitch and Chaikoff, 1960) and protein (Schimke et al., 1968) metabolising enzymes respectively. Induction of the drug metabolising enzymes following the intake of foreign compounds appears to be analagous. It differs in being directed specifically towards the removal from the body of substances of no nutrient value rather than to the utilisation of nutrient materials, but in view of the rapidity with which lipid-soluble foreign compounds might otherwise accumulate within the body (cf Butler, 1958), such a function may be equally vital to the well being of the organism.
It is important to recognise, however, that owing to the low substrate specificity of the drug metabolising enzyme system, an increase in its activity may alter the rate of metabolism not only of a wide range of foreign compounds but also of certain physiologically active endogenous substances, notably steroid hormones and vitamin D (Brooks et al., 1972; Hunter, 1974; Janz and Schmidt, 1974; Parke, 1972; Silver, 1974). Moreover, drug metabolism is not always synonymous with drug detoxication: in a number of instances metabolites possess greater reactivity than the parent compound and the hazard from such substances may be increased by prior exposure to enzyme inducing agents (De Matteis et al., 1974; Gillette et al., 1974; Mitchell et al., 1973; Wright & Prescott, 1973). These considerations would indicate that although drug metabolising enzyme induction may be regarded as an adaptive phenomenon in so far as the inducing agent is concerned, there are situations in which it may not be entirely without adverse consequences for the organism.

Significance of Liver Enlargement

Whilst it is well known that induction of drug metabolising enzymes is usually accompanied by enlargement of the liver, it is noteworthy that this change also accompanies the protein (Hurvitz and Freedland, 1968) and carbohydrate (Allen and Leahy, 1966; Bender et al., 1972) mediated enzyme induction referred to above. During pregnancy and lactation in the rat the maternal liver enlarges and this effect, both morphologically and in terms of chemical composition, shares several features in common with the changes occurring in drug-induced liver enlargement (Wilson et al., 1970). The extent of liver enlargement in nephrosis is proportional to the degree of albuminuria and presumably reflects a compensatory stimulation of hepatic albumin...
synthesis (Lipmann et al., 1952; Marsh and Drabkin, 1958). It seems reasonable to conclude, therefore, that in such situations enlargement of the liver is primarily the result of an increased functional capacity (Goss, 1967). The observed temporal and dose-relationships between increases in drug metabolising enzyme activity and liver weight (Argyris and Magnus, 1968; Gilbert and Golberg, 1965; Hoffman et al., 1970, 1971; Seifert and Vacha, 1970) suggest that in the presence of enzyme induction and the absence of histopathological change, drug-induced liver enlargement may also be considered primarily as a consequence of increased functional capacity. The results of the present long-term studies with BHT are consistent with such a view. In fact, the absence of histopathological change and the ready reversibility of the liver enlargement indicates that, although the precise nature of the stimulus remains unknown, the liver growth produced by BHT cannot \textit{per se} be regarded as an adverse effect.

An enlarged but histologically normal liver may also result from exposure to compounds which do not cause an induction of the drug metabolising enzymes. In such circumstances there is no obvious hyperfunctional basis for the liver response since there is no evidence that compounds producing this response are only those whose metabolism is mediated via enzyme systems other than the microsomal drug metabolising enzymes. The latter, on the contrary, are known to play an important role in the metabolism of several such compounds, e.g. safrole (Casida et al., 1966); coumarin (Feuer, 1970; Gibbs et al., 1971) and dieldrin (Parke, 1968). Moreover, pathological changes in the liver are known to occur following the chronic administration of a number of compounds producing liver enlargement in the absence of drug metabolising enzyme induction (see Grasso et al., 1974). It seems clear,
therefore, that liver enlargement in the absence of enzyme induction cannot be regarded as adaptive or hyperfunctional in the sense outlined earlier.

The results obtained with safrole and Ponceau MX support this contention, for in both cases the dissociation of liver enlargement from drug metabolising enzyme induction was paralleled by the appearance of histochemical changes indicative of cytological injury (Grasso et al., 1974). Similarly, cytological evidence of damage involving mitochondrial changes was first noted in the enlarged liver produced by dieldrin at a stage where drug metabolising enzyme activity had fallen to control levels (Hutterer et al., 1969). It is also noteworthy that pathological changes do not develop in the enlarged liver produced by 4-methylcoumarin - a potent inducer of the drug metabolising enzymes (Feuer and Granda 1970; Feuer et al., 1973) - whilst the reverse is true for the parent compound, coumarin.

Such results raise the possibility that the apparent inability of the liver to increase - or to sustain an increase in - its capacity for the metabolism of these compounds may be causally related to their hepatotoxicity. Additional evidence that this may be so derives from dose-response studies with some inducers of the drug metabolising enzymes. In these studies (Gilbert and Golberg 1965; Golberg 1967; Hoffmann et al., 1970, 1971), the degree of liver enlargement and enzyme induction increased linearly with the dose until a level of administration was reached above which no further increases occurred. At this point, the liver concentration of the administered compound rose sharply and symptoms of toxicity were observed. An analogy might be drawn between this situation and that obtaining after the demise of the enzyme induction produced by safrole and dieldrin or that resulting from
the initial absence or inadequacy of enzyme induction as in the case of coumarin and Ponceau MX. Such an argument was advanced by Golberg (1970) but to establish its validity some comparative pharmacokinetic data for inducers and non-inducers of the drug metabolising enzymes, and some knowledge of the mechanisms underlying the absence or failure of this enzyme induction would be necessary. It would also be useful to establish whether or not changes in the activity of extra-hepatic sites of drug metabolism parallel the disturbance of the equilibrium between intake and excretion that may accompany the demise of the enzyme induction produced by safrole. It may be, of course, that the changes - or lack of changes - in drug metabolising enzyme activity play no role in the pathogenesis of the lesions produced by the compounds in question, or that the primary biochemical lesion involves other enzyme systems. It will be of particular interest in this context to determine whether pathological changes develop when these compounds are administered at levels below those necessary to produce liver enlargement. Despite these uncertainties, however, the available evidence suggests that liver enlargement in the absence of drug metabolising enzyme induction may be indicative of incipient hepatotoxicity and is to be contrasted with the 'hyperfunctional' enlargement discussed earlier. Moreover, it is evident that the biochemical and histochemical criteria employed in the present studies may provide a means of distinguishing between these two at an early stage when both present histologically as enlarged but otherwise normal livers.

The possible value of the measurement of induced changes in drug metabolising enzyme activity as an index of hepatotoxicity has recently been suggested (De Matteis et al., 1974; Feuer et al., 1974) and the present results provide some additional support for this view.
However, the biphasic response produced by safrole - and that reported for dieldrin (Hutterer et al., 1969) - emphasises that very short observation periods as advocated by De Matteis et al. and Feuer et al. may give rise to misleading results and clearly illustrates the fallacy of the assumption made by Platt and Cockrill (1967) that the changes they observed after 14 days would be representative of the changes occurring after chronic administration. Furthermore, it is possible that the conclusions drawn in the preceding discussion may apply only to the rat. Several agents known to produce sustained stimulation of drug metabolism in mouse liver, e.g. phenobarbitone and dieldrin, do give rise to gross pathological changes in this organ (Thorpe & Walker, 1973; Wright et al., 1972). Although the metabolism of dieldrin, for example, is known to be qualitatively different in the mouse and the rat (Baldwin et al., 1972), it remains to be seen whether the type of results obtained in the present study can be reproduced in other species less prone to the development of liver pathology than the mouse.

Hepatic Nodules

In addition to histopathological changes in the liver, including fatty change, hydropic degeneration and cell necrosis, both safrole and Ponceau MX gave rise to proliferative lesions appearing grossly as hepatic nodules. Such lesions have been regarded by some pathologists as neoplastic (Bonser and Roe, 1970; Ikeda et al., 1966) but by others as hyperplastic (Grasso 1970; Grasso et al., 1969). In this context it is important to note that cell necrosis was observed prior to the appearance of the nodules induced by safrole and Ponceau MX. A parallel might therefore be drawn between this situation and the induction of
cirrhosis by carbon tetrachloride in the rat (McLean et al., 1969; Hartroft, 1964) in which case cell necrosis is evident several weeks before the development of cirrhotic nodules. In this experimental model the nodules vary in size but are uniformly distributed throughout all lobes of the liver and accompanied by fibrosis. In contrast, the nodules induced by safrole and Ponceau MX were selectively sited in the major lobes, were few in number and were not usually accompanied by extensive fibrosis. The reasons for the selective siting of these lesions are not known. It seems significant, however, that the cell degeneration produced by Ponceau MX and safrole was irregularly distributed whereas in the case of carbon tetrachloride, cell necrosis occurs with regularity in all lobules. These considerations suggest that the nodules induced by safrole and Ponceau MX represent a reactive hyperplasia and possess a pathological significance which is not dissimilar to that of cirrhotic nodules. As with cirrhotic nodules in the human (Willis, 1967), the hyperplastic nodules in the rat are prone to the development of neoplasia, although this event appears to be rare in the case of Ponceau MX (Grasso et al., 1975). The majority of the nodules appeared to be the result of controlled growth, both because of the uniform arrangement of the cells in cord-like structures resembling normal liver, and because the mitotic activity appeared, in general, to be of the same order as that seen in untreated controls. Nevertheless, the heterogeneous enzyme histochemical appearance of the nodules suggests that they comprise a number of differing cell populations. The possibility cannot be excluded that the enzyme-deficient foci within the nodules may be in some way analogous with those observed at an early stage in the livers of rats treated with several established hepatocarcinogens (Goldfarb, 1973; Kitigawa and Sugano, 1973; Scherer et al., 1972).
The significance of the biochemical changes observed in the nodules induced by safrole and Ponceau MX is not clear. In the earliest Ponceau MX nodules studied (56 weeks) however, the apparent inverse relationship between drug metabolising enzyme activity in the nodule and in the adjacent liver is suggestive of a biochemically 'regenerative' role for the nodules. This would imply that the development of nodules represents a response not only to the total loss of functional units (i.e. the cell necrosis referred to above) but also to the reduced functional efficiency of the remaining units. Although no such inverse relationship was observed at weeks 66, 70 and 80, the fairly consistently higher substrate metabolising capacity of the Ponceau MX nodules in comparison with the adjacent liver is equally consistent with a 'regenerative' function and contrasts with the very low levels of drug metabolising enzyme activity reported for a number of rat liver tumours (see below). It is noteworthy, however, that rapidly growing non-neoplastic liver tissue e.g. in the neonate (see review by Done, 1964) or following partial hepatectomy (Fouts et al., 1961) is also characterised by reduced levels of drug metabolising enzyme activity. The implication, therefore, is that the nodules (at least those induced by Ponceau MX) represent a fairly stable (i.e. non-dividing) cell population and this is supported both by the histological evidence discussed earlier and by the biochemical results which show little change in the characteristics of the nodules in terms of their drug metabolising enzyme activity between weeks 56-80 (see Tables 5.4-5.6).

The above considerations must be tempered by the fact that when expressed on a per gram tissue basis, the substrate metabolising capacity of the nodules was not appreciably greater - and in the case of the safrole nodules was in fact less than that of the adjacent liver.
Moreover, certain components of the drug metabolising enzyme system were present at very low levels in the nodules, resulting in a departure from the normal quantitative interrelationships between different compounds of the system. Whether or not these quantitative abnormalities lead to qualitative differences in the manner in which foreign compounds are metabolised in the nodule has not been resolved. If this were the case, it is possible that alteration of the metabolism of the nodule-inducing compound might result in the increased production of a carcinogenically active derivative, thereby predisposing the nodule to the development of cancer. Another factor likely to influence the drug metabolic capacity of the nodule is the blood supply it receives, for this will govern the bioavailability within the nodule of the agent responsible for its induction. It would be of interest, therefore, to determine whether the blood supply to the nodules is atypical either in terms of total flow rate or in the ratio between its venous and arterial afferent components. For a better assessment of the functional significance of the nodules, a method for gauging their metabolic activity in vivo is clearly necessary. In this context a useful clue might be provided by 2-acetylaminofluorene (AAF) which binds to cellular macromolecules only after two activation steps, one involving the drug metabolising enzyme system and the other occurring in the cytosol. Thus, nodule-bearing rats could be administered with suitably labelled AAF and nodular and non-nodular liver subsequently analysed for covalently bound fluorene residues. The value of such an approach would, of course, be reduced if there were qualitative differences between nodule and adjacent liver in their metabolism of AAF.
It was not possible to make a direct comparison of the safrole- and Ponceau MX-induced liver nodules with frankly neoplastic liver. However, as mentioned in 6.1, the hyperplastic liver nodules induced by AAF have been implicated as direct precursors of the malignant liver tumours produced by this compound. Such nodules differed from those produced by safrole and Ponceau MX both in the time of their appearance (8-16 weeks vs. 45-65 weeks) and in their biochemical and morphological characteristics. In both the nodular and the adjacent non-nodular liver from AAF-treated rats, all components of the drug metabolising enzyme system were present at markedly reduced levels - expressed either on a per gram tissue or on a specific activity basis. The contrast between this situation and that obtaining in Ponceau MX-induced nodules is illustrated by Fig. 8.1. This apparent lack of differential between nodular and adjacent non-nodular liver in the AAF-treated rats probably reflects the fact that in these animals, the liver adjacent to the large nodules was itself largely composed of microscopic areas of hyperplasia.

Existing publications indicate that drug metabolising enzyme activity in a number of tumour systems, including several so-called minimal deviation hepatomas, is either very low or absent (Adamson and Fouts, 1961; Brown et al., 1971; Hart et al., 1965; Neubert and Hoffmeister, 1960). Thus the results obtained with the AAF-induced hyperplastic nodules appear to resemble those reported for neoplastic liver. On the other hand, the high specific activity of several pathways of drug metabolism in the Ponceau MX nodules and the apparent preservation in the safrole nodules of the unusual interrelationships between components of the drug metabolising enzyme system typically produced in 'normal' liver by safrole, are in
Comparison of Some Biochemical Characteristics of Hyperplastic Liver Nodules Induced by AAF or Ponceau MX

Means of 6 values for liver nodules and adjacent non-nodular liver are expressed as % of values for liver from untreated control rats. Dashed line (100%) represents control levels. A = Units per g tissue B = Units per mg microsomal protein
PONCEAU MX

% CONTROL

ANILINE 4-HYDROXYLASE

BIPHENYL 4-HYDROXYLASE

CYTOCHROME P450

MICROSOMAL PR
contrast with such results. Morphologically, the nodules produced by each of the compounds were similar in so far as all were composed of relatively well ordered, enlarged hepatocytes. However, mitotic figures were prominent in the AAF nodules and in the male rats, the cells of those nodules contained enlarged vesicular nuclei. Although their precise significance is uncertain, similar nuclear changes are produced in rat liver by a number of hepatocarcinogens (Jackson 1974; Rouiller, 1964).

Overall, the results obtained might be construed as evidence that the liver nodules induced by safrole and Ponceau MX and those induced by AAF are entities of fundamentally different biological significance. In particular, the difference in their respective times of appearance, both in absolute terms and in relation to the appearance of other pathological changes in the liver (see Table 8.1), support this construction. With regard to the biochemical results, however, it is recognised that measurements of absolute levels of enzyme activity may bear little relation to the degree to which these nodular lesions deviate from normal liver (Wu, 1967). A potentially more valuable approach would involve the study of metabolic regulation within the nodules since loss of normal regulatory function is a characteristic of neoplastic tissue (Pitot, 1966). The inducibility of drug metabolising enzymes has been studied in a number of tumour systems. Conney and Burns (1963) demonstrated induction of azo-dye N-demethylase by 3-methylcholanthrene in Morris hepatoma but no such response could be evoked in primary azo-dye-induced hepatomas (Conney et al., 1956). According to Hart et al. (1965), phenobarbitone elicited increases in drug metabolising enzyme activity in a series of highly differentiated hepatocellular carcinomas, although these increases were much smaller than in the surrounding liver tissue and the
metabolism of several substrates remained unaffected. It would be of interest to extend this type of observation to the nodules induced by safrole and Ponceau MX and to compare the results so obtained with those of parallel observations on AAF-induced hyperplastic nodules and hepatocellular carcinomas. In the present study, drug metabolising enzyme activity in the nodules was in fact measured in the presence of an effector of the system, i.e. the test compound. The possible significance of this might have been gauged by comparing levels of drug metabolising enzymes in nodules from rats maintained on the test compound until immediately prior to determination of enzyme activity, with those in rats allowed a prior period of recovery on control diet.

However, a characterisation of the biochemical properties of the safrole-and Ponceau MX-induced nodules must clearly have a broader basis than studies on the drug metabolising enzyme system. Other aspects of liver function require investigation. Information on the regulation of intermediary metabolism would be valuable particularly since data of this type has been collected for a number of tumour systems (see Pitot, 1966) and for the AAF-induced hyperplastic nodules (see Farber, 1973b). In the specific case of the latter, Epstein et al. (1967) have reported that glycogen in the nodule cells is relatively more stable both to a 24 hour fast and to the administration of glucagon than in the cells of the surrounding parenchyma. On the other hand, Teebor and Seidman (1970) found that whilst the specific activity of glucose-6-phosphatase was lower in 12-week nodules than in the surrounding liver, the percentage induction of activity following a 24 hour fast or the administration of triamcinolone was very similar in both tissues. In the interpretation of such results the duration of the AAF pretreatment is likely to be a critical factor.
A number of other known properties of the AAF-induced nodules might afford a basis for comparisons of these nodules with those induced by safrole and Ponceau MX. Thus, Epstein et al. (1969/70) have reported that DNA in the cells of the nodules shows a number of abnormalities not present in DNA from the surrounding liver parenchyma. Slifkin et al. (1972) found that cells from the nodules, in contrast to those in the adjacent parenchyma, could be successfully grown in vitro. Transplantation of the AAF nodules is not possible until islands of malignancy can be seen histologically within the nodules (Farber, 1973b; Reuber and Firminger, 1963): nothing is known of the transplantability of the safrole and Ponceau MX nodules at any stage in their development. Finally, it has recently been shown that a specific antigen is reproducibly associated with the liver nodules induced by AAF but not with the surrounding parenchyma or with proliferating liver tissue following partial hepatectomy (Farber, 1974). Although the significance of this remains to be established, it would be of interest to determine whether any immunological changes are associated with the development of nodules in the livers of rats treated with safrole or Ponceau MX.

Despite the limitations of the present study, the results obtained point to certain major differences in the characteristics of the liver nodules induced by safrole and Ponceau MX and those induced by the hepatocarcinogen, AAF (see Table 8.1). These differences exist not only at the pathological and biochemical level but also in the behaviour of the nodules after withdrawal of the test compound. Thus, it is known that despite cessation of treatment, quite a high proportion of the early hyperplastic nodules induced by AAF progress to frank malignancy (Epstein et al., 1967; Teebor and Becker, 1971).
## Summary of Hepatic Changes Produced by Safrole, Ponceau MX and AAF

<table>
<thead>
<tr>
<th></th>
<th>Safrole/Ponceau MX</th>
<th>AAF (Male)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Liver enlargement</td>
<td>+</td>
<td>-</td>
</tr>
<tr>
<td>Induction of drug metabolising enzymes</td>
<td>+ then -</td>
<td>-</td>
</tr>
<tr>
<td>Histopathological changes</td>
<td>+</td>
<td>+</td>
</tr>
<tr>
<td>Time of appearance of nodules</td>
<td>45-65 weeks</td>
<td>8-16 weeks</td>
</tr>
<tr>
<td>Time of appearance in relation to other histopathological changes</td>
<td>after/concurrent</td>
<td>before/concurrent</td>
</tr>
<tr>
<td>Drug metabolising enzyme activity in nodules</td>
<td>high</td>
<td>low</td>
</tr>
<tr>
<td>Nuclear changes in nodules</td>
<td>-</td>
<td>+</td>
</tr>
<tr>
<td>Mitotic figures in nodules</td>
<td>rare</td>
<td>frequent</td>
</tr>
<tr>
<td>Histology of nodules</td>
<td>regular</td>
<td>regular</td>
</tr>
</tbody>
</table>
On the contrary, the nodules induced by Ponceau MX - which in any case appear at a much later stage - do not appear to progress (Grasso et al. 1975). No similar studies on progression have been reported for safrole-induced nodules but their similarity to the Ponceau MX nodules, both biochemically and pathologically, suggests that their behaviour would resemble more probably these nodules rather than those induced by AAF.

Since pathological changes in the liver are produced both by AAF and by safrole and Ponceau MX, the significance of these changes in the development of tumours is difficult to discern. However, it is known that, on cessation of administration, AAF-induced pathological changes (apart from the nodules) regressed whereas the nodules progressed to malignancy (Reuber, 1965). In this case therefore, the possibility arises that malignant transformation may be independent of the other pathological changes produced. This appears less likely in the case of safrole and Ponceau MX. With these compounds, the early biochemical evidence of impaired functional capacity and the subsequent appearance of histopathological changes suggests that the ultimate development of nodules may represent a response to chronic, toxic liver injury. In such a situation it should be possible to establish a 'no-effect' level which would preclude the development of these nodules. Furthermore, if, as appears to be the case, the development of nodules is a necessary prerequisite for the development of tumours, then the same 'no-effect' level would also preclude the production of tumours. Further experimental work is clearly required to establish more firmly this proposed sequence of events. If this were established it would indicate that the neoplasia induced by safrole and Ponceau MX arises indirectly rather than as a consequence of 'chemical carcinogenesis' in the sense in which this term is commonly used. Such a distinction in the mechanisms by which chemicals induce neoplasia is envisaged in a recent publication from the W.H.O. (1974).
Key to Tables

This section contains the data from the long-term feeding studies on which were based Figs. 3.2, 4.2, 5.2, 6.3 and 6.4 and Tables 3.3, 4.3, 5.3 and 6.3. Included also are the data which formed the basis of Tables 4.4, 5.4 - 5.6 and 6.4 relating to the liver nodules induced by safrole, Ponceau MX and 2-acetylaminofluorene.

Unless stated otherwise, all values represent the mean and standard error of the mean of observations on groups of 4 rats. Significant differences between values for control and treated groups are indicated as follows: - a = p<0.05, b = p<0.01 and c = p<0.001. Values not so marked do not differ significantly at the 5% (p<0.05) level. In Tables A6 - A10, where values for liver nodule tissue are compared both to values for adjacent, non-nodular liver and for untreated control liver, the suffix ns is used to indicate means not differing significantly from each other.

The following units are used throughout: -

Body weight - g
Liver weight - g
Relative liver weight - g liver/100 g body weight
Succinate dehydrogenase - µmol INT reduced/g liver/minute
Glucose-6-phosphate dehydrogenase - µmol NADP reduced/g liver/minute
Ethylmorphine N-demethylase - µmol formaldehyde produced/g liver/hour
Aniline 4-hydroxylase - µmol 4-aminophenol produced/g liver/hour
Biphenyl 4-hydroxylase - µmol 4-hydroxybiphenyl produced/g liver/hour

continued overleaf...
Biphenyl 2-hydroxylase - nmol 2-hydroxybiphenyl produced/g liver/hour
NADPH-cytochrome c reductase - μmol cytochrome c reduced/g liver/minute
Cytochrome P450 - nmol/g liver
Cytochrome b5*- nmol/ g liver
455nm absorption maximum - ΔOD 455-490nm/g liver or /g microsomal protein
Microsomal protein - mg/g liver
Total liver protein - mg/g liver
**TABLE A1**
Measurements of Some Drug Metabolising Enzymes and Related Parameters at Intervals
During the Chronic Administration of BHT to Female Rats

<table>
<thead>
<tr>
<th>Parameter</th>
<th>Control</th>
<th>BHT</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Liver Weight</strong></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>3.86 ± 0.15</td>
<td>5.41 ± 0.10</td>
</tr>
<tr>
<td>BHT</td>
<td>5.06 ± 0.38</td>
<td>8.02 ± 0.57</td>
</tr>
<tr>
<td>Relative Liver Weight</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.94 ± 0.06</td>
<td>2.38 ± 0.05</td>
</tr>
<tr>
<td>BHT</td>
<td>3.95 ± 0.10</td>
<td>3.53 ± 0.18</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>13.7 ± 1.3</td>
<td>12.5 ± 0.7</td>
</tr>
<tr>
<td>BHT</td>
<td>26.7 ± 1.1</td>
<td>19.4 ± 0.4</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>1.45 ± 0.07</td>
<td>1.25 ± 0.20</td>
</tr>
<tr>
<td>BHT</td>
<td>1.61 ± 0.13</td>
<td>1.87 ± 0.10</td>
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<tr>
<td>Biphenyl 4-hydroxylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>2.32 ± 0.10</td>
<td>2.16 ± 0.09</td>
</tr>
<tr>
<td>BHT</td>
<td>4.43 ± 0.16</td>
<td>4.15 ± 0.13</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.11 ± 0.01</td>
<td>0.03 ± 0.01</td>
</tr>
<tr>
<td>BHT</td>
<td>0.23 ± 0.00</td>
<td>0.14 ± 0.01</td>
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</table>

*Note: b, c indicate significant differences from control values.*
<table>
<thead>
<tr>
<th></th>
<th>Control</th>
<th>BHT</th>
<th>Control</th>
<th>BHT</th>
<th>Control</th>
<th>BHT</th>
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<th>BHT</th>
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<th>Control</th>
<th>BHT</th>
<th>Control</th>
<th>BHT</th>
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</thead>
<tbody>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>1.01 ± 0.10</td>
<td>1.35 ± 0.07</td>
<td>1.40 ± 0.08</td>
<td>1.68 ± 0.06</td>
<td>1.29 ± 0.08</td>
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<tr>
<td></td>
<td>1.33 ± 0.05</td>
<td>2.00 ± 0.15</td>
<td>2.20 ± 0.09</td>
<td>2.15 ± 0.07</td>
<td>2.20 ± 0.19</td>
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<tr>
<td>Cytochrome P450</td>
<td>21.3 ± 1.0</td>
<td>20.1 ± 1.1</td>
<td>20.5 ± 1.5</td>
<td>24.6 ± 1.0</td>
<td>24.5 ± 1.0</td>
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<td></td>
<td>38.8 ± 0.6</td>
<td>38.9 ± 0.7</td>
<td>38.7 ± 2.3</td>
<td>40.9 ± 1.5</td>
<td>49.9 ± 2.6</td>
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<tr>
<td>Cytochrome b5</td>
<td>11.6 ± 0.6</td>
<td>11.7 ± 0.7</td>
<td>14.0 ± 0.6</td>
<td>15.7 ± 0.1</td>
<td>15.7 ± 0.4</td>
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<tr>
<td></td>
<td>20.7 ± 0.5</td>
<td>27.6 ± 1.1</td>
<td>27.0 ± 1.2</td>
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<td>29.6 ± 1.3</td>
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<tr>
<td>Microsomal protein</td>
<td>21.6 ± 0.5</td>
<td>21.2 ± 0.8</td>
<td>26.0 ± 0.5</td>
<td>28.9 ± 0.5</td>
<td>29.3 ± 1.5</td>
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<tr>
<td></td>
<td>32.3 ± 0.5</td>
<td>33.7 ± 1.4</td>
<td>34.3 ± 1.7</td>
<td>35.4 ± 1.0</td>
<td>38.5 ± 2.1</td>
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<tr>
<td>Succinate dehydrogenase</td>
<td>4.88 ± 0.20</td>
<td>4.25 ± 0.56</td>
<td>5.41 ± 0.18</td>
<td>4.99 ± 0.12</td>
<td>4.87 ± 0.19</td>
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<td>3.80 ± 0.28</td>
<td>3.00 ± 0.40</td>
<td>4.18 ± 0.21</td>
<td>3.44 ± 0.23</td>
<td>3.73 ± 0.25</td>
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<tr>
<td>Glucose-6-phosphate dehydrogenase</td>
<td>5.60 ± 0.53</td>
<td>5.92 ± 0.83</td>
<td>4.87 ± 0.08</td>
<td>5.13 ± 0.58</td>
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<td></td>
<td>5.05 ± 0.20</td>
<td>3.81 ± 0.09</td>
<td>3.73 ± 0.05</td>
<td>3.27 ± 0.03</td>
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<tr>
<td>Total protein</td>
<td>225 ± 1</td>
<td>221 ± 3</td>
<td>235 ± 3</td>
<td>206 ± 7</td>
<td>208 ± 0</td>
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<td>209 ± 1</td>
<td>192 ± 5</td>
<td>239 ± 3</td>
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Measurements of Some Drug Metabolising Enzymes and Related Parameters at Intervals During the Chronic Administration of 2-Acetylaminofluorene (AAF) to Male Rats*

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<td>0.21 ± 0.05</td>
<td>0.02 ± 0.00</td>
<td>0.02 ± 0.00</td>
</tr>
<tr>
<td>AAF</td>
<td>0.29 ± 0.06</td>
<td>0.09 ± 0.00</td>
<td>0.09 ± 0.01</td>
</tr>
<tr>
<td><strong>NADPH-Cytochrome c reductase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>0.68 ± 0.03</td>
<td>1.25 ± 0.04</td>
<td>1.24 ± 0.06</td>
</tr>
<tr>
<td>AAF</td>
<td>0.67 ± 0.04</td>
<td>1.21 ± 0.04</td>
<td>1.02 ± 0.07</td>
</tr>
<tr>
<td><strong>Cytochrome P450</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>19.0 ± 2.6</td>
<td>29.1 ± 2.3</td>
<td>34.8 ± 2.1</td>
</tr>
<tr>
<td>AAF</td>
<td>14.9 ± 2.0</td>
<td>17.5 ± 0.4</td>
<td>14.2 ± 0.8</td>
</tr>
<tr>
<td><strong>Cytochrome b5</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>10.6 ± 0.7</td>
<td>13.5 ± 0.3</td>
<td>17.1 ± 1.1</td>
</tr>
<tr>
<td>AAF</td>
<td>14.3 ± 0.9</td>
<td>12.7 ± 0.3</td>
<td>14.2 ± 0.6</td>
</tr>
<tr>
<td><strong>Microsomal protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>29.1 ± 1.5</td>
<td>31.3 ± 1.1</td>
<td>29.0 ± 1.0</td>
</tr>
<tr>
<td>AAF</td>
<td>23.2 ± 0.5</td>
<td>25.6 ± 0.6</td>
<td>24.9 ± 0.8</td>
</tr>
<tr>
<td><strong>Succinate dehydrogenase</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>5.52 ± 0.51</td>
<td>4.76 ± 0.37</td>
<td>4.44 ± 0.11</td>
</tr>
<tr>
<td>AAF</td>
<td>5.31 ± 0.45</td>
<td>4.28 ± 0.07</td>
<td>3.04 ± 0.13</td>
</tr>
<tr>
<td><strong>Total protein</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Control</td>
<td>244 ± 17</td>
<td>220 ± 3</td>
<td>209 ± 4</td>
</tr>
<tr>
<td>AAF</td>
<td>218 ± 14</td>
<td>198 ± 4</td>
<td>178 ± 5</td>
</tr>
</tbody>
</table>

See also Tables A10 and A11


TABLE A6

Measurements of Some Drug Metabolising Enzymes and Related Parameters in Liver from Control Rats and in Non-Nodular and Nodular Liver from Rats Given a Diet Containing 0.25% Safrole for 85 Weeks

Values are means from 4 control and 6 treated rats and nodular tissue from 5 of the treated rats.

<table>
<thead>
<tr>
<th></th>
<th>CONTROL</th>
<th>NON-NODULAR</th>
<th>NODULAR</th>
<th>CONTROL</th>
<th>NON-NODULAR</th>
<th>NODULAR</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>16.2 ± 1.8</td>
<td>17.2 ± 1.9 ns</td>
<td>9.6 ± 1.6aa</td>
<td>617 ± 58</td>
<td>515 ± 69ns</td>
<td>424 ± 33aa</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>1.78 ± 0.10</td>
<td>1.30 ± 0.14b</td>
<td>0.96 ± 0.06b,ns</td>
<td>68.8 ± 5.0</td>
<td>39.8 ± 6.8a</td>
<td>44.8 ± 5.1a</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>2.90 ± 0.06</td>
<td>2.88 ± 0.57ns</td>
<td>2.57 ± 0.15ns,ns</td>
<td>112 ± 2</td>
<td>90 ± 22ns</td>
<td>120 ± 12ns</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>0.01 ± 0.00</td>
<td>0.09 ± 0.01c</td>
<td>0.07 ± 0.01c,ns</td>
<td>0.42 ± 0.04</td>
<td>2.80 ± 0.37c</td>
<td>3.09 ± 0.20</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>0.67 ± 0.05</td>
<td>1.55 ± 0.18b</td>
<td>0.92 ± 0.18a,b</td>
<td>25.5 ± 2.1</td>
<td>44.4 ± 2.8b</td>
<td>40.3 ± 4.0a</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>19.0 ± 0.9</td>
<td>26.4 ± 1.0b</td>
<td>6.5 ± 0.6c,c</td>
<td>0.74 ± 0.04</td>
<td>0.78 ± 0.06ns</td>
<td>0.30 ± 0.02</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>11.1 ± 0.6</td>
<td>33.4 ± 2.0c</td>
<td>11.1 ± 1.3ns,c</td>
<td>0.43 ± 0.02</td>
<td>0.97 ± 0.03c</td>
<td>0.50 ± 0.01</td>
</tr>
<tr>
<td>455nm Absorption Maximum</td>
<td>-</td>
<td>0.680 ± 0.087</td>
<td>0.108 ± 0.011c</td>
<td>-</td>
<td>1.59 ± 0.19</td>
<td>0.40 ± 0.05</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>26.1 ± 0.9</td>
<td>34.5 ± 2.3a</td>
<td>22.2 ± 2.3ns,b</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Total protein</td>
<td>235 ± 5</td>
<td>225 ± 7ns</td>
<td>217 ± 2 b,ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>4.44 ± 0.32</td>
<td>4.07 ± 0.39ns</td>
<td>4.05 ± 0.28ns,ns</td>
<td>-</td>
<td>-</td>
<td>-</td>
</tr>
</tbody>
</table>

* Compared statistically to values for control liver
+ ' ' ' ' ' ' ' non-nodular liver
### TABLE A7

Measurements of Some Drug Metabolising Enzymes and Related Parameters in Liver from Control Rats and in Non-Nodular and Nodular Liver Tissue from Rats Given a Diet Containing 1% Ponceau MX for 66 Weeks

Values are means from 5 control and 5 treated rats and nodular tissue from 4 of the treated rats.

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>CONTROL</th>
<th>NON-NODULAR</th>
<th>NODULAR</th>
<th>Units per mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td>CONTROL</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>14.3 ± 1.9</td>
<td>13.5 ± 1.0ns</td>
<td>13.6 ± 3.0ns,n</td>
<td>531 ± 57</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>1.76 ± 0.07</td>
<td>1.04 ± 0.06c</td>
<td>1.25 ± 0.27ns,ns</td>
<td>66.1 ± 5.5</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>2.42 ± 0.05</td>
<td>2.06 ± 0.13ns</td>
<td>2.85 ± 0.61ns,ns</td>
<td>90.7 ± 3.8</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>0.019± 0.000</td>
<td>0.032± 0.010ns</td>
<td>0.043± 0.013ns,ns</td>
<td>0.71± 0.03</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>0.73 ± 0.06</td>
<td>0.65 ± 0.04ns</td>
<td>0.44 ± 0.07a,a</td>
<td>27.0 ± 1.1</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>21.6 ± 1.8</td>
<td>22.0 ± 1.2ns</td>
<td>10.5 ± 1.7b,c</td>
<td>0.81± 0.06</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>12.8 ± 1.4</td>
<td>15.7 ± 1.2ns</td>
<td>7.6 ± 1.5ns,b</td>
<td>0.47± 0.03</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>26.8 ± 1.2</td>
<td>22.5 ± 0.9a</td>
<td>13.5 ± 1.2c,c</td>
<td>-</td>
</tr>
<tr>
<td>Total protein</td>
<td>202 ± 1</td>
<td>187 ± 1c</td>
<td>162 ± 4c,c</td>
<td>-</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>5.82 ± 0.23</td>
<td>5.44 ± 0.03ns</td>
<td>3.67 ± 0.24c,c</td>
<td>-</td>
</tr>
</tbody>
</table>

* Compared statistically to values for control liver
† " " " " " " non-nodular liver
## TABLE A8

Measurements of Some Drug Metabolising Enzymes and Related Parameters in Liver from Control Rats and in Non-Nodular and Nodular Liver Tissue from Rats Given a Diet Containing 1% Ponceau MX for 70 Weeks

Values are means from 4 control and 6 treated rats and nodular tissue from each of the treated rats

<table>
<thead>
<tr>
<th></th>
<th>Units per g liver</th>
<th>Units per mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>NON-NODULAR</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>23.9 ± 3.1</td>
<td>18.2 ± 1.8ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>1.69 ± 0.10</td>
<td>0.99 ± 0.09b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>1.99 ± 0.19</td>
<td>1.73 ± 0.14ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>0.02 ± 0.00</td>
<td>0.06 ± 0.01b</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>NADPH-cytochrome c reductase</td>
<td>0.98 ± 0.06</td>
<td>0.79 ± 0.06ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>18.5 ± 1.1</td>
<td>17.7 ± 1.2ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Cytochrome b₅</td>
<td>11.0 ± 1.3</td>
<td>14.2 ± 1.3ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>34.3 ± 0.5</td>
<td>30.9 ± 1.3ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Total protein</td>
<td>221 ± 6</td>
<td>245 ± 6a</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>5.07 ± 0.19</td>
<td>4.45 ± 0.31ns</td>
</tr>
<tr>
<td></td>
<td></td>
<td></td>
</tr>
</tbody>
</table>

* Compared statistically to values for control liver

† " " " " " " non-nodular liver
**TABLE A9**

Measurements of Some Drug Metabolising Enzymes and Related Parameters in Liver from Control Rats and in Non-Nodular and Nodular Liver Tissue from Rats Given a Diet Containing 1% Ponceau MX for 80 Weeks

Values are means from 4 control and 6 treated rats and nodular liver tissue from 4 of the treated rats

<table>
<thead>
<tr>
<th>Enzyme/Parameter</th>
<th>Units per g liver</th>
<th>Units per mg microsomal protein</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>CONTROL</td>
<td>NON-NODULAR</td>
</tr>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>19.3 ± 2.1</td>
<td>17.1 ± 1.1ns*</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>1.83 ± 0.08</td>
<td>1.24 ± 0.10c</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>4.29 ± 0.24</td>
<td>3.03 ± 0.17b</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>0.07 ± 0.01</td>
<td>0.12 ± 0.01a</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>1.23 ± 0.03</td>
<td>0.93 ± 0.13a</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>23.8 ± 1.3</td>
<td>24.2 ± 1.6ns</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>18.8 ± 0.8</td>
<td>19.6 ± 1.5ns</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>32.8 ± 1.5</td>
<td>30.2 ± 1.1ns</td>
</tr>
<tr>
<td>Total protein</td>
<td>206 ± 7</td>
<td>182±5a</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>5.13 ± 0.18</td>
<td>437 ± 0.21a</td>
</tr>
</tbody>
</table>

* Compared statistically to values for control liver

† " " " " " " non-nodular liver
TABLE A10

Measurements of Some Drug Metabolising Enzymes and Related Parameters in Liver from Control Rats and in Non-Nodular and Nodular Liver from Male Rats Given a Diet Containing 0.06% 2-Acetylaminofluorene for 22 Weeks

Values are means from 6 control and 6 treated rats and nodular tissue from each of the treated rats

<table>
<thead>
<tr>
<th>Enzyme</th>
<th>Control (Units per g liver)</th>
<th>Non-Nodular (Units per g liver)</th>
<th>Nodular (Units per mg microsomal protein)</th>
</tr>
</thead>
<tbody>
<tr>
<td>Ethylmorphine N-demethylase</td>
<td>41.0 ± 1.5</td>
<td>11.2 ± 2.5*</td>
<td>15.6 ± 2.4*, ns</td>
</tr>
<tr>
<td>Aniline 4-hydroxylase</td>
<td>3.03 ± 0.13</td>
<td>0.74 ± 0.05c</td>
<td>0.48 ± 0.07c, a</td>
</tr>
<tr>
<td>Biphenyl 4-hydroxylase</td>
<td>4.04 ± 0.12</td>
<td>2.24 ± 0.21c</td>
<td>2.07 ± 0.08c, ns</td>
</tr>
<tr>
<td>Biphenyl 2-hydroxylase</td>
<td>0.05 ± 0.01</td>
<td>0.06 ± 0.00ns</td>
<td>0.06 ± 0.05ns, ns</td>
</tr>
<tr>
<td>NADPH-Cytochrome c reductase</td>
<td>0.99 ± 0.08</td>
<td>0.57 ± 0.03b</td>
<td>0.59 ± 0.02b, ns</td>
</tr>
<tr>
<td>Cytochrome P450</td>
<td>30.8 ± 0.9</td>
<td>8.5 ± 0.4c</td>
<td>7.3 ± 0.7c, ns</td>
</tr>
<tr>
<td>Cytochrome b5</td>
<td>16.6 ± 1.3</td>
<td>8.8 ± 0.7b</td>
<td>8.6 ± 0.4b, ns</td>
</tr>
<tr>
<td>Microsomal protein</td>
<td>32.6 ± 1.3</td>
<td>20.8 ± 1.3c</td>
<td>21.6 ± 1.1c, ns</td>
</tr>
<tr>
<td>Total protein</td>
<td>248 ± 5</td>
<td>198 ± 4c</td>
<td>202 ± 4c, ns</td>
</tr>
<tr>
<td>Succinate dehydrogenase</td>
<td>5.53 ± 0.19</td>
<td>3.48 ± 0.21c</td>
<td>3.62 ± 0.22c, ns</td>
</tr>
</tbody>
</table>

* Compared statistically to values for control liver

† " " " " " non-nodular liver
### Table A11

**Liver Weights of Rats Killed for Biochemical Studies on Liver Nodules (Tables A6-A10)**

<table>
<thead>
<tr>
<th></th>
<th>Liver Weight</th>
<th>Relative Liver Weight</th>
</tr>
</thead>
<tbody>
<tr>
<td><strong>Control</strong></td>
<td>7.42 ± 0.78</td>
<td>2.17 ± 0.12</td>
</tr>
<tr>
<td><strong>Safrole (wk. 85)</strong></td>
<td>18.80 ± 3.83</td>
<td>8.02 ± 1.91</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>7.97 ± 0.23</td>
<td>2.01 ± 0.17</td>
</tr>
<tr>
<td><strong>Ponceau MX (wk. 66)</strong></td>
<td>16.71 ± 0.96</td>
<td>6.49 ± 0.40</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>7.80 ± 0.22</td>
<td>2.15 ± 0.07</td>
</tr>
<tr>
<td><strong>Ponceau MX (wk. 70)</strong></td>
<td>23.80 ± 3.47</td>
<td>8.25 ± 1.27</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>8.71 ± 0.53</td>
<td>2.42 ± 0.16</td>
</tr>
<tr>
<td><strong>Ponceau MX (wk. 80)</strong></td>
<td>23.90 ± 2.99</td>
<td>10.31 ± 1.35</td>
</tr>
<tr>
<td><strong>Control</strong></td>
<td>11.17 ± 0.39</td>
<td>2.18 ± 0.04</td>
</tr>
<tr>
<td><strong>AAF (Male wk. 22)</strong></td>
<td>14.92 ± 0.85</td>
<td>5.67 ± 0.18</td>
</tr>
</tbody>
</table>
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