

**NADPH DEPENDENT CYTOCHROME P-450
REACTIONS: MODE OF INHIBITION BY THE
N-BUTANOL FRACTION OF
*DESMODIUM ADSCENDENS***

A THESIS SUBMITTED

BY

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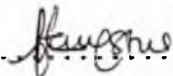
TO THE DEPARTMENT OF BIOCHEMISTRY, FACULTY OF SCIENCE,
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DECLARATION

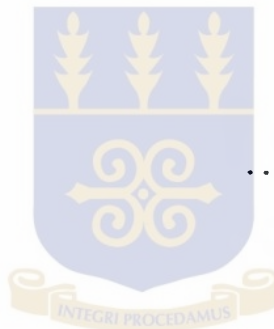
THE EXPERIMENTAL WORK DESCRIBED IN THIS PROJECT WAS DONE BY ME,
AT THE DEPARTMENT OF BIOCHEMISTRY, UNIVERSITY OF GHANA, LEGON,
UNDER THE SUPERVISION OF PROF. M.E. ADDY.

REFERENCES CITED IN THIS WORK HAVE BEEN FULLY ACKNOWLEDGED.

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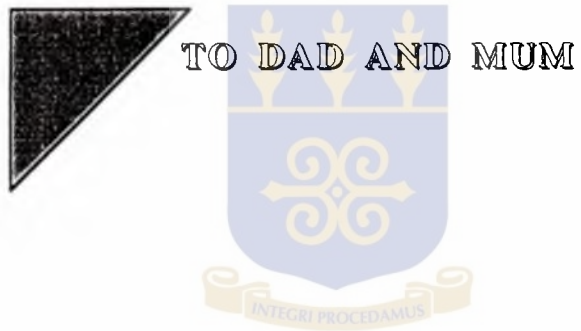


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DEDICATION



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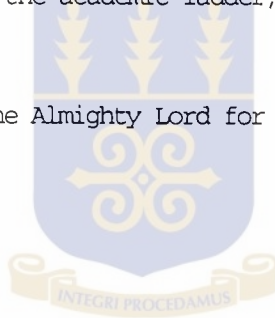


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LIST OF ABBREVIATIONS

AA	arachidonic acid
BIEM	- ENF-injected black mice
BIWM	- ENF-injected white mice
ENF	β -naphthoflavone
CO	- carbon monoxide
CYP	- cytochrome P-450
DAF	- <i>Desmodium adscendens</i> fraction
DNA	- deoxyribonucleic acid
EDTA	ethylenediaminetetraacetic acid
EET	- epoxyeicosatrienoic acid
ER	- endoplasmic reticulum
EROD	- ethoxyresorufin O -deethylase
GSH	- glutathione (reduced)
HEIE	- hydroxyeicosatetraenoic acid
LTD ₄	- leukotriene D ₄
3-MC	3-methylcholanthrene
MI	- metabolic intermediate
mRNA	- messenger RNA
NADH	nicotinamide adenine dinucleotide (reduced)
NADPH	- nicotinamide adenine dinucleotide phosphate (reduced)
nBF	- n- butanol fraction

NIEM	-	Non-induced black mice
NIWM	-	Non-induced white mice
PAH	-	polycyclic aromatic hydrocarbon
PB	-	phenobarbital
PCB	-	polychlorinated biphenyls
PCN	-	pregnenolone α - carbonitrile
PGE ₂	-	prostaglandin E ₂
PGF _{2α}	-	prostaglandin F _{2α}
PGG ₂	-	prostaglandin G ₂
PGH ₂	-	prostaglandin H ₂
PGs	-	prostaglandins
RNA	-	ribonucleic acid
SDDW	-	sterile double-distilled water
SER	-	smooth endoplasmic reticulum
TC	-	ternary complex
THIQ	-	tetrahydroisoquinoline
WR	-	White Wistar rats
WRL	-	White Wistar rat liver
WRK	-	White Wistar rat kidney

ABSTRACT

The n-butanol fraction (nBF) of *Desmodium adscendens*, a plant used for the management of asthma, is an inhibitor of NADPH-dependent cytochrome P-450 (CYP) reactions. Its mechanism of action as an inhibitor is however not known.

In this study, flavoprotein reductase activity, spectral changes associated with binding and spectral properties of reduced cytochrome c and CYP were used to investigate the mode of inhibition.

nBF reduced cytochrome c but not CYP directly. In the presence of NADPH, the rate of formation of CYP²⁺ (the reduced form of CYP) was enhanced by the addition of nBF. These effects were observed in reactions without substrate, indicating that in the absence of a substrate, nBF does not prevent the NADPH reduction of CYP, but rather enhances it.

In the presence of a substrate, as exemplified by a spectrophotometric assay of the EROD reaction, nBF was found to change the spectrum of oxidized CYP. The results indicate that in the presence of nBF, the binding site of CYP is altered to prevent substrate binding.

Therefore, the mode of inhibition of nBF relates to substrate binding. nBF could be inhibiting the NADPH-dependent CYP reactions by binding to CYP and preventing substrate binding. nBF could also inhibit the flow of electrons from NADPH to substrate-bound CYP by interfering with the flavoprotein reductase activity when it is bound to CYP. On the other hand, nBF could have the same effect on the rate of reduction of CYP to CYP²⁺ in the presence of a substrate as it did in the absence of a substrate, leading to the formation of the CYP-O₂-substrate ternary complex, in which case the effect of nBF would be to prevent the breaking up of the ternary complex to form products, the build-up of which causes the substrate to remain unmetabolized.

INTRODUCTION AND LITERATURE REVIEW

1.1 GENERAL INTRODUCTION

Without appropriate means, living organisms would not be able to eliminate from their systems the lipophilic compounds which are either produced by their own metabolism (i.e. steroid hormones, fatty acids, prostaglandins), or are accidentally or voluntarily absorbed from their environments (i.e. food additives, drugs, pesticides, pollutants etc.). Living organisms have thus developed a number of enzymic systems which transform these substances into more polar and therefore hydrophilic metabolites which can be easily excreted via the urine or faeces. Such enzyme systems must not be interfered with, since they are very vital to the survival of the organism.

One of the steps in the metabolic transformation of lipophilic compounds to more polar ones is usually catalysed by cytochrome P-450 (CYP)-dependent monooxygenases. These microsomal multienzyme complexes oxidize a great variety of endogenous as well as exogenous substrates. The reaction involves the formation of a ternary complex between the iron centre of the cytochrome, the substrate and molecular oxygen. Within the ternary complex, electrons donated by reduced nicotinamide adenine dinucleotide phosphate (NADPH), through a flavoprotein reductase, are received by CYP and passed on to molecular oxygen. CYP, therefore, goes through a ferric-ferrous cycle. The reducing power supplied by the electrons as they pass

through CYP is used to split molecular oxygen, one atom of which gets inserted into the substrate and the other atom is reduced to water. With this reaction occurring in the liver, xenobiotics are rendered more polar and therefore can be excreted through the aqueous bile.

The biochemical and biological properties of these enzyme complexes have been extensively studied in laboratory animals (Lu and West, 1980). The different types of cytochrome P-450 are characterized by their substrate specificity (Haugen *et al.*, 1975). Their quantitative and qualitative proportions in a given tissue may vary largely as a function of physiological, pharmacological and pathological parameters (Lu and West, 1980). This phenomenon is particularly important with respect to drugs because for many drugs, the nature and toxicity of their different metabolites which are often synthesized by various types of CYP, are not identical. It is also important with respect to environmental pollutants. Different CYPs are induced by different pollutants and these differences form the basis for the nomenclature of the CYPs.

Apart from pollutants, drugs, other xenobiotics and endogenous substrates are also transformed by the monooxygenase enzyme complex. Arachidonic acid (AA), usually derived from membrane phospholipids as a result of phospholipase A₂ activity, is one of the endogenous compounds metabolized by the monooxygenase enzyme complex. It is thus a major substrate for the NADPH-dependent CYP enzyme complex in a reaction referred to as the third

pathway of AA metabolism (Capdevila *et al.*, 1982) after the cyclooxygenase pathway giving rise to prostaglandins (PGs), prostacyclins (PGI₂) and thromboxane (TX) (Samuelsson *et al.*, 1978), and the lipoxygenase reaction giving rise to leukotrienes and lipoxins (Samuelsson *et al.*, 1980).

The discovery of renal cytochrome P-450 monooxygenase was related to arachidonic acid (AA) metabolism. The renal system metabolizes AA by three types of reactions:

- i) allylic oxidation leading to the formation of hydroxyeicosatetraenoic acids (HETEs);
- ii) olefin epoxidation leading to the formation of four different epoxyeicosatrienoic acids (EETs); and
- iii) oxidation at ω - and ω -1 positions to form the 20-HETEs and 19-HETEs respectively (Schwartzman *et al.*, 1985a).

Some of the products of this NADPH-dependent CYP pathway, like 5,6 EET and 11,12(R) HETE, are known to affect the Na⁺/K⁺ ATPase activity and to cause diseases associated with changes in volumes of body fluids. One of such diseases is renal hypertension. In epithelial cells isolated from the thick ascending limb of Henle's loop of the rabbit kidney, AA is specifically metabolized by a CYP-dependent pathway to products which affect Na⁺/K⁺ ATPase activity and vascular tone (Schwartzman *et al.*, 1985). Compounds which can inhibit the CYP monooxygenase enzyme complex so as to prevent the formation of the products which affect the renal Na⁺/K⁺ ATPase

activity could be effective agents for the treatment of hypertension.

An n-butanol fraction (nBF) of an aqueous extract of *Desmodium adscendens* was shown to inhibit AA metabolism by this monooxygenase pathway (Addy and Schwartzman, 1992). Salsolinol (Appendix AI:b) was found to inhibit AA metabolism by the NADPH-dependent CYP monooxygenase enzyme complex of the endoplasmic reticulum, just as the plant extract did (Addy and Schwartzman, 1992).

With AA as substrate, salsolinol inhibited the formation of the epoxides, EETs and the HETEs. Salsolinol is the 6,7-dihydroxy analogue of salsoline (Appendix AI:a), a tetrahydroisoquinoline (THIQ) compound in the plant already listed as an anti-hypertensive agent (Stecher, 1968).

The nBF was also found to inhibit ethoxyresorufin-o-deethylase activity (Brookman-Amisah, 1994), an enzyme activity specific for one of the pollution-induced isozymes of CYP. nBF has been shown to act as a reducing agent (Addy and Schwartzman, 1995) and it is possible that its reducing ability plays a role in the monooxygenase enzyme system.

This inhibition of the monooxygenase enzyme by nBF could therefore be beneficial for its anti-hypertensive role. However, if all the CYP isozymes, including the constitutive ones are inhibited, it will be potentially dangerous because apart from AA, which is not free but usually

bound to membrane phospholipids, steroids also serve as endogenous substrates for several different forms of P-450 related enzyme complexes.

A unique set of P-450 isozymes is localized in steroidogenic tissues, i.e., adrenal cortex, testis and ovary. Lipids other than steroids also serve as substrates for these P-450 proteins. Rat liver CYP enzymes are able to catalyze the hydroxylation of fatty acids such as lauric acid (dodecanoic) in both ω - and ω -1 positions (Das *et al.*, 1968; Bjorkhem and Danielsson, 1970). In addition, studies carried out using porcine and rat kidney cortex microsomes (Ichihara *et al.*, 1971; Jakobsson *et al.*, 1970) suggest that cytochrome P-450 enzymes are involved in the hydroxylation of lauric acid in these tissues as well.

The overall aim of the research reported here was to find out the mechanism of this inhibition so as to understand how the monooxygenase enzyme activity can be regulated. With this, one can predict the safety of nBF on other monooxygenase reactions that are of relevance to the general metabolism of the cell.

1.2 CYTOCHROME P-450 MONOOXYGENASES

The cytochrome P-450 dependent monooxygenase system is found throughout nature, from bacteria to man, where it is involved in the oxidation of many organic compounds. The requirement of both a reducing agent and molecular oxygen places the reaction within the external mixed function oxidase

classification of Mason (1957, 1965). The terminology monooxygenase means that the enzyme catalyzes the consumption of one molecule of oxygen per molecule of substrate with one atom of oxygen appearing in the product and the other undergoing two-equivalent reduction. Direct support of this view was given by Posner *et al.* (1961), who employed $^{18}\text{O}_2$ and H_2^{18}O to show that the oxygen utilized in the hydroxylation of acetanilide was derived from molecular oxygen rather than from water. The essential components of the monooxygenases have been identified as cytochrome P-450, NADPH-cytochrome P-450 reductase (probably the same as, or very similar to NADPH-cytochrome c reductase), and phospholipid (Imai, Y. 1976; Levin *et al.*, 1974; Yasukochi and Masters, 1976). Following the discovery of multiple forms of the cytochrome P-450s and in depth investigation of at least 2 of these enzymes in pure form, it became apparent that slight different spectral properties existed for each isoenzyme catalyzing different reactions.

Cytochrome P-450 monooxygenases catalyze a wide variety of oxidations with a vast number of substrate types. Indeed, many authorities suggest that this fact supports the purported multiplicity of P-450 monooxygenase (Haugen *et al.*, 1975). It is thought that few and perhaps no liver microsomal P-450 monooxygenases catalyze a single reaction, but certain cytochromes will have greater activity of a certain reaction type than others.

Due to the substrate-nonspecificity of the functional monooxygenase enzyme system in the microsomes of liver and other tissues, determination of its activity depends on the choice of substrate (Burke and Mayer, 1974). This determination is usually decided purely by convenience of assay. Because of the original interest in this enzyme complex as a detoxifying and drug-metabolizing system, most commonly used substrates are drugs or environmentally encountered chemicals.

It has become increasingly evident that several forms of CYP exist and may account for the multiple monooxygenase activities in liver microsomes (Haugen *et al.*, 1975; Ryan *et al.*, 1975). This is based upon the knowledge that different spectral (Werringloer and Estabrook, 1975), and catalytic (Lu *et al.*, 1972) forms of this cytochrome can be induced by chemicals (Haugen *et al.*, 1976). A nomenclature based on gene sequence information of P-450 forms described a gene superfamily and organized all sequences known by then into P450 gene families. This was later revised to include chromosomal localization (Nebert *et al.*, 1991). A selection of mammalian gene families is provided in Table 1.

There are 27 clearly related species of CYP present in the liver endoplasmic reticulum in rats, each with a wide and somewhat overlapping substrate specificity, that act on a wide variety of drugs, carcinogens and other xenobiotics in addition to endogenous compounds such as certain steroids. This number keeps increasing with new findings.

Table 1. Selected gene families of CYP

Gene Family	Gene Subfamily	Other Name Species	Common Inducers
CYP1	CYP1A	P-450 _c rat P ₁ -450 mouse	PAH, PCB, BNF etc.
CYP2	CYP2B	P450 _b rat Form 2 rabbit	PB
CYP3	CYP3A	P-450 FCN, rat	FCN
CYP4	CYP4A	P-452 rat	Clofibrate

PAH = polycyclic aromatic hydrocarbon; PCB = polychlorinated biphenyls;
PB = phenobarbital; FCN =pregnenolone - α - carbonitrile

1.3 REACTION MECHANISM

Cytochrome P-450 monooxygenases bind substrate and interact with a flavoprotein reductase and molecular oxygen in a two-step (2-electron sequence resulting in the activation of oxygen (Estabrook *et al.*, 1971). Ultimately, one oxygen atom is incorporated into the substrate and the second is reduced to water. Formation of a CYP-substrate-O₂ ternary complex helps to explain the generally suggested substrate specificity imparted by the different cytochrome monooxygenases. It also helps to explain the wide variety of oxidative reactions known to be catalyzed by monooxygenases from liver microsomes which include the following:

- i) oxidative deamination of amphetamine;
- ii) O-, N-, and S- dealkylations of substances like 7-ethoxyresorufin, 7-ethoxycoumarin, aminopyrin, ethylmorphine and thioesters like methylmercaptan;
- iii) hydroxylation of alkyl and aryl hydrocarbons like n-propylbenzene, valproic acid, pentobarbital, debrisoquine and acetanilide;
- iv) epoxidation of substances like benzene and benzo(α) pyrene;
- v) N-hydroxylation;
- vi) N- and S- oxidation of aniline, amphetamine and thioethers;
- vii) oxidative dehalogenation;
- viii) oxidation of ethanol (Brodie *et al.*, 1958).

Hepatic microsomal cytochrome P-450 dependent metabolism is not restricted to oxidative reactions. A wide variety of azo dyes are cleaved reductively to aromatic amines, and nitro compounds such as chloramphenicol and nitrobenzene are reduced to primary amines. Reductive dehalogenation also occurs in the liver microsomes (Mannering, 1972).

Although the CYP-catalyzed reaction requires two electrons to accomplish its task of heme iron reduction, oxygen binding and oxygen cleavage, a basic mechanistic problem is the direct and simultaneous transfer of electrons from NADPH to the CYP. Pyridine nucleotides are two electron donors, but CYP with its single heme prosthetic group, accepts only one electron at a time. Thus, a protein that serves to transfer electrons from

NADPH to the CYP molecule must have the capacity to accept two electrons but serve as a one electron donor. This problem is solved by the presence of an NADPH-dependent flavoprotein reductase, which accepts two electrons from NADPH simultaneously but transfers the electrons individually to an intermediate iron-sulfur protein or directly to CYP. The electron transport systems reside exclusively in either mitochondria or endoplasmic reticulum (Okita and Masters, 1992).

The possible coupling of this reductive process with an NADH mediated electron transfer explains the often suggested synergistic role of NADH as well as cytochrome b_5 and cytochrome b_5 reductase (Nilsson and Johnson, 1963) as shown in Fig. 1.1. A coupling/decoupling role for hydrogen peroxide has been proposed (Thurman *et al.*, 1972). Thus NADPH (and NADPH-generating systems) can be replaced by hydrogen peroxide and a variety of organic peroxides in the oxidation of certain xenobiotics by microsomal mixtures (Ellin and Orrenius, 1975; Hrycay *et al.*, 1976; Rahimtula and O'Brien, 1975;) and cytochrome P-450 LM_2 (Nordblom *et al.*, 1976) in particular. However, the role of hydrogen peroxide in intact hepatocyte may be of lesser importance (Jones *et al.*, 1978).

The details of the cytochrome P-450 monooxygenase reaction involving the cytochrome P-450 is shown in Fig. 1.2. As indicated in the scheme, substrate binding to native ferric P-450 is followed by reduction to the ferrous state, thereby allowing oxygen binding. A second reduction results

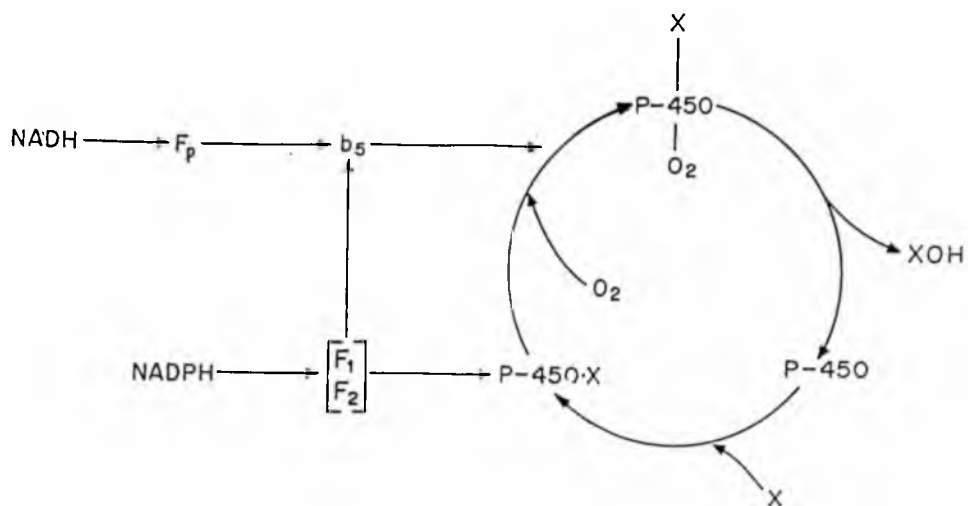


FIG. 1.1 A SCHEME FOR MICROSOMAL ELECTRON TRANSPORT REACTIONS OF CYP. [Fp, NADPH-cytochrome b_5 reductase; F_1F_2 , NADPH-cytochrome P-450 reductase; X, hydroxylatable substrate]. The ferrous oxycytochrome can also decompose to yield H_2O_2 (not shown).

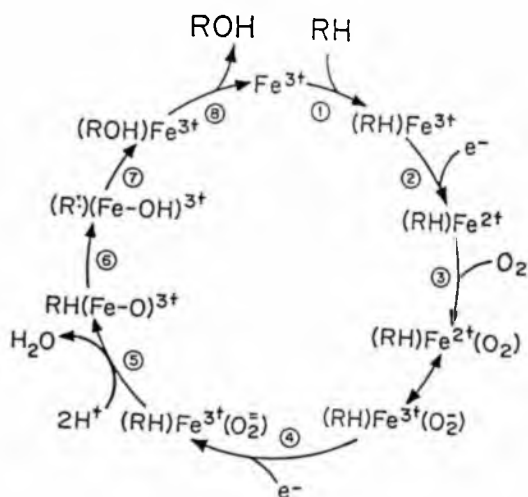


FIG. 1.2 PROPOSED SCHEME FOR THE MECHANISM OF ACTION OF CYP IN HYDROXYLATION REACTIONS.
RH = substrate; ROH = hydroxylatable product

in splitting of the oxygen-oxygen bond, one atom being lost as water. The other oxygen atom, presumably now an "activated-oxygen" is inserted into a carbon-hydrogen bond of the substrate to produce the corresponding alcohol which is then released with regeneration of the resting ferric state of the enzyme and completion of the catalytic cycle (White and Coon, 1980).

The first step in Fig. 1.2 is the binding of the substrate to the active site of the enzyme. The binding of the substrate to the cytochrome system has been shown to be hydrophobic (Griffin and Peterson, 1972), and is facilitated by the perturbation of equilibrium between the high and low spin state of the heme of the ferric iron to favor the latter form. Binding of the substrate, therefore, converts the heme from a low spin state to a high spin state. Step 2 of the reaction cycle is an electron reduction of the substrate-bound CYP and this reducing equivalent ultimately comes from NADPH. After reduction, the heme is now able to bind an oxygen molecule (step 3) which is the first actual step in oxygen activation.

Step 4 is mandatory for the reaction stoichiometry and is also required in the position shown in the reaction sequence as indicated previously in Fig. 1.1. A branch exists at this point in the CYP catalytic cycle and it is interesting to mention here that active turnover by the liver microsomal CYP involves the production of hydrogen peroxide (H_2O_2); as much as 55% of consumed oxygen appears as H_2O_2 in the presence of substrate and

essentially 100% in its absence (Nordblom and Coon, 1977). Thus, in addition to the hydroxylase activity, CYP also exhibits a concurrent oxidase activity with the formation of H_2O_2 .

Hilderbrandt and Estabrook (1971) suggested that cytochrome b_5 may supply the second electron at this stage, i.e step 4. Further evidence was provided by Werringloer and Kawani (1980) who measured both the kinetics and extent of reduction of CYP and cytochrome b_5 in a carbon monoxide (CO) atmosphere, and found that these hemoproteins participate in reversible electron transfer reactions when associated with the microsomal membrane. Electron transfer was examined from cytochrome b_5 to P-450 in the presence of NADPH; the equilibria were independent of the reducing agent used. Their observations indicate that the redox properties of cytochrome b_5 are favourable for the reduction of ferrous oxy-P450 in the membrane. The cleavage of the oxygen-oxygen bond in the complex (step 5) can occur in 2 ways; i.e either homolytically or heterolytically. The nature of the substrate to be oxidized undoubtedly plays an important role at this step of the reaction sequence. Heterolysis would lead to the formation of an iron-oxenoid complex. Oxene addition to a double bond can form arene oxides or epoxides in the case of AA metabolism. The electrophilic oxygen intermediate may attack an electron-rich carbon atom resulting in hydrogen abstraction (step 6) and oxygen insertion (step 7). Dissociation of the product alcohol from the cytochrome P-450 active site (step 8) completes the cycle.

Homolysis of the oxygen-oxygen bond is probably the favoured route when an organic peroxide, such as cumene hydroperoxide, is used (Estabrook *et al.*, 1982). Studies of benzo (α) pyrene or the radical-mediated oxidation of amino pyrene are two examples of this type of reaction. Thus one can visualize that the cleavage of the oxygen-oxygen bond of the peroxide may be assisted by the donation from the substrate. As a result water is formed as well as a radical species of the substrate.

1.4 CYTOCHROME P-450s

Cytochrome P-450 (CYP), earlier referred to as the CO-binding pigment, was first described by Klingenberg (1958), and Garfinkle (1958). Omura and Sato (1962) later characterized it as a hemoprotein. The term cytochrome P-450 refers to a family of heme proteins present in all mammalian cell types, except mature blood cells and skeletal muscle cells which catalyze the oxidation of a wide variety of structurally diverse compounds. CYP also occurs in prokaryotes. The designation of a protein as CYP originated from its spectral properties before its catalytic function was known. This group of proteins has a unique absorbance spectrum that is obtained by adding a reducing agent, such as sodium dithionite, to a resuspension of endoplasmic reticulum vesicles, frequently referred to as microsomes, followed by the bubbling of CO gas into the solution. The CO is bound to the reduced heme protein which produces an absorbance spectrum with a peak at 450 nm; thus the name P-450 for a pigment with an absorbance at 450 nm.

Specific forms of CYP differ in their maximum absorbance wavelength, with a range between 446 and 452 nm. The many forms of CYP are classified, according to their sequence similarities, into various gene subfamilies; this system of nomenclature is being adopted almost universally (Nebert *et al.*, 1991).

In mammalian cells, CYPs serve as electron acceptors in electron transport systems, which are present either in the ER or inner mitochondrial membrane. The CYP protein contains a single iron protoporphyrin IX prosthetic group, and the resulting heme protein contains binding sites for both an oxygen molecule and the substrate.

1.4.1 Isoenzymes

Attempts by Sladek and Mannering (1966) to measure some of the differences between the two types of microsomal drug metabolizing system synthesized as a result of stimulation by polycyclic aromatic hydrocarbons (PAHs) and phenobarbital (PB) led to the conclusion that PAHs cause the synthesis of a modified CYP. For lack of a more suitable nomenclature for the microsomal hemoprotein, it was named cytochrome P₁-450.

Soon after this evidence for the existence of cytochrome P₁-450, Alvares *et al.*, (1967) and Hilderbrandt *et al.*, (1968) showed that the λ_{max} of reduced microsomal protein bound to CO obtained after administration of PAHs differed slightly from that observed in microsomes from untreated animals.

Alvares *et al.*, observed a λ_{max} at 448 nm, therefore cytochrome P₁-450 is sometimes called P-448. Hilderbrandt and associates observed a λ_{max} at 446 nm, thus cytochrome P₁-450 is referred to as P-446 by some investigators. The shift from 450 nm to 448 nm and 446 nm is slight, but real.

The administration of PAHs causes the biosynthesis of cytochrome P₁-450, a molecular species of CYP not normally detected in appreciable amounts in microsomes of untreated or phenobarbital-treated animals. This does not exclude the possibility that small amounts of cytochrome P₁-450 may be found in untreated animals.

In the broadest sense, the various P-450 isozymes can be classified as: (1) those forms which metabolize physiological or endogenous substrates and (2) those forms which metabolize xenobiotic or exogenous substrates. It is important to note a fundamental difference between these two classes of P-450. Those P-450 isozymes which metabolize exogenous substrates are inducible *in vivo* and exposure to xenobiotics can lead to increased levels of specific forms of P-450 and/or their respective enzymatic activities. As has been shown in the case of several different xenobiotic metabolizing forms of P-450, changes in their levels result largely from changes in the amount of mRNA encoding these proteins (Adesnick *et al.*, 1981). In contrast, while those P-450 isozymes which metabolize endogenous substrates have the capacity to be induced, the constitutive levels of these enzymes are generally optimal for the particular function they serve. There are

instances where a specific form of P-450 contains diverse activities; one involved in xenobiotic metabolism and the other in metabolism of endogenous substrates. An example is the products of AA generated by P-450-mediated reactions via a lipoxygenase-like mechanism (Capdevila *et al.*, 1981). Such products have been shown to exhibit chemotactic activity and to be involved in inflammatory responses (Samuelsson, 1980). The major phenobarbital-inducible form of P-450 in liver has been found to possess this activity.

The level of individual isozymes are strikingly dependent on the animal's age, sex and history of exposure to foreign compounds which can serve as monooxygenase inducers. Immunochemical and catalytic studies on the sex specificity of P-450 isozymes expression have established that P-450 2c is male specific and undergoes a developmental induction at puberty (Waxman, 1984). P-450 2d is developmentally induced in female rats although it is also expressed at significant levels in immature males at 3-4 weeks of age (Waxman *et al.*, 1985)

1.4.2 Conversion to P-420

Membrane-bound P-450 is affected by surface active agents, chelating agents, sulfhydryl reagents, and lipophilic substances. These agents convert P-450 to an inactive form, P-420 (Omura and Sato, 1964). The diversity of such effective agents appears to have caused confusion in the interpretation of the state of P-450.

P-450 in liver microsomes acts as a site of both oxygen and substrate activation for drug hydroxylation (Imai and Sato 1966). Since only those drugs possessing high solubility in lipid solvents are hydroxylated by the microsomal system, it seems reasonable to assume that the reactive site of P-450, i.e. the vicinity of the heme, is in contact with, or buried in a highly hydrophobic part of the P-450 protein or the lipids of the microsomal membrane.

The unusual spectral properties of CYP are ascribed to a hydrophobic interaction of the heme with nearby components. Since the conversion of P-450 to P-420 is always accompanied by the inactivation of the hydroxylase systems, and P-420 is incapable of reacting with the hydroxylatable substrates in the same way as P-450 (Imai and Sato, 1966), it is likely that the integrity of the unusual state of P-450 is essential for its function. Depending on the agent employed, the conversion of P-450 to P-420 would result from the disturbance of the hydrophobic environment around the heme either by primary action of the agent or by secondary effects caused by conformational changes in the hemoprotein.

The conversion of P-450 to P-420 induced by neutral salts proceeds more rapidly in the reduced form of the hemoprotein than in the oxidized form (Imai and Sato, 1967). Cytochrome P-420 formed as a result of treatment of microsomes with the detergent, sodium cholate, was converted back to cytochrome P-450 by polyols and reduced glutathione (Ichikawa and Yamano,

1967). Dialysis also produces partial reversal.

1.4.3 Spectral Characteristics

Cytochrome P-450 (CYP) is measured by the difference spectrum seen when it is reduced, usually with sodium dithionite, and carbon monoxide (CO) is bubbled through the reduced microsomal suspension. NADH and NADPH reduce CYP only in the absence of molecular oxygen, the pigment being autooxidizable. About 50% inhibition of hydroxylation reactions are obtained when the CO/O₂ ratio is about one.

Determination of the absolute spectrum of CYP is complicated by the presence of cytochrome b₅ and cytochrome P-420. The problem has been solved to a large degree by removing cytochrome b₅ from the microsomes and by stabilizing CYP with glycerol. In Mason's laboratory hepatic microsomes were treated with the non-ionic detergent, Lubrol WX, to produce subparticles which gave an absolute spectrum for CYP with no apparent interference from cytochrome b₅ and very little interference from cytochrome P-420 (Miyake *et al.*, 1968). A method was proposed by Kinoshita and Horie (1967) for determining the absolute spectrum of CYP in microsomes which had not been treated to remove cytochrome b₅. This method is based on the knowledge that cytochrome b₅ is the only microsomal cytochrome other than CYP and that the administration of inducing agents such as phenobarbital causes a large increase in the concentration of CYP in microsomes without affecting appreciably the cytochrome b₅.

1.4.4 Binding to Substrates

CYP catalyzes hydroxylation reactions of steroids, drugs and other compounds. It has been found that CYP combines with these compounds frequently causing spectral effects. Studies by Imai and Sato (1966), showed that drugs and other foreign compounds combine with hepatic CYP to produce spectra of two general types, type I and type II. Compounds giving type I or II difference spectra with hepatic microsomes have come to be known as type I and type II compounds (or drugs). Type I compounds give difference spectra with a λ_{\max} in the general range of 385-390 nm and a λ_{\min} in the general range of 418-427 nm; the λ_{\max} and λ_{\min} given by type II compounds are 425-435 nm and 390-405 nm respectively (Schenkman *et al.*, 1967). The characteristic difference spectra observed when type I or type II drugs are added to microsomes can be seen when the absolute spectra of CYP are recorded before and after the addition of hexobarbital (type I) or aniline (type II). Besides these two groups of drugs, there is a third class of compounds which bind CYP to give a spectrum known as reverse type I (Schenkman *et al.*, 1967). This class of compounds is the mirror image of the type I spectral change (λ_{\max} 420nm, λ_{\min} 385nm) (Jefcoate, 1978) (Fig. 1.3). Depending on the experimental conditions amines interact with CYP with difference spectra that may vary continuously between two extreme spectra, termed type IIa (λ_{\max} 425nm, λ_{\min} 390nm) and type IIb (λ_{\max} 432nm, λ_{\min} 410nm) (Jefcoate and Gaylor, 1969). However there are a group of related drugs which do not give any spectral change when bound to CYP (Wilson and Harding, 1970). Imai and Sato (1967a) pointed out that

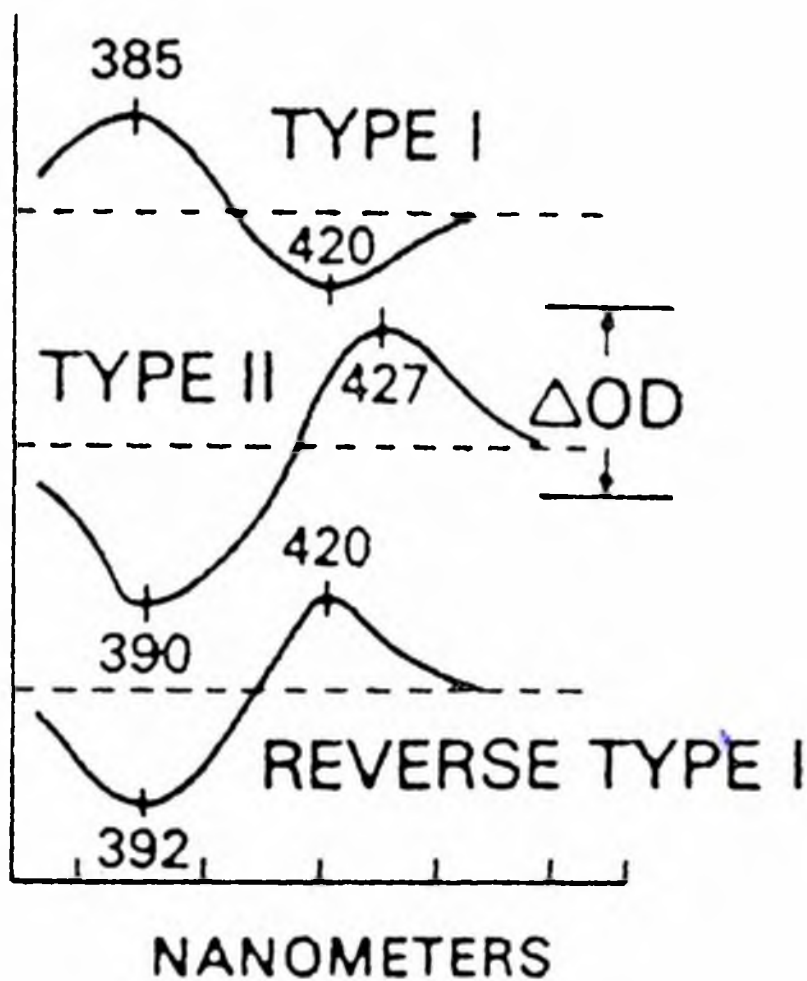


FIG. 1.3 DIFFERENCE SPECTRA OF TYPE I, TYPE II AND REVERSE TYPE I COMPOUNDS

barbital and benzene are hydroxylated by microsomes although they do not produce binding spectra. It is also to be noted that the ability of compounds to form binding spectra with CYP does not guarantee their metabolism; many n-alkylamines combine avidly with CYP to produce type II spectra, but are not metabolized (Jefcoate *et al.*, 1969).

With but one exception, type I and type II compounds produced their characteristic binding spectra consistently regardless of the source of the microsomes. The exception is phenobarbital (PB), which gave a type I binding spectrum with microsomes from rats, but a type II binding spectrum with microsomes from rabbits (Mannering, 1972).

CYP can be oxygenated only when in the reduced form and must therefore be in the reduced state when the activated oxygen reacts with the substrate. Spectrophotometric studies have shown that in the presence of both oxygen and NADPH, CYP is mostly in the oxidized state. The reduction of CYP is believed to be the rate-limiting step in the overall process of microsomal metabolism. Both type I and type II substrates protect CYP against destruction and react with the ferric forms (Schenkman *et al.*, 1969). Type I substrates accelerate while type II substrates decelerate the reduction to the ferrous form (Kupfer and Orrenius, 1970); type I substrates do not affect re-oxidation while type II substrates may accelerate it (Gigon *et al.*, 1968). Schenkman (1970) adduced evidence that most type II changes are accompanied by type I changes and become enlarged and symmetrical if

corrected for the type I changes.

Leibman and co-workers (1969) believed that many of the inconsistencies in the kinetics of binding and metabolism of CYP could be explained by the interconvertibility of type I and type II CYP complexes. This conclusion was drawn partly from kinetic studies in which either a type I or II compound (modifier) was added to a suspension of microsomes placed in both cuvettes of a dual beam spectrophotometer. Graded amounts of either a type I or type II compound (substrate) were added to the sample cuvette and the difference spectrum recorded. The sum of the absorbance change at wavelengths of the peaks and troughs were determined. When the modifier was a type II compound and the substrate a type I compound, competitive inhibition was observed. When the modifier and substrate were type II compounds, inhibition was never competitive, and in certain cases kinetics were analogous to classical non-competitive inhibition. Both competitive and non-competitive inhibition were seen when the modifier and substrate were type I compounds. When the modifier was a type I substance, and the substrate a type II compound, "stimulation" rather than inhibition was seen.

1.5 OTHER COMPONENTS OF THE MICROSOMAL MONOOXYGENASE SYSTEM

1.5.1 Reductases

NADPH-cytochrome c reductase and NADPH-cytochrome P-450 reductase are 2 reductases purported to be involved in drug metabolism. NADPH-cytochrome c

reductase is thought to reduce CYP directly or indirectly through a non-heme iron protein or some other unidentified carrier. Because cytochrome c is not present in microsomes, and there is no other natural substrate for the reductase in these organelles, NADPH-cytochrome c reductase was considered particularly eligible to play a role in the transfer of electrons from NADPH to CYP. The enhancement of NADPH-cytochrome c reductase activity seen in microsomes when microsomal drug metabolizing activity is caused to be increased as a result of phenobarbital administration provided another indirect association of the reductase with the microsomal hydroxylating system (Remmer and Merker, 1965; Orrenius and Ernster, 1964).

NADPH-CYP reductase contains both flavin adenine dinucleotide (FAD) and flavin mononucleotide (FMN) as prosthetic groups. It is the only mammalian flavoprotein known to contain both FAD and FMN (Masters and Okita, 1992). The FAD serves as the entry point of electrons from NADPH, and FMN serves as the exit point, transferring electrons individually to CYP. Because the flavin molecule may exist as one or two electron reduced forms, and 2 flavin molecules are bound per reductase molecule, the enzyme may receive electrons from NADPH and store them between the 2 flavin molecules before transferring them individually to the heme iron for O₂ binding (first electron) and cleavage of the oxygen molecule (second electron) (Fig 1.2).

In certain reactions catalyzed by the microsomal P-450, the transfer of the 2 electrons may not be directly from NADPH-CYP reductase, but may occur from cytochrome b_5 which is also present in the ER (Fig 1.1). Cytochrome b_5 is reduced either by NADPH-CYP reductase or another microsome-bound flavoprotein, NADH-cytochrome b_5 reductase, which is specific for NADH.

NADPH-CYP reductase activity measurement is based on the knowledge that CO forms a complex with reduced, but not oxidized CYP to give a Soret peak at 450 nm. From a variety of experiments that showed the rate of drug metabolism to be more closely related to the rate of cytochrome P-450 reductase than the total amount of CYP or the rate of cytochrome c reduction, it was concluded that NADPH-CYP reductase activity is rate-limiting in the overall reaction involving microsomal drug metabolism. Of considerable interest was the observation that type I binding compounds stimulated NADPH-CYP reductase activity whereas type II binding compounds either inhibited or had no effect on the reductase (Gigon *et al.*, 1968, 1969).

NADPH-CYP reductase activities of microsomes from male and female rats were also determined with the idea that an explanation might be found for the well known sex difference in drug metabolism (Gigon *et al.*, 1968, 1969). Only a slight difference in the reductase activity was found and this was probably due to the small difference in CYP content of the microsomes. However, ethylmorphine (type I compound) caused a much greater stimulation

of NADPH-CYP reductase in microsomes from males than it did in microsomes from females. This suggested to the authors that the CYP in microsomes from female rats is less capable of participating in the oxidation of substrates than that in microsomes from male rats.

Nothing is known about NADPH-CYP reductase other than its activity. As the reduction of CYP is relatively difficult to assay directly, a simplified determination of enzyme activity is widely used, utilizing exogenous cytochrome c (oxidized, ferric form) as an artificial electron acceptor. Accordingly, the reduction of cytochrome c by NADPH-cytochrome c (P-450) reductase mirrors the reduction of CYP. The assumption is that NADPH-cytochrome (c) reductase reflects the activity of NADPH-CYP reductase, or of some other flavoprotein, as it functions as part of the microsomal multi-enzyme hydroxylase complex. The principle of this method is that oxidized (ferric) cytochrome c has a characteristic absorption spectrum as does the reduced (ferrous) form. However, the reduced form has a characteristic absorption band at 550 nm, a band that is absent in the oxidized form. Therefore the enzyme activity can be conveniently assayed by measuring the increase in absorbance at 550 nm as a function of time.

1.5.2 **Lipid Factor**

Lu and others (Lu and Coon, 1968; Lu *et al.*, 1969) separated solubilized hepatic microsomes into three fractions containing CYP, an NADPH reductase and a heat stable lipid factor. The combination of all three fractions was

required for maximal drug metabolizing activity.

Heating the lipid factor for 2 hours at 100°C at neutral pH or for 10 minutes at 50°C in 0.1M HCl or H₂SO₄ did not affect it. It was however destroyed by ashing (Lu *et al.*, 1969a). Attempts to replace the lipid factor fraction with other phospholipid preparations including rat liver lecithin or phosphatidyl ethanolamine, egg yolk lecithin, bovine phosphatidyl ethanolamine, proved unsuccessful. The lipid factor could therefore be considered as acting physically in such a way as to provide access of the drug to CYP.

1.5.3 **Non-heme Iron Protein**

Adrenodoxin, a non-heme iron protein, is a component of the electron transport system that functions in the hydroxylation of steroids by adrenal microsomes and mitochondria (Suzuki and Kimura, 1965, Kimura and Suzuki, 1965). In this system, adrenodoxin acts as the electron carrier between flavin enzyme (adrenodoxin reductase) and CYP. Because of the similarities of the microsomal hydroxylase reactions of the adrenal gland and liver, it has been assumed that adrenodoxin or some other non-heme iron protein is involved in the microsomal drug metabolizing system. In the presence of NADPH, a mixture of adrenodoxin and adrenodoxin reductase caused the reduction of CYP contained in submicrosomal liver particles, but the essentiality of adrenodoxin was not determined (Miyake *et al.*, 1968). Kimura (1968) concluded that tissues capable of producing steroid hormones

(adrenals, testis, ovary) contain adrenodoxin, but liver and other tissues do not. This does not however exclude the possibility that a different non-heme protein may function in the hepatic hydroxylase system.

1.6 INHIBITORS OF CYTOCHROME P-450s MEDIATED REACTIONS

Investigations concerning the inhibition of hepatic mixed-function monooxygenases are of direct relevance to toxicology and pharmacology in anticipating and predicting the safety of the environment and of drug therapy. Studies in this area is also of relevance to the concentrations of essential endogenous compounds produced by this enzyme complex. For these reasons, it is essential to understand the possible interactions between various inhibitors and the enzyme complexes at the molecular level. By the use of conventional enzyme kinetic studies and techniques, interactions have been classified as competitive or non-competitive; but many instances have arisen where the results do not conform to either of these classifications. In many cases, the lack of conformity has been attributed to the multi-enzyme nature of mixed-function oxygenation reactions or the inability or unsuitability of using conventional concepts for an enzyme located in a membrane (endoplasmic reticulum).

CYP monooxygenases are differently inhibited by a variety of substances including CO, 2-diethylaminoethyl-2-diphenylvalarate (SKF 525-A), metyrapone, ethyl-isocyanate, the Lilly compound, 2,4-dichloro-6-phenolphenoxy-ethylamine (DPEA) (Imai and Sato, 1967b; Omura and Sato,

1964) salsolinol and the n-butanol fraction (nBF) of *Desmodium adscendens* (Addy and Schwartzman, 1992). Indeed varying inhibitory effects on different microsomal preparations are suggested as underlying support for multiple forms of P-450 monooxygenases. However inhibitor studies must be interpreted with caution, especially with heterogenous preparations. For example, microsomal hydroxylase activity is widely thought to be insensitive to cyanide. However, CYP systems have been found to be inhibited by cyanide in a concentration-dependent manner.

1.6.1 **Formation of Metabolic Intermediate Complexes**

In the last two decades, a facet of mixed-function oxygenases inhibition has become apparent which yield an interpretation of some previously unexplained observations and provides an insight into a new concept of mixed-function oxygenase inhibition. This involves compounds which are not substrates for the mixed-function oxygenase reaction, but intermediates in the reaction, or products of the reaction which do not leave the enzyme. This entity (intermediate or product) binds tightly to the enzyme, preventing its further participation in mixed function oxygenation (Franklin, 1977). These compounds form complexes with CYP which can be detected in a ferrous state by an absorbance maximum in the Soret region.

No product of a mixed-function oxygenase reaction has been found which, upon addition to ferrous CYP, immediately shows the same amount of complex which can be obtained during metabolism. Much of the information presently

available suggests that an intermediate generated during the oxygenation reaction forms the complex with CYP (Franklin, 1977), and thus the term "metabolic intermediate (MI) complex" is used to describe such compounds.

The ability to detect spectrophotometrically (given certain conditions) the existence of the enzyme-intermediate complex has facilitated the rapid investigation of this aspect of oxidative drug metabolism.

The prerequisites for MI complex are conditions necessary for mixed-function oxygenation reactions to occur. Thus, O_2 and NADPH are essential, with the exception of organic peroxides which can provide the equivalent of both (Elcombe *et al.*, 1975). NADH substitutes only very poorly as a source of reducing equivalents compared with NADPH (Franklin, 1974).

The MI complex is probably a combination of a metabolic intermediate and the heme iron of CYP. The MI complex exhibits an absorbance maximum in the Soret region between 448 and 456 nm (for compounds examined to date) when the heme iron is in the reduced (ferrous) state (Werringloer and Estabrook, 1975). Observation of a 450 nm absorbance maximum needs careful scrutiny, however, since generation of CO during prolonged aerobic incubation of microsomes with NADPH can occur. Binding of the CO thus formed with CYP, will become apparent under near anaerobic conditions, as produced by dithionite addition. The MI is not displaced from the complex by CO and, thus, interferes with the determination of CYP. The MI complex, once formed, inhibits mixed-function reactions in a non-competitive manner.

Compounds capable of forming MI complexes can be divided into 2 main categories; non-nitrogenous and nitrogenous. A few other compounds not readily fitting into these categories (e.g. N-2-ethylhexyl-5-norbornene-2, 3-dicarboximide [MGK-264] and fluorine) have also been observed to form MI complexes (Ullrich and Schnabel, 1973). The non-nitrogenous group consists predominantly of methylenedioxybenzene derivatives whose complexes show an absorbance maximum at 427 nm, in addition to that at 455 nm, when in the ferrous state (Franklin, 1971).

As a group, nitrogenous MI complexes differ from the non-nitrogenous in several respects. Foremost is their instability in the ferric state. However, in contrast to non-nitrogenous MI complexes, the nitrogenous MI complexes stabilize the CYP in the ferrous state and thus, after *in vivo* formation, are immediately visible in the microsomes, that is, they do not require dithionite addition to show the presence of an absorbance maximum at 455 nm. The main classes associated with the nitrogenous MI forming compounds are the amphetamine, SKF-525-A, dithionite unstable and nitroso related compounds (Franklin, 1974).

While the substrate, the metabolic route, and the source of microsomes all play a role in determining the kinetics of inhibition, a key factor is pre-incubation of the inhibitor prior to substrate addition. For the SKF 525-A class of compounds the effects of omitting preincubation are clear and

indicate straight forward competitive inhibition (similar V_{max} , dissimilar K_m). Preincubation of SKF 525-A and its analogues with hepatic microsomes change the kinetics of inhibition from competitive inhibition to non-competitive inhibition. In particular, clear non-competitive inhibition is seen with SKF 525-A and its secondary amine derivative (Testa and Jenner, 1981).

1.7 N-BUTANOL FRACTION (nBF) OF *DESMODIUM ADSCENDENS*

Desmodium adscendens is a plant used in herbal medicine for treating asthma, to aid in child birth, to improve lactation and to relieve the pain associated with dysmenorrhea. In studies to elucidate the scientific basis for the therapeutic effects of the plant, it was found that an extract from dried stem leaves of *D. adscendens*, made by solvent extraction with n-butanol, activated the cyclooxygenase enzyme and increased prostaglandin (PG) production (Addy and Schwartzman, 1995). Tyramine and hordenine, phenolic compounds of the β -phenethylamine type, which have been isolated from the plant, were evaluated alongside the plant extract as modulators of the cyclooxygenase enzyme. Both compounds were also found to activate the enzyme and increase PG production, especially prostaglandin E_2 (PGE_2), depending on the enzyme concentration and availability of co-enzymes. They were found to activate the enzyme more than the extract of the plant. Increasing the concentration of the nBF of the plant extract did not increase its activation of the cyclooxygenase enzyme. It was inferred from these results that the plant material contained compounds other than the β -

phenethylamines which affected the type and quantity of the second messengers produced by the cyclooxygenase pathway of arachidonic acid (AA) metabolism. An attempt was therefore made at evaluating other compounds known to be present in the plant extract. Salsoline (Appendix AI:a), a 6-hydroxytetrahydro-isoquinoline derivative reported to be an alkaloid present in *D. adscendens* (Asante-Poku *et al.*, 1988), was not available during the experimental period. Therefore salsolinol (1-methyl-6,7-dihydroxy-1,2,3,4-tetrahydroisoquinoline), a 6,7-dihydroxy analogue was used.

Salsolinol decreased the amount of PGE₂ formed and increased that of PGH₂ and PGF_{2α} in either the presence or absence of GSH. It was also a much more effective activator of the cyclooxygenase enzyme as indicated by a higher metabolism of AA in its presence compared to that of nBF, tyramine or hordenine in the presence of GSH. The increased metabolism was in favor of PGH₂ and PGF_{2α} production.

In the cyclooxygenase pathway of AA metabolism (Fig. 1.4), the AA is converted to an endoperoxide 15-hydroperoxide prostaglandin G₂ (PGG₂). PGG₂ is then converted to the hydroxyl derivative PGH₂ by a GSH-dependent peroxidase. This is an indication that formation of PGH₂ from PGG₂ requires a reducing agent. This product of the cyclooxygenase pathway is converted to prostaglandin D, E and F as well as thromboxane (TX) or prostacyclin (PGI₂) by different specific enzymes (Fig. 1.4), whose

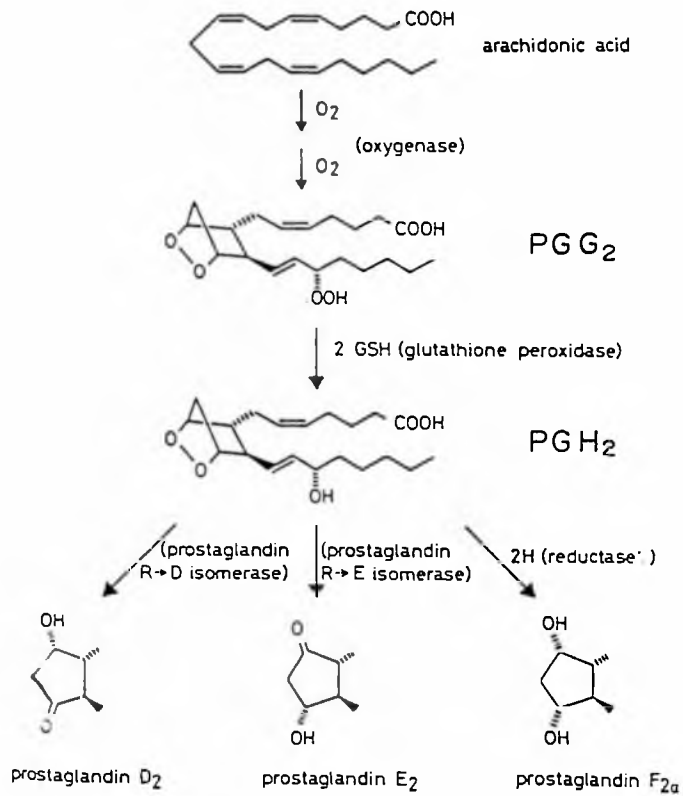


FIG. 1.4 PATHWAY OF PROSTAGLANDIN BIOSYNTHESIS

presence varies depending upon the cell type and tissue (Grew, 1992). The conversion of PGH_2 to $\text{PGF}_{2\alpha}$ utilizes a reductase implying that an additional reductant is required as a co-enzyme by the cyclooxygenase enzyme system to convert AA to $\text{PGF}_{2\alpha}$. Co-enzymes known to be utilized by the cyclooxygenase enzyme for AA metabolism to produce various PGs include phenolic compounds, catecholamines, indole compounds and other reducing agents (Lands *et al.*, 1971). The effect of the nBF and other compounds on PG synthesis indicates that the fraction contains the type of reductant required for the reduction of hydroperoxide when PGG_2 goes to PGH_2 and that for the reduction of the carbonyl when PGH_2 is converted to $\text{PGF}_{2\alpha}$. These results suggest that salsoline, with the same basic structure as salsolinol is likely to be the compound in the plant which acts as a reducing co-enzyme in the redox enzyme systems in this pathway of AA metabolism.

The nBF has also been shown to inhibit AA metabolism by the CYP monooxygenase pathway (Addy and Schwartzman, 1992). Salsolinol, the 6, 7-dihydroxy analogue of salsoline, the tetrahydroisoquinoline compound in the plant, was found to inhibit AA metabolism by the NADPH-dependent CYP monooxygenase enzyme complex of the ER, just as the plant extract did. With AA as substrate, salsolinol inhibited the formation of the epoxides, EETs and HETEs in this NADPH-dependent CYP monooxygenase reaction.

The CYP monooxygenase-catalysed reaction starts with substrate binding to native ferric P-450 (Fig. 1.2) and this is followed by reduction to the

ferrous state, thereby allowing oxygen binding. If the CYP must be in the ferric (Fe^{3+}) state to bind the substrate, a reducing agent capable of reducing the Fe^{3+} of the cytochrome will prevent substrate binding and inhibit the reaction. With the reducing property indicated in the cyclooxygenase reactions, the nBF of *D. adscendens* could contain such an inhibitor.

The extract could also contain a type of inhibitor which interferes with the flow of electrons from NADPH to CYP, the rate determining step that activates molecular oxygen for this type of oxygenation reaction. The extract may also react with either the substrate, making it unavailable, or act as a co-substrate, replacing the NADPH as the electron donor/co-reductant in the mixed-function oxygenase reaction.

In a previous study in which an attempt was made to find out the mode of action of salsolinol in this inhibition of all the products of the NADPH-dependent AA oxygenation (Addy and Schwartzman, 1992), the tetrahydroisoquinoline (THIQ) compound was found to reduce cytochrome c directly, with a reducing potential similar to that of reduced glutathione (GSH) (Kamassah, 1992). It was inferred from the results that the mode of action of salsolinol and hence salsoline in the plant, was to reduce CYP and keep it in the reduced state, thereby interfering with the oxidation/reduction cycle required for its function.

The hepatic CYP1A1 isoenzyme which is inducible by β -naphthoflavone was also shown to be non-competitively inhibited by the nBF of an aqueous extract of *D. adscendens* when 7-ethoxyresorufin was used as an artificial substrate in an EROD assay (Brookman-Amisshah, 1994). In this reaction, 7-ethoxyresorufin is deethylated to resorufin, as shown in Appendix AII.

The CYP isoenzyme responsible for AA oxygenation is different from CYP1A1. Therefore, inhibition of both by nBF signifies a mode of inhibition more related to the general reaction mechanism such as the reduction from Fe^{3+} to Fe^{2+} . The study reported here was designed to test the reducing properties of nBF and its interactions with CYP and substrates using spectral changes as the method of investigation.

MATERIALS AND METHODS**2.1 MATERIALS****2.1.1 Chemicals and Reagents**

Butanol, sodium potassium tartrate, potassium chloride, sodium chloride, sodium carbonate, sodium hydroxide, dithiothreitol (DTT), glycerol (87%), silica gel 60, methanol and potassium dihydrogen phosphate were purchased from Fluka Chemie AG, Switzerland. Sodium dithionite, sodium dihydrogen orthophosphate and chloroform were obtained from British Drug House Chemical Ltd., England. Ethylenediaminetetra-acetic acid (EDTA) and Folin-Ciocalteu's phenol reagent were purchased from Hopkins and Williams, England. Copper sulphate (anhydrous) was obtained from Merck Darmstadt, FRG. Bovine serum albumin, β -naphthoflavone, glutathione (reduced), nicotinamide adenine dinucleotide phosphate (NADPH), salsolinol, resorufin, cytochrome c and ethoxyresorufin were obtained from Sigma Chemical Co., U.S.A. Tris-(hydroxymethyl) amino methane was obtained from Eastman Kodak Co., U.S.A. Carbon monoxide gas was purchased from Union Carbide, Belgium. Soyabean oil was obtained from the local market. Dry stem leaves of *Desmodium adscendens* were obtained under cocoa trees at the Cocoa Research Institute of Ghana (CRIG). All chemicals were of the highest commercial grade available.

2.1.2 **Animals**

White Wistar rats, black C57BL/ks J dbm and white DDY mice were bred at the Animal Experimentation Unit of the Noguchi Memorial Institute for Medical Research (NMIMR) at Legon. All animals were fed on pelleted feed from the NMIMR. The composition of the feed was 40% wheat bran, 40% maize, 2% fish meal, 17% cod liver oil, and 1% sodium chloride.

2.2 **METHODS**

2.2.1 **Preparation of Buffers and Solutions**

Buffers and solutions were prepared as described in Appendix B.

2.2.2 **Preparation of n-butanol fraction of *D. adscendens***

Crude aqueous extract of *D. adscendens* was prepared by boiling the pulverized dry leaves for approximately 4 hours in water and allowing to cool. The mixture was centrifuged to remove solid particles. The supernatant was collected and the pellets discarded. The supernatant was concentrated to approximately 20.0ml by a Rotary Evaporator.

The crude extract was shaken three times with 3 volumes of water-saturated n-butanol. The organic layer was pooled after 3 extractions, the solvent removed (by evaporation) and the residue taken up into 10.0ml of water. It was then freeze-dried on an Eyela Freeze Dryer FD-1. The freeze-dried n-butanol fraction of *D. adscendens* (nBF) was stored in a refrigerator when not in use.

2.2.3 Fractionation of nBF

Approximately 0.4g of the freeze-dried nBF was dissolved in 5.0ml of water and coated on 1.6g of silica gel 60 (40-60 μ m, 230-400 mesh). The silica gel was dried in a rotary evaporator and added to the top of a column (30 x 500mm) containing 33g of silica gel (same mesh) packed in n-butanol. 150 ml of five different n-butanol based solvents of graded polarity were flashed through the column sequentially. Each eluent was collected in bulk, evaporated to get rid of the solvent, and taken up in equal volumes (approx. 5ml) of double distilled water.

The solvents used in the flash chromatography were as follows: (1) n-butanol; (2) water-saturated n-butanol; (3) 5% MeOH in water-saturated n-butanol; (4) 10% MeOH in water-saturated n-butanol; (5) 20% MeOH in water-saturated n-butanol. The scheme used in the fractionation of the crude aqueous extract from *Desmodium adscendens* is shown below in Fig. 2.1.

Values in parentheses are yields expressed as percentage of weight of material taken for the step in the fractionation scheme. Fractions with reported bioactivity (Addy, 1989) are marked with an asterisk. The method used in this separation is that according to Still *et al.*, (1978).

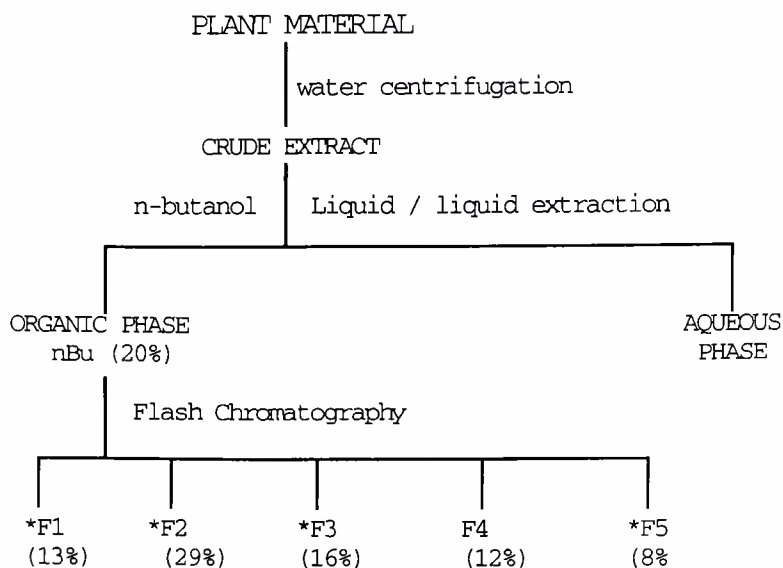


FIG. 2.1 SCHEME USED IN THE FRACTIONATION OF THE CRUDE EXTRACT FROM *D. ADSCENDENS*

2.2.4 Induction of CYP Monooxygenase in Mice

Eight mice were injected intraperitoneally once daily for 3 days with β -naphthoflavone (ENF) (80 mg/kg body weight), prepared by sonication in soyabean oil. The mice were sacrificed on the fourth day. The control animals were injected with the same volume of soyabean oil minus β -naphthoflavone.

2.2.5 Preparation of Microsomes

A modified method of Omura and Sato, 1964 was used in this preparation. The experimental animals were killed by strangling. Their livers and kidneys

were removed and weighed. The tissues were excised into small pieces with a pair of scissors, rinsed in 0.9% NaCl and blotted dry. 2 times volume of homogenizing buffer (Appendix BI) was added in a centrifuge tube and the excised tissues were homogenized with a polytron kinematic homogenizer (6 strokes).

The homogenate was centrifuged at 10,000g for 10min. at 4°C using a Hitachi 20FR-52D Centrifuge. The pellet was discarded and the supernatant centrifuged at 16,800g for 10min. at 4°C. The supernatant obtained, often referred to as the S-9 fraction, was centrifuged at 40,000rpm (105,000g) for 1 hour in a Hitachi 80P-7 automatic preparative Ultracentrifuge (Rotor RP65T-453) at 4°C. When rats were used as the experimental animals, the pellets were washed in homogenizing buffer to remove any hemoglobin not removed earlier and to clean microsomes of cytochrome b_5 contamination which often interferes with CYP spectral studies. The washed pellets were then re-homogenized in 1 volume of resuspension buffer (Appendix BII) and distributed into cryotubes and stored at -84°C until they were used. Preparation of microsomes was carried out on ice to prevent enzyme degradation.

2.2.6 Protein Concentration Estimation

Protein was determined as described by Lowry *et al.*, (1951) in which absorbances of coloured complexes resulting from a reaction between alkaline copper-phenol reagent and tyrosine and tryptophan are measured at

750 nm.

Protein reaction was carried out as follows: 0.4ml microsomal preparation (diluted 1:20 with 0.5M NaOH) was made up to a final volume of 1.0ml with 0.5M NaOH. 5.0ml of alkaline copper reagent (Appendix BIII) were added, mixed thoroughly by vortexing, and allowed to stand for 10 minutes. 0.5ml of a 1:1 diluted solution of Folin-Ciocalteu phenol reagent (1N) was added and mixed immediately and completely using a Vortex Mixer. After 30 minutes of incubation, the absorbance was read at 750 nm on a Shimadzu UV-190 double beam Spectrophotometer, after zeroing with a blank containing all the reagents except the microsomes. This determination was done in duplicate. The microsomal protein concentration was directly interpolated from a standard curve constructed with bovine serum albumin (BSA).

2.2.7 **Ethoxyresorufin O-Deethylase (EROD) Assay**

The EROD assay was performed in order to ascertain the induction of CYP1A1. The EROD measurements were performed according to the method of Mayer and Burke (1974). The instrument was standardized with resorufin and an emission scan for resorufin was run to determine the wavelength of maximum fluorescence. The EROD reaction was carried out in a fluorometer cuvette as follows: 1.975ml of EROD buffer, 10 μ l 7-ethoxyresorufin (0.41 μ M) and 5 μ l of the liver microsomal preparation from the non-injected white mice (NIWM) were mixed thoroughly in the cuvette. 10 μ l of NADPH (10mM) were added to the mixture, and the amount of resorufin formed with time was recorded for

a time interval close to 2 minutes. The reaction was spiked with 10 μ l of the working solution of resorufin (17.9 μ M), and the change in fluorescence recorded for a time interval close to 1 more minute. This served as the control experiment. The reaction was repeated using 5 μ l of the microsomal preparation from the BNF-injected white mice (BIWM). The volume of the buffer was adjusted accordingly to give a final reaction mixture of 2.0ml.

EROD activities of microsomal preparations from the non-induced black mice (NIEM) and BNF-injected black mice (BIEM) - both of the C57 BL/ksJ dm strain were also determined. Two different volumes of microsomal preparations 4.6 and 4.750 μ l were used for the BIEM and NIEM respectively.

The velocity of the reaction using induced CYP monooxygenase was

calculated as follows:
$$\text{Velocity} = \frac{S \times c}{t \times R}$$

A diagram of a printed tracing from the fluorimeter is shown in Fig. 2.2.

In order to evaluate the inhibitory effect of certain reducing agents on this system, the EROD assay was repeated using the same volumes and concentrations of reagents as in the control assay. After running for about 1 minute, 50 μ l of 50mM salsolinol were added to the reaction cuvette. The reaction was run for a further 1 minute before spiking with the working solution of resorufin. The assay was repeated, varying the volumes (concentrations) of salsolinol. Different volumes of the DAF1-5 of the nBF

obtained after Flash Chromatography were also used. Microsomal preparations from the two different strains of mice were used for the inhibition studies.

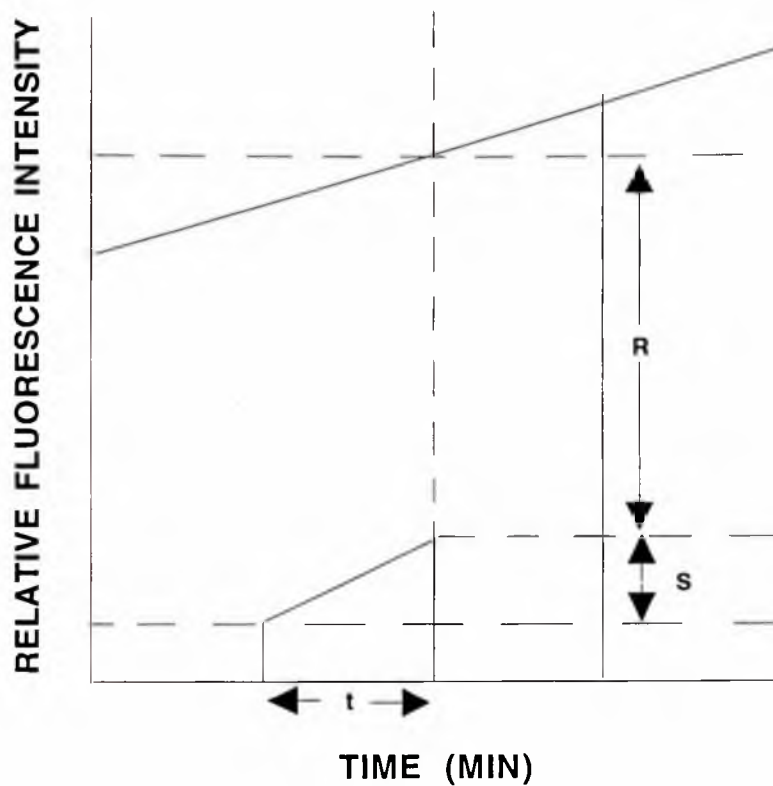


FIG. 2.2 DIAGRAM OF PRINTED TRACING FROM SFM-25
 FLUORIMETER PRINTER

S = fluorescence change due to reaction, measured in mm.

R = fluorescence change due to internal spike, measured in mm.

c = moles of resorufin in internal spike (179pmol)

t = time in min.

2.2.8 Determination of Cytochrome P-450 Content

Microsomes were diluted in the resuspension buffer (Appendix BII) to a final protein concentration of approximately 2mg/ml. A 5ml sample of the suspension was equally divided into 2 cuvettes with a 1-cm light path. Carbon monoxide was gently bubbled through the sample and reference cuvettes for approximately 4 minutes at a flow rate of approximately one bubble per second. After establishing a baseline by scanning between 400 nm and 500 nm at a speed of 0.2mm/sec, a few grains of solid sodium dithionite ($\text{Na}_2\text{S}_2\text{O}_4$) were added to the sample cuvette. The spectrum was then re-scanned from 400 nm to 500 nm.

The absorbances at 420, 450, and 490 nm were noted and these were used in calculating the CYP and P-420 concentrations as described by Omura and Sato (1964), using values of 91 and 110 $\text{mM}^{-1} \text{cm}^{-1}$ for molar extinction coefficient between 450 and 490 nm, and between 420 and 490 nm respectively.

2.2.9 Reduction of CYP

An attempt was made to ascertain whether the substrates used in this research, could reduce CYP directly. The experiments were performed as described above, but in place of sodium dithionite as the reducing agent, L-salsolinol, GSH and nBF were used.

In order to determine the rate of reduction of CYP, experiments were carried out as described in section 2.2.8 with 0.4mM NADPH solution as the reducing agent. After scanning and determining the CYP and P-420 contents, varying concentrations of L-salsolinol and nBF were added immediately. After 25 minutes, the CYP and P-420 contents were again determined after re-scanning the spectrum from 400 nm to 500 nm. In another set of experiments, varying concentrations of nBF were pre-incubated with the microsomal fraction before NADPH addition, and the rate of reduction of CYP determined for 25 minutes at 5 minutes interval. An equal volume of distilled water was used in place of the reducing agents as controls. In these experiments, after determining the rate of reduction of CYP, the spectrum was re-scanned from 400 nm to 500 nm and the CYP and P-420 contents determined.

2.2.10 **Reduction of Cytochrome c**

To 1.5ml of TRIS-HCl buffer, pH 7.4 and 1.9ml of cytochrome c solution (11.5 μ M), 0.1ml of saturated Na₂S₂O₄ was added. The absorption spectrum (between 330-700 nm) of the mixture was obtained using a Shimadzu UV-190 double beam spectrophotometer against a blank of 3.4ml TRIS-HCl buffer and an equal amount of saturated Na₂S₂O₄ (i.e 0.1ml). This absorption spectrum was recorded over one obtained without the addition of Na₂S₂O₄. The second scanning was done 15 minutes after the addition of the reducing agent. Different concentrations of nBF, salsolinol and DAF1-5 were used in place of sodium dithionite as reducing agents.

2.2.11 **Cytochrome (c) P-450 Reductase Assay**

The method used in this experiment is a modification of that according to Masters *et al.*, (1967). Microsomal preparation (approximately 10mg/ml) was used in this assay. 2.15ml of 0.1M phosphate buffer, pH 7.4 (Appendix BI), 250 μ l of oxidized cytochrome c (5mg/ml), and 0.1ml of the microsomal preparation were mixed in a spectrophotometer cuvette. The reaction was initiated by addition of 25 μ l (2% NADPH), and the absorbance change at 550 nm measured for 3 minutes at 30 seconds intervals on a Shimadzu double-beam Spectrophotometer UV-190, against a blank containing the phosphate buffer instead of NADPH. The effect of nBF on the reductase activity was studied by pre-incubating varying concentrations of the nBF for 3 minutes before the addition of NADPH. The reductive role of nBF was also investigated by initiating the reaction with nBF instead of NADPH.

2.2.12 **Binding studies**

The oxidized spectrum of the microsomal preparation was obtained and its interaction with the substrate 7-ethoxyresorufin (7-ER) and plant extract investigated. In these binding studies, microsomal preparations from mice pre-treated with ENF were used because CYP1A1, which is induced by ENF is specific for EROD activity. The metabolism of 7-ER to resorufin was used as a model for the binding studies. The spectrophotometric method of Klotz *et al.*, 1984 was used. For this method, a mixture consisting of 14.5 μ l (0.41 μ M) of 7-ER, 7.5 μ l microsomal preparation (10mg/ml) and 15 μ l NADPH

(100.5 μ M) in 3ml of 0.1M EROD buffer, pH 7.4 was matched against a control containing all the above except NADPH. The spectrum of the enzymatic product, resorufin was obtained by scanning the solution between 400-700 nm. Varying concentrations of nBF were added after product formation to determine its effect on resorufin after it has been formed.

In another set of experiments, varying concentrations of nBF were pre-incubated with the microsomes and substrate for 3-5 minutes before NADPH addition. nBF was added to both cuvettes to offset absorbances due to the plant extract. The spectrum of the microsomes and 7-ER pre-incubated with nBF was obtained before and after NADPH addition.

The spectrum of resorufin and 7-ER was obtained by scanning between 300-700 nm. Resorufin and 7-ER were dissolved in 0.1M Tris-HCl pH 8.0 containing 0.1M NaCl to give a final concentration of 1.6 μ M resorufin and 8.4 μ M 7-ER.

RESULTS

3.1 PROTEIN CONCENTRATION

The protein concentrations of the liver microsomal preparations were calculated from the calibration curve shown in Fig. 3.1 using a dilution factor of 1:20. The λ_{750} absorbances and the resultant protein concentrations are shown in Table 3.1.

Table 3.1 Absorbance measurements at 750 nm and protein concentrations of the microsomal preparations from the liver and kidney of the different laboratory animals

Sample	Absorbance	Protein concentration (mg/ml)
NIEML	0.196	17.0
BIEML	0.235	20.5
NIWML	0.187	16.14
BIWML	0.217	18.9
WWRL	0.177	15.51
WWRK	0.121	10.2

NIEML = non-induced black mice liver; BIEML = ENF-injected black mice liver; NIWML = non-induced white mice liver; BIWML = ENF-injected white mice liver; WWRL = white wistar rat liver microsomes; WWRK = white wistar rat kidney microsomes.

The amount of protein per gm. wet weight of liver for the microsomal preparations from the mice are presented in Fig. 3.2.

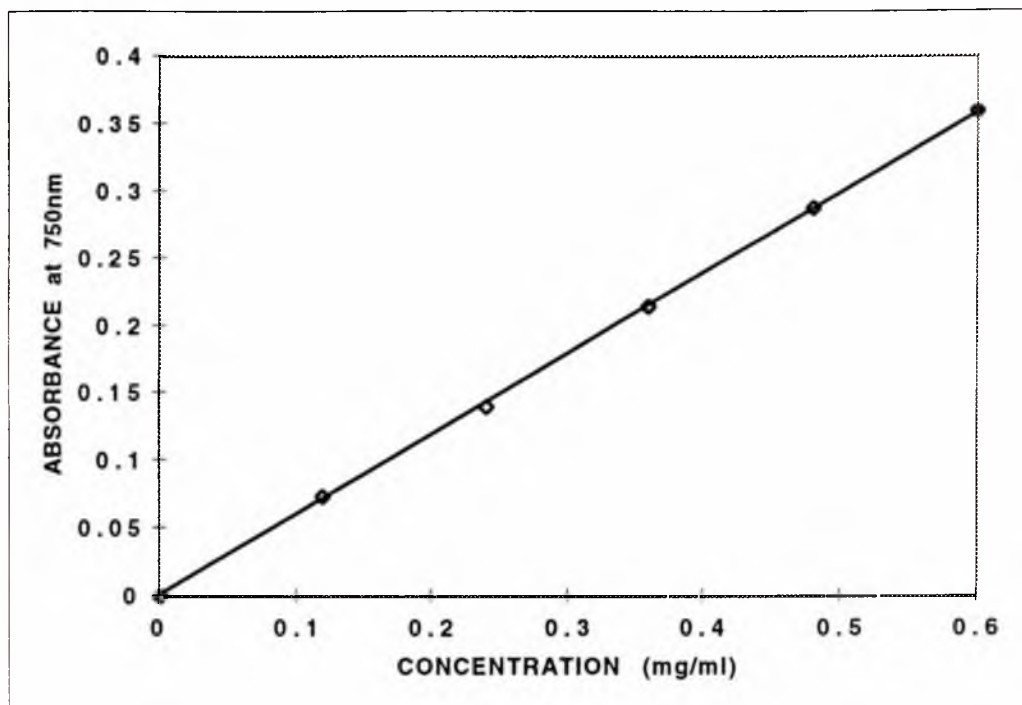
3.2 CYP AND P-420 CONTENT

Cytochrome P-448, a molecular species of CYP is induced by 3-methylcholanthrene and other polycyclic hydrocarbons (e.g. ENF). Its

reduced cytochrome-CO complex absorbs maximally at 448 nm. CYP of the ENF-injected mice (BIEM and BIWM) refers to P-448.

FIG. 3.1

CALIBRATION CURVE FOR PROTEIN DETERMINATION

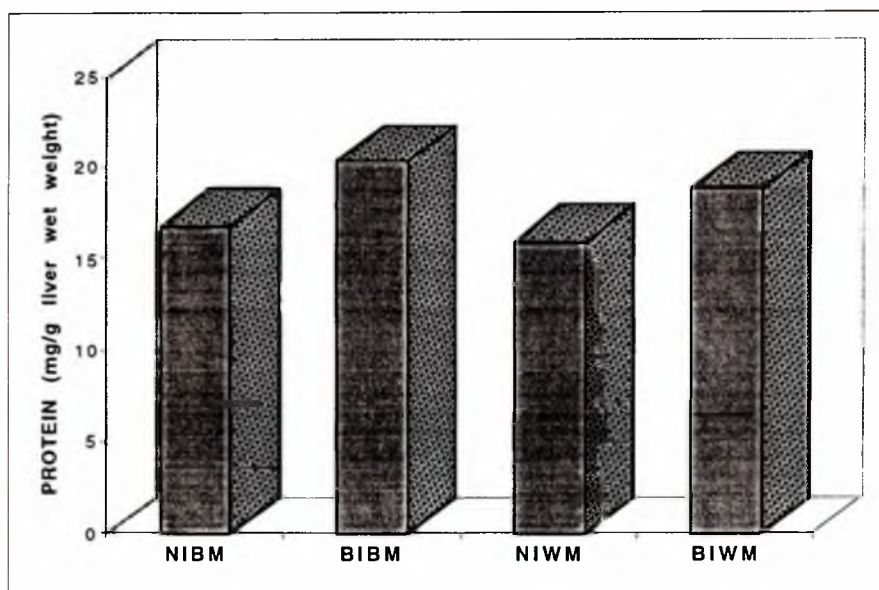


The CYP contents of the microsomal preparations were calculated using an extinction coefficient of $91 \text{ mM}^{-1} \text{ cm}^{-1}$ for the wavelength couple 450 (or 448) - 490 nm with dithionite as reducing agent. The results are shown in Fig. 3.3. As shown in Fig. 3.3, administration of ENF to the mice resulted in a greater than 2 fold elevation of the CYP content in both strains of

mice. The non-induced CYP values in both strains of mice were comparable. There appeared to be a higher induction of CYP in the black mice.

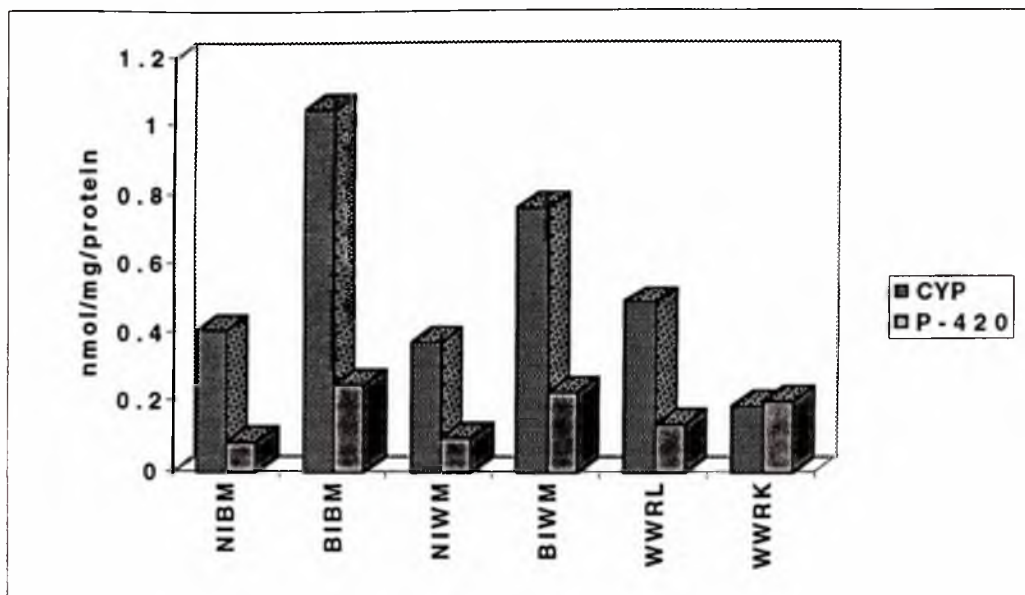
FIG. 3.2

TOTAL LIVER MICROSOMAL PROTEIN



- NIBM - Non-induced black mice
- BIBM - BNF-injected black mice
- NIWM - Non-induced white mice
- BIWM - BNF-injected white mice

FIG. 3.3

CYP AND P-420 CONTENTS OF MICROSOMAL PREPARATIONS

- NIBM** - Non-induced black mice
BIBM - BNF - injected black mice
NIWM - Non-induced white mice
BIWM - BNF-injected white mice
WWRL - White wistar rat (liver)
WWRK - White wistar rat (kidney)

The amount of cytochrome P-420 present in the microsomal preparations was also determined using an extinction coefficient of $110 \text{ mM}^{-1} \text{ cm}^{-1}$ for the wavelength couple 420 - 490 nm. (See Appendix C for sample calculation). The difference spectra of the dithionite reduced CYP-CO complex of the different microsomal preparations are shown in Fig. 3.4. There was a lower

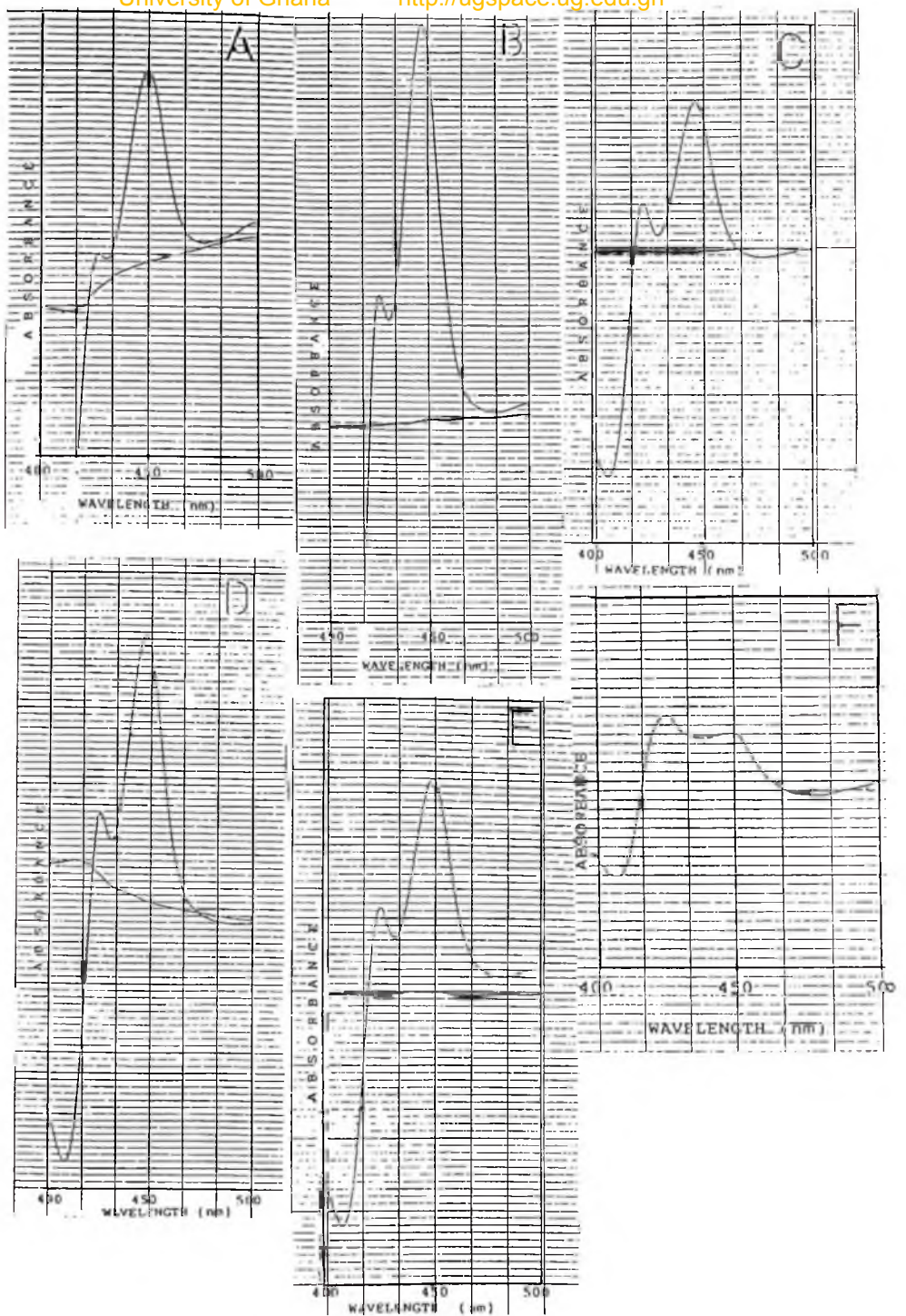


FIG. 3.4 CO-DIFFERENCE SPECTRA OF MICROSOMAL PREPARATIONS USING SODIUM DITHIONITE AS REDUCING AGENT. A: NIBML; B: BIBML; C: NIWML; D: BIWML; E: WWRL; F: WWRK

concentrations of CYP in the kidney as compared to that in the liver of the white Wistar rats.

3.3 EROD ACTIVITY

The excitation and emission maxima for the resorufin used were found to be 560 nm and 583 nm respectively. An emission scan is shown in Fig. 3.5. There was no response ($S=0$, Table 3.2) in the EROD assay when microsomal preparations from the non-induced mice, both black C57BL/ks J dbm and white DDY strains were used. Characteristic tracings are shown in Fig. 3.6 (Panels A&B). The tracings of the EROD reaction using the microsomal preparations from the ENF-injected mice are shown in Fig. 3.6 (Panels C&D). These showed an obvious increase in the amount of resorufin formed in the cuvettes, suggestive of a good enzyme activity.

Table 3.2 Values obtained from EROD assay using microsomal preparation from (A) black C57BL/ks J dbm mice, and (B) white DDY mice

Microsomal preparation	t (min)	S (nm)	R (nm)	Specific activity (pmol/min/mg. protein)
A: NIEM	1.1	0	27	0
BIEM	1.1	15	27.5	1441.3
B: NIWM	2.05	0	37	0
BIWM	1.95	23	40	1117.5

S = change in fluorescence due to the reaction; R = change in fluorescence due to the working standard; t = duration (time) of reaction.

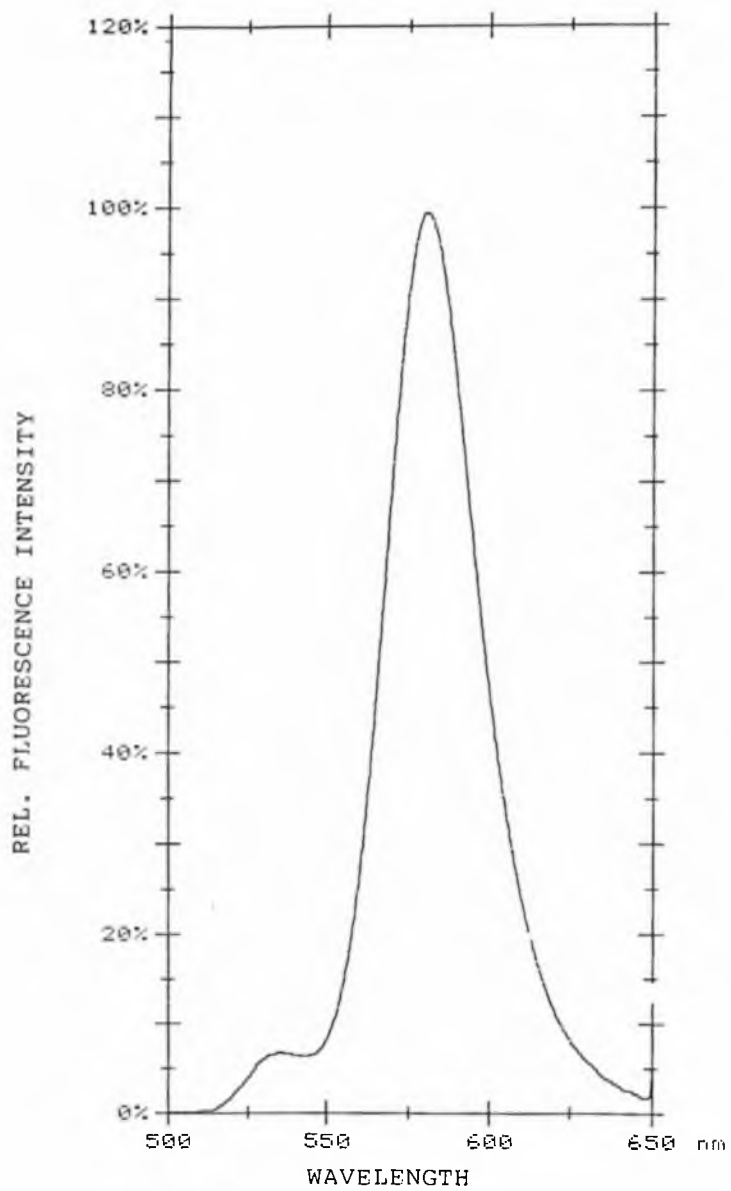


FIG. 3.5 EMISSION SCAN OF RESORUFIN

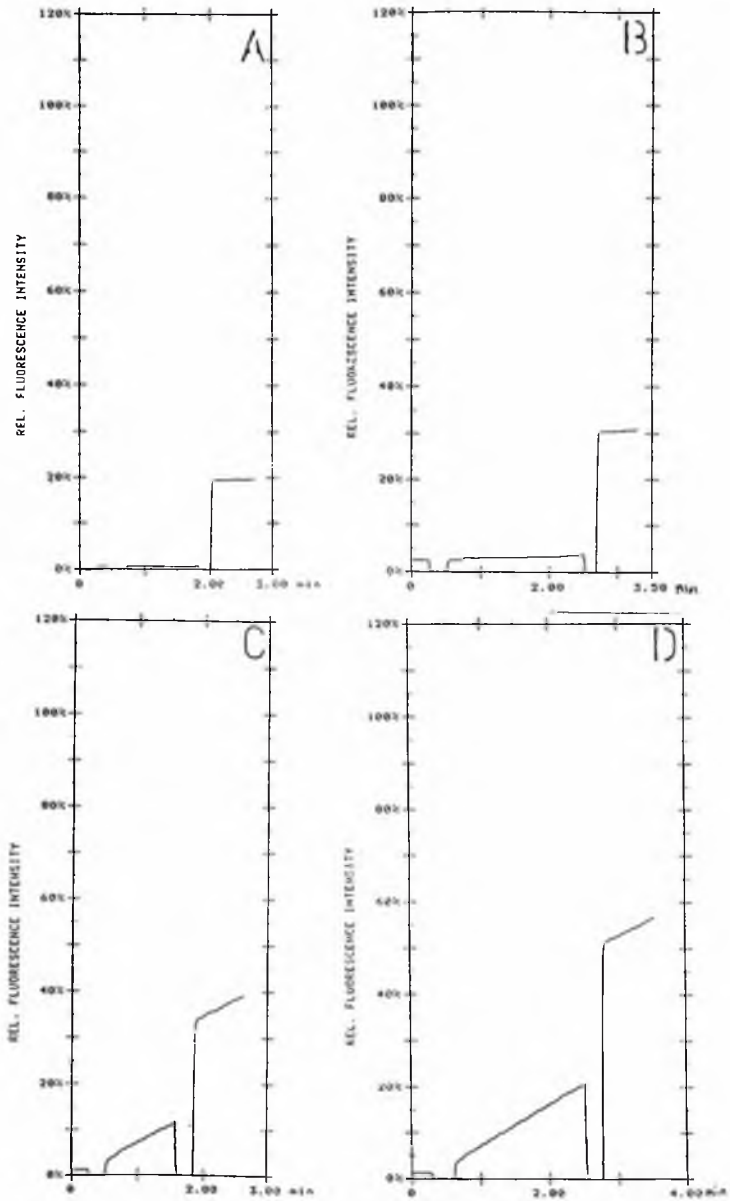


FIG. 3.6 FLUORESCENCE (EROD) RESPONSE OF A:NIBM; B:NIWM; C:BIBM; D:BIWM MICROSOMAL PREPARATIONS

3.3.1 **Effect of Flash Chromatography fractions of nBF**

Characteristic tracings of some flash chromatography fractions of nBF on the EROD activity of the microsomal preparations are shown in Fig. 3.7. Fig. 3.8 shows the percentage inhibition of EROD activity by the flash chromatography fractions of nBF. As can be seen in the figure, the inhibition by both DAF1 and DAF2 were biphasic for both BIWM and BIEM. The overall % inhibition was greater for DAF1 as compared to DAF2 even though concentrations of DAF2 were higher. Also the effect of DAF1 and DAF2 on the inhibition of EROD activity in BIWM was greater than in BIEM. For DAF3, the % inhibition increased with increasing concentration for both BIEM and BIWM even though the concentrations were lower compared to those of DAF1 and DAF2. These inhibitions were greater than those of DAF1 and DAF2.

3.3.2 **Effect of salsolinol**

Characteristic tracings of the effect of salsolinol on the EROD activity of the microsomal preparations are shown in Fig. 3.9A. Fig. 3.9B shows the percentage inhibition of EROD activity by the different concentrations of salsolinol. As shown in the figure, inhibition increased with increasing concentration of salsolinol. At the lower concentrations inhibition was greater in BIWM but as the concentration increased, that of BIEM became greater.

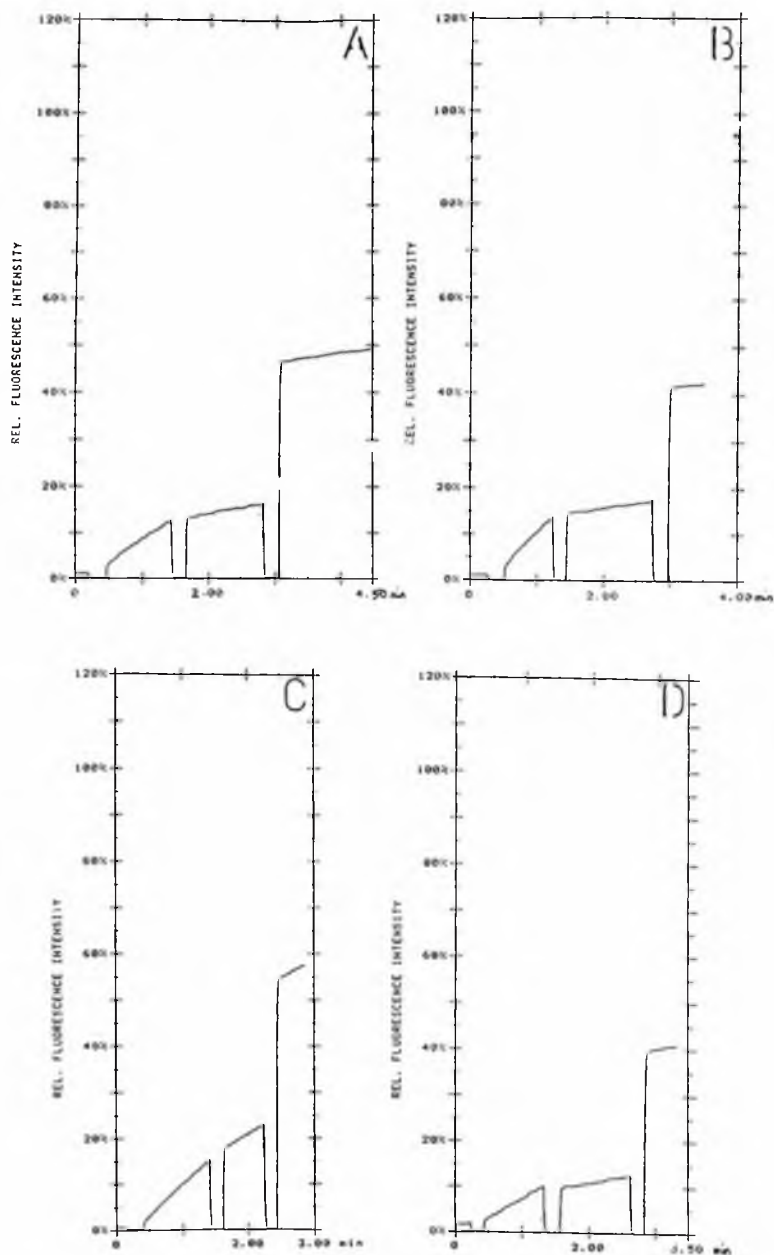
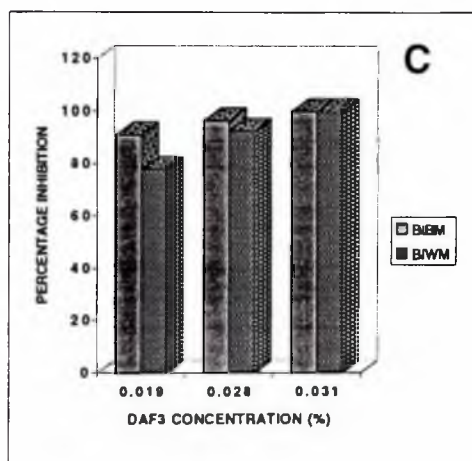
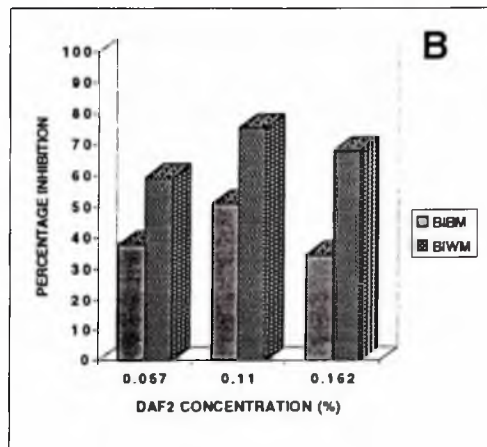
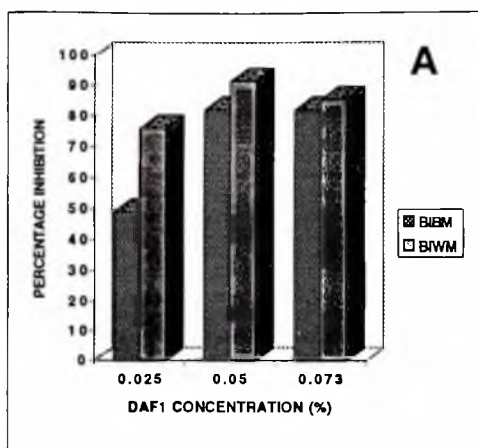


FIG. 3.7 EFFECT OF DAF1 (0.073%) AND DAF2 (0.11%) ON THE FLUORESCENCE (EROD) RESPONSE OF A: BIBM (DAF1); B: BIWM (DAF1); C: BIBM (DAF2); D: BIWM (DAF2) MICROSOMAL PREPARATIONS

FIG. 3.8

PERCENTAGE INHIBITION OF EROD ACTIVITY BY DIFFERENT CONCENTRATIONS OF (A) DAF1, (B) DAF2 AND (C) DAF3



BIBM - BNF - Injected black mice
BIWM - BNF - Injected white mice

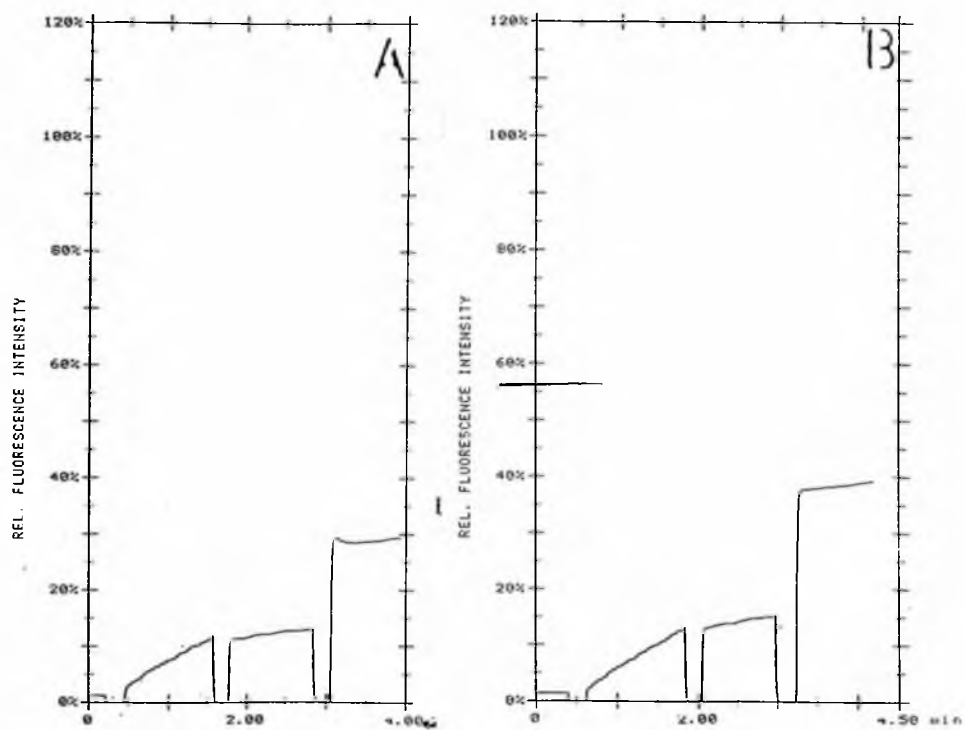
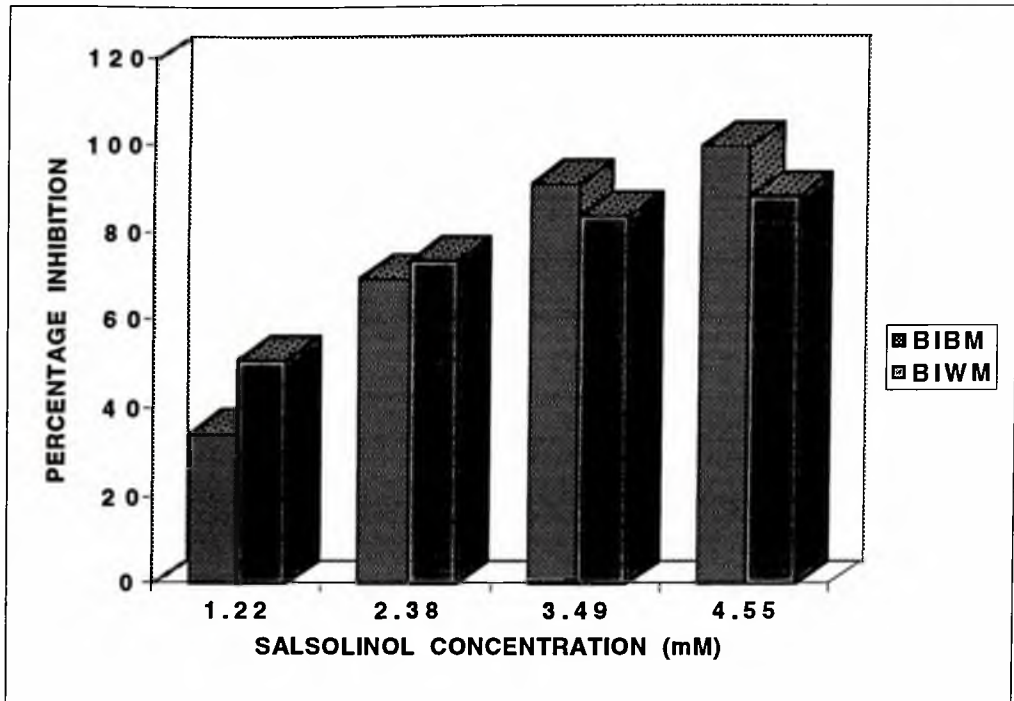


FIG. 3.9A EFFECT OF SALSOLINOL (2.38mM) ON THE FLUORESCENCE (EROD) RESPONSE OF A: BIBM; B: BIWM

FIG. 3.9B

**PERCENTAGE INHIBITION OF EROD ACTIVITY BY
DIFFERENT CONCENTRATIONS OF SALSOLINOL**

BIBM - BNF-injected black mice
BIWM - BNF-injected white mice

3.4 REDUCTION OF CYTOCHROME C

The addition of a reducing agent to an oxidized solution of cytochrome c leads to reduction of the cytochrome with a characteristic peak at 550 nm. Fig. 3.10 (Panel A) shows the spectra of the oxidized and reduced forms of cytochrome c, with characteristic bands at 408 and 530 nm for the oxidized

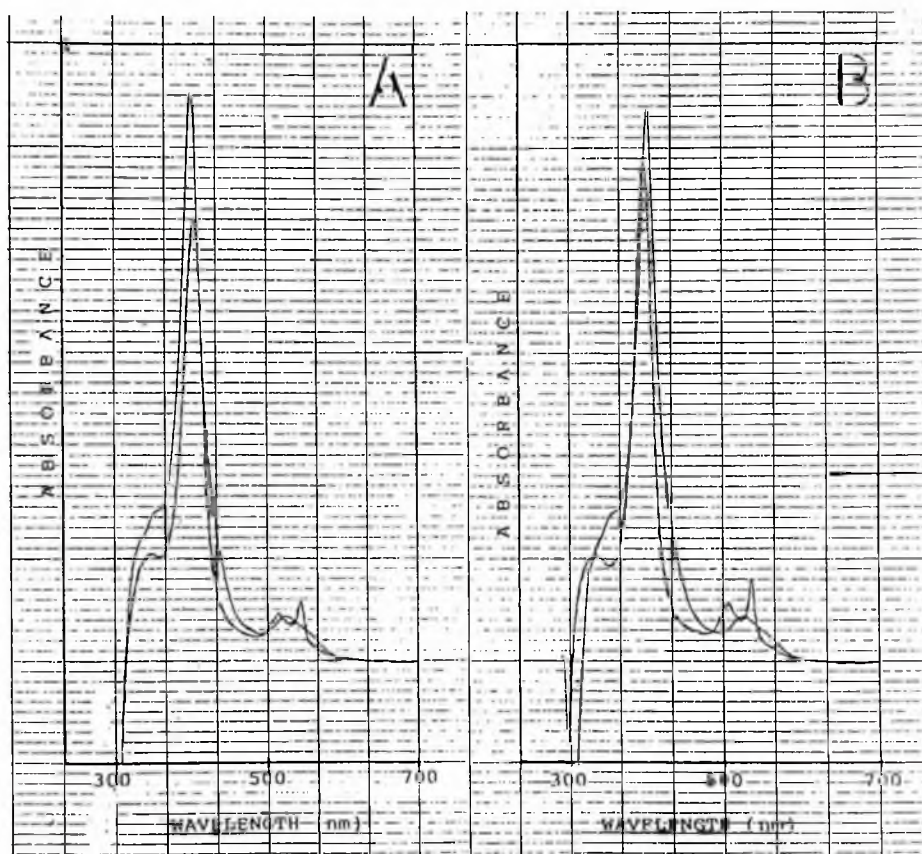


FIG. 3.10 SPECTRA OF OXIDIZED (—) AND REDUCED (----) CYTOCHROME C USING A: SATURATED SODIUM DITHIONITE (100 μ l) AND B: 1.43mM SALSOLINOL AS REDUCING AGENTS

(A) and 415, 520, and 550 nm for the reduced (B) form. The reducing agent used was saturated sodium dithionite (100 μ l). Fig. 3.10 (Panel B) shows the spectra of the oxidized and the reduced forms of cytochrome c, using 1.43mM salsolinol as the reducing agent. The α -band at 550 nm, is characteristic of reduced cytochrome c.

3.4.1 **Effect of nBF**

At lower nBF concentrations (0.006% and 0.012%) the oxidized solutions of cytochrome c did not show any reduction. However, at higher concentrations of 0.02 and 0.17%, there was a reduction. Fig. 3.11 shows the spectra of the reductions. At the lower concentrations, the prominent 550 nm peak peculiar to the reduced form of cytochrome c is clearly missing.

3.4.2 **Effect of Flash Chromatography fractions of nBF**

With the exception of Fig. 3.12 (Panels A&B) when 0.015% and 0.022% of DAF1 were used, all the other fractions did not show any reduction when added to the oxidized cytochrome c. A characteristic spectrum of cytochrome c before and after addition of DAF3 (0.018%) (one of the fractions which did not give any reduction), is shown in Fig. 3.12 (Panel C).

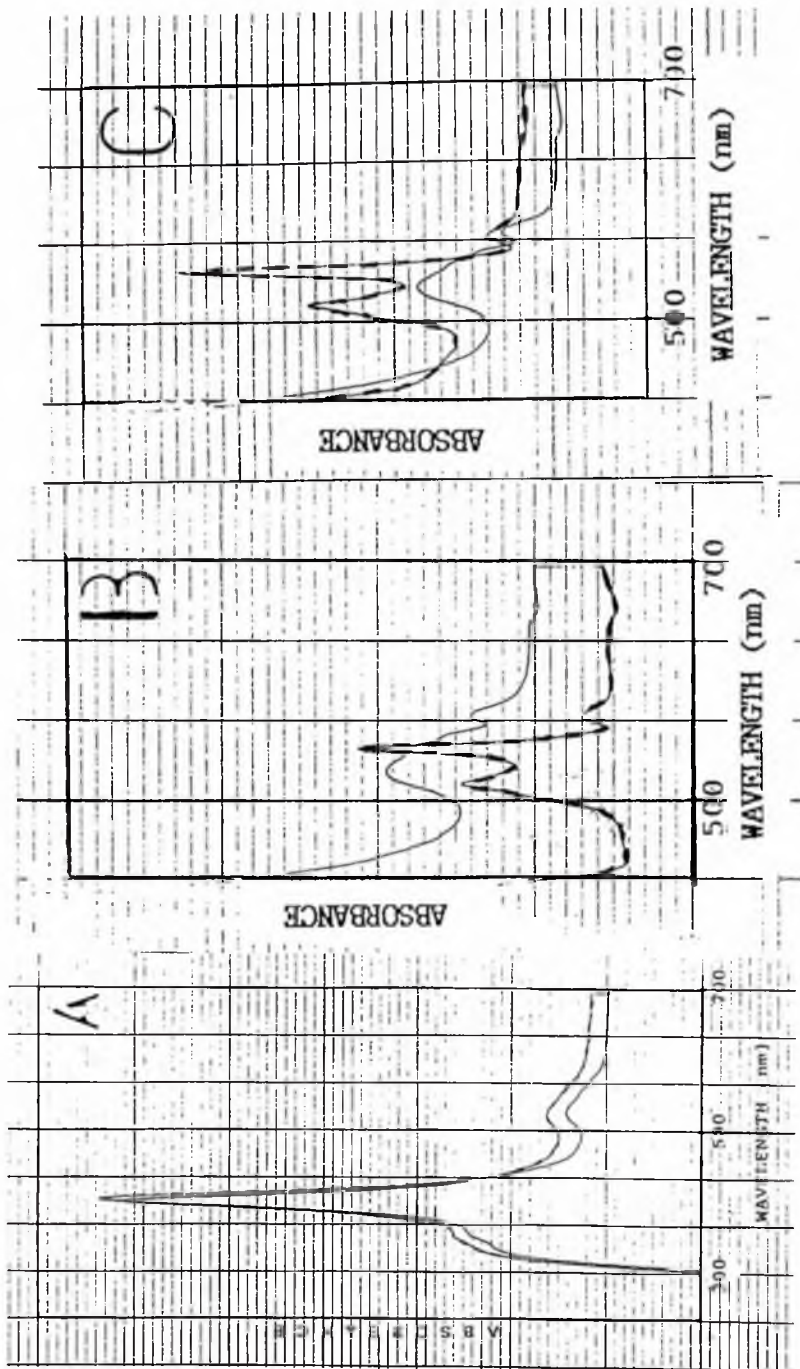


FIG. 3.11 SPECTRA OF OXIDIZED (—) AND REDUCED (- - -) CYTOCHROME C USING A:0.012%; B:0.02%; C 0.17% OF NBF AS REDUCING AGENT

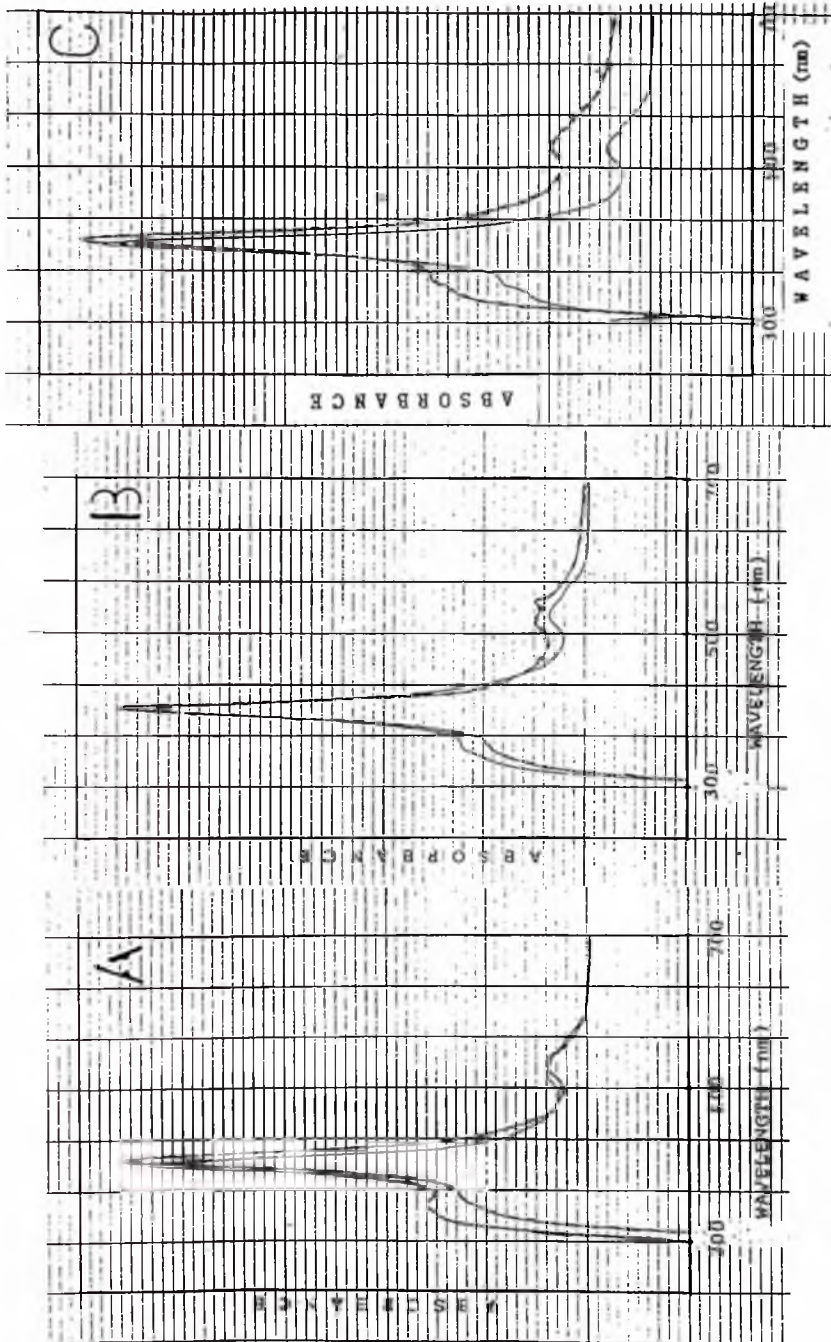


FIG. 3.12 SPECTRA OF OXIDIZED (—) AND REDUCED (- - -) CYTOCHROME C USING A:0.015% DAF1; B:0.022% DAF1; C:0.018% DAF3 AS REDUCING AGENT

3.5 REDUCTION OF CYP

3.5.1 Direct Reduction of CYP

The 450 nm peak resulting from the interaction between CO and reduced CYP was not observed, when different concentrations of nBF were used in place of sodium dithionite. Salsolinol and GSH, known to reduce cytochrome c, were also used and found not to produce the characteristic 450 nm peak. The difference spectra of CYP using nBF and these other compounds (salsolinol and GSH) as reducing agents are shown in Fig. 3.13 (Panels A-C).

3.5.2 Indirect effect on the Rate of Reduction of CYP

In these experiments, the CYP was reduced by the addition of NADPH and the effect of nBF on this reduction estimated. Both CYP and P-420 content were determined with CO binding. In one set of the experiments, the plant extract (nBF) was added after NADPH has been added and the amount of CYP and P-420 have been determined. The reduction was allowed to proceed for an additional 25 minutes, after which the CYP and P-420 were again determined. For comparison, the experiments were repeated with salsolinol in place of nBF.

Fig. 3.14 A&B show the difference spectra of the microsomal preparations before and after addition of different concentrations of nBF for NIEM and BIEM respectively. The levels of CYP and P-420 before and after addition of nBF are shown in Fig. 3.15A and 3.15B.

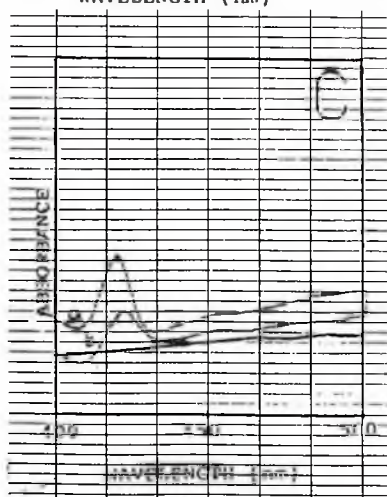
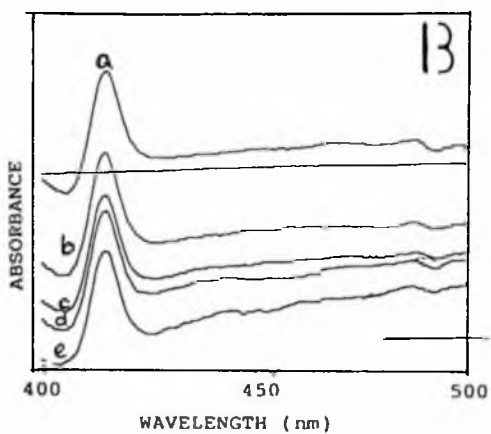
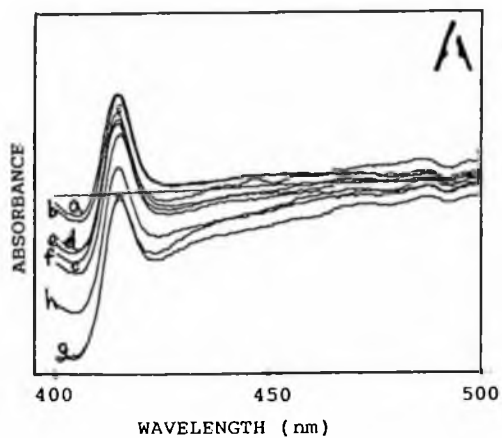


FIG. 3.13 CO-DIFFERENCE SPECTRA OF MICROSOMAL PREPARATIONS USING A: SALSOLINOL; B: GSH; C: nBF AS REDUCING AGENT. a---->h: INCREASING CONCENTRATION OF REDUCTANT

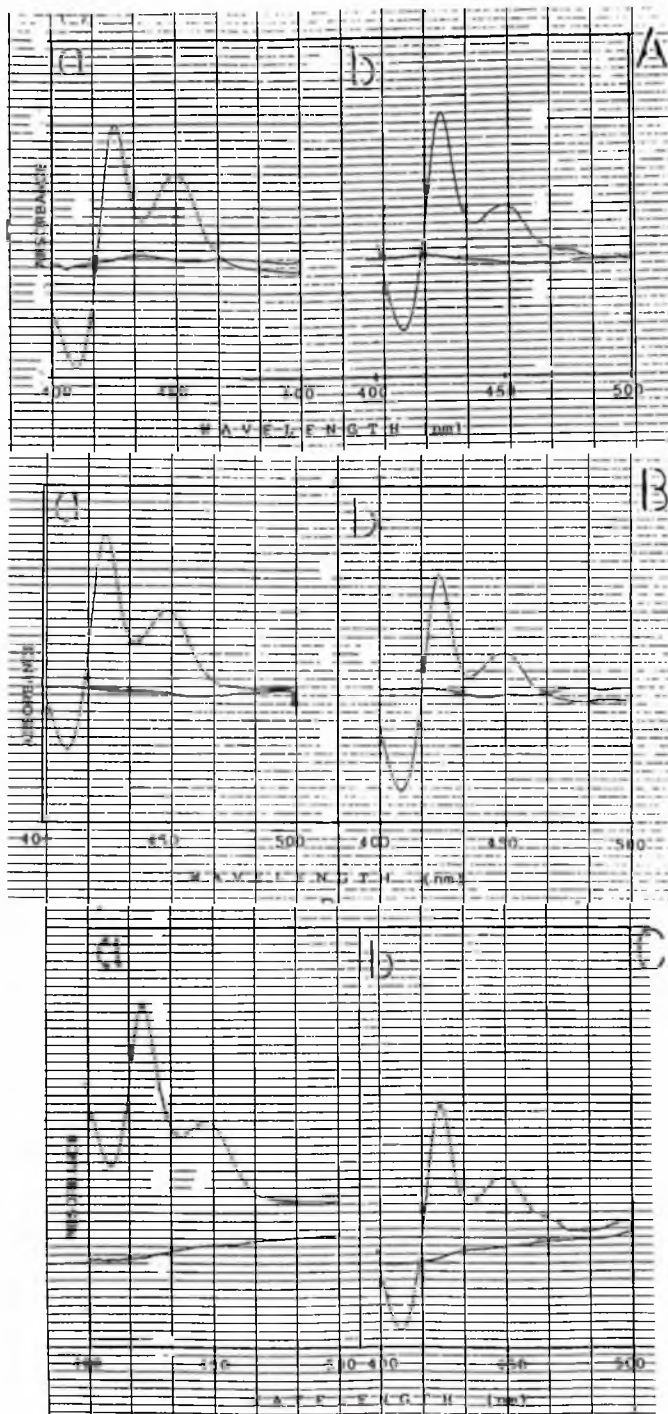


FIG. 3.14A CO-DIFFERENCE SPECTRA OF MICROSOMAL PREPARATIONS FROM NIBM (a) AFTER AND (b) BEFORE A:0.002%; B:0.004%; C:0.011% NBF ADDITION

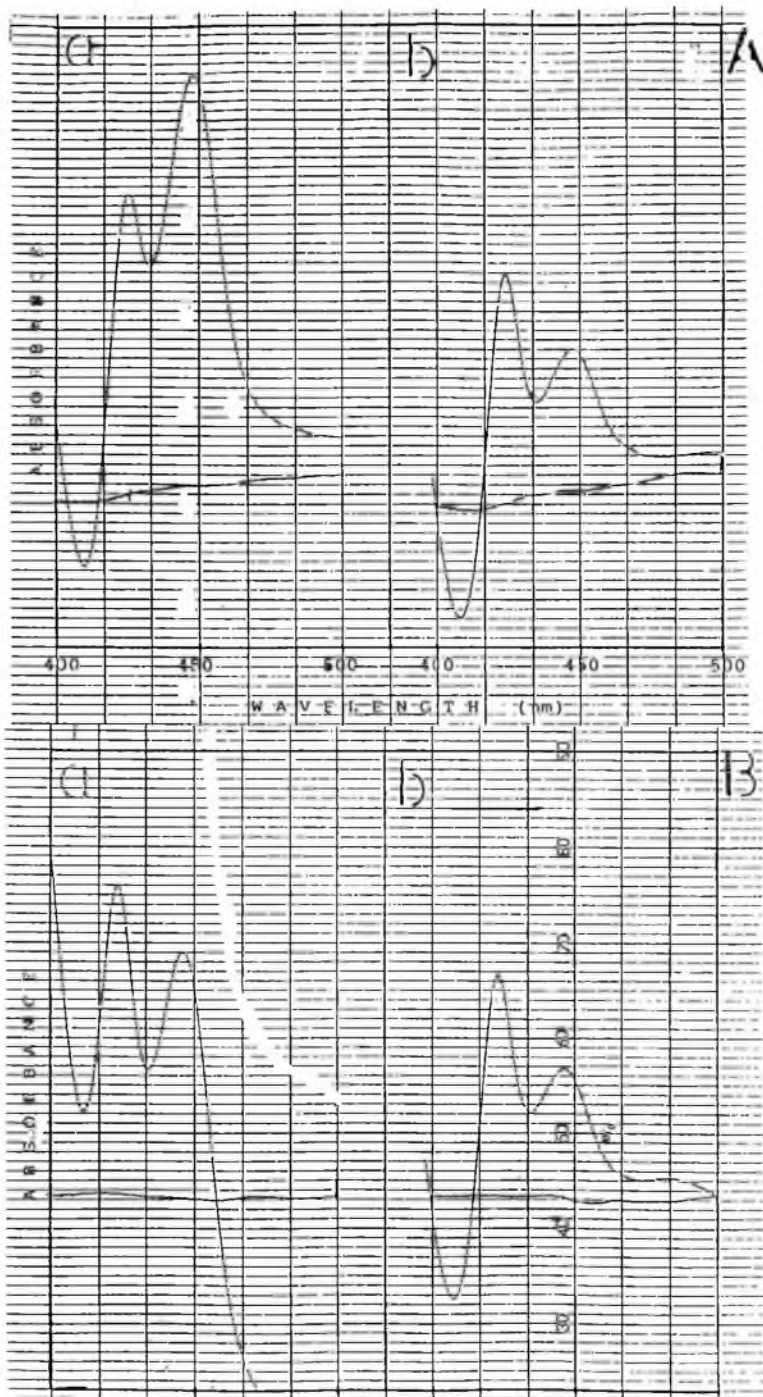


FIG. 3.14B CO-DIFFERENCE SPECTRA OF MICROSOMAL PREPARATIONS FROM BIBM (a) AFTER AND (b) BEFORE A:0.011%; B:0.04% nBF ADDITION

FIG. 3.15A

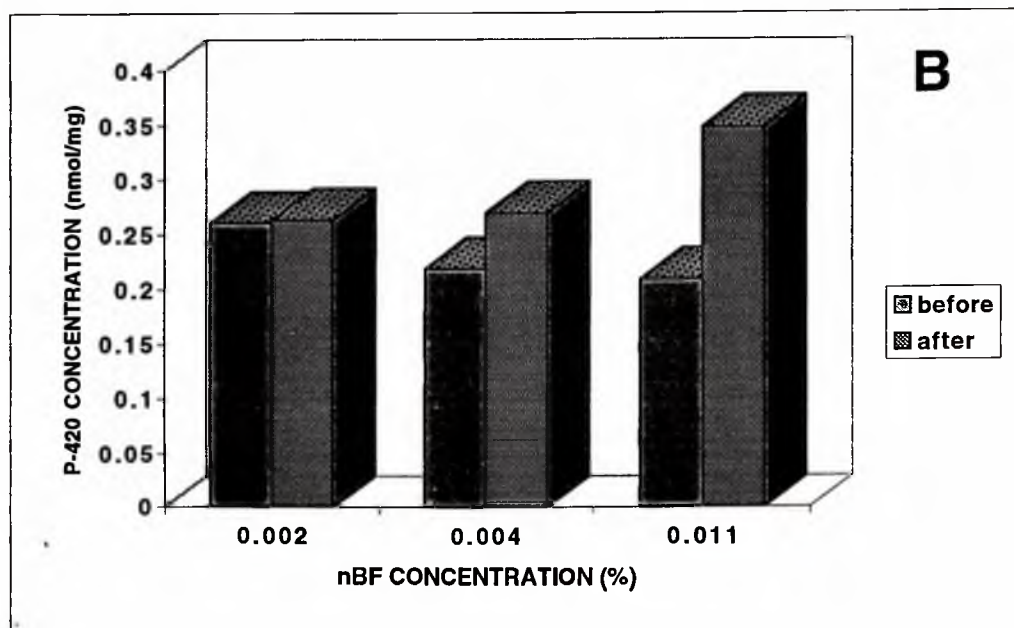
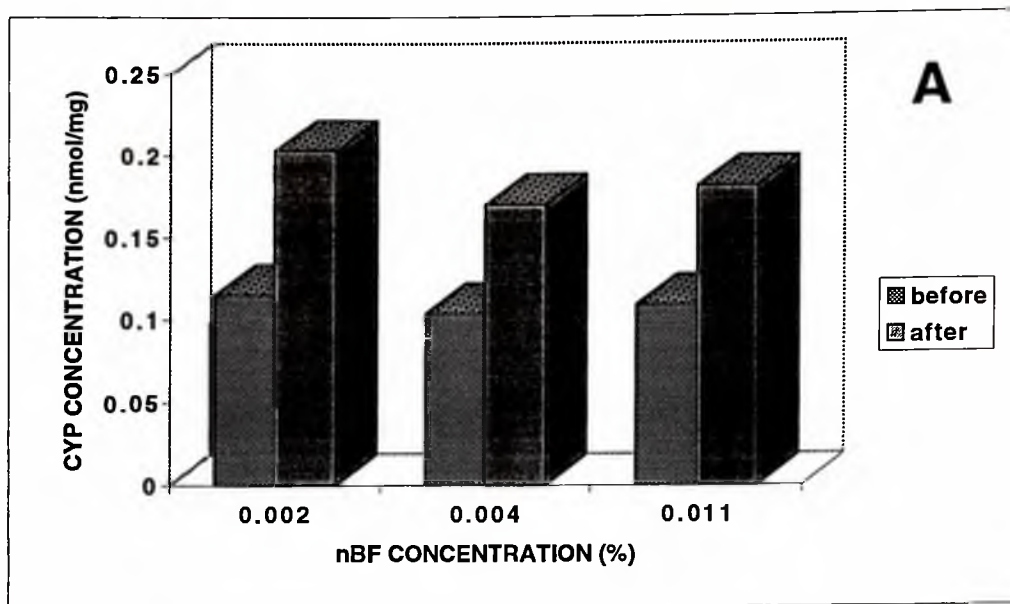
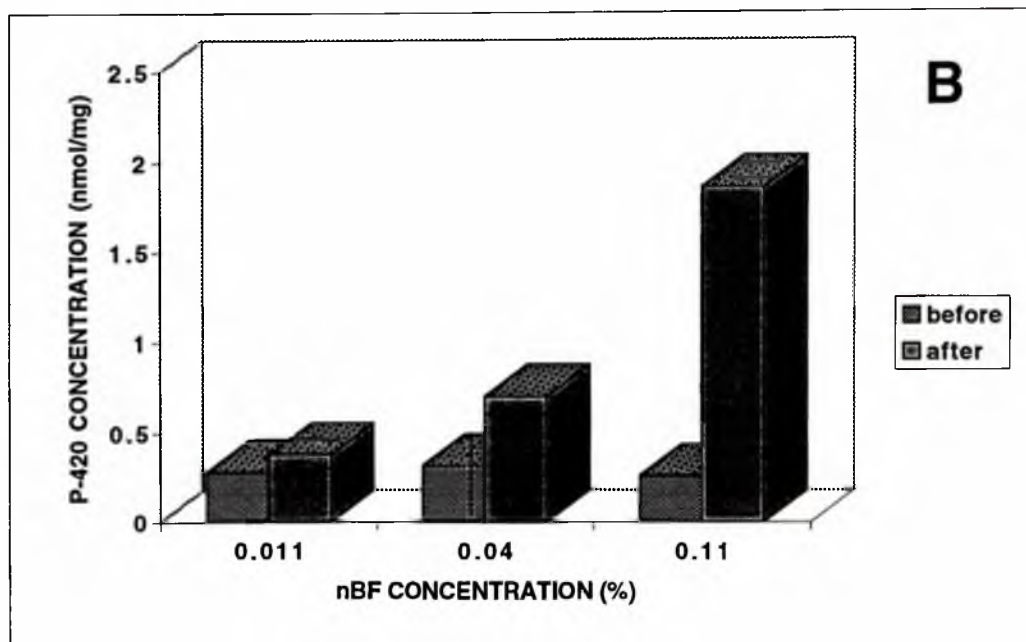
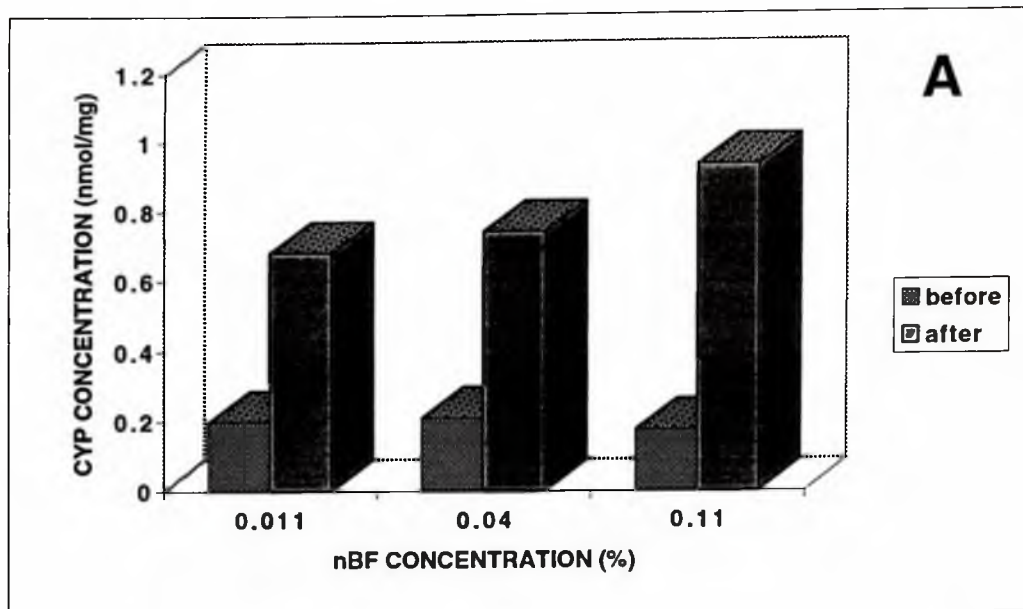
**LEVELS OF (A) CYP AND (B) P-420 IN NIBM LIVER
MICROSOMES BEFORE AND AFTER nBF ADDITION**

FIG. 3.15B

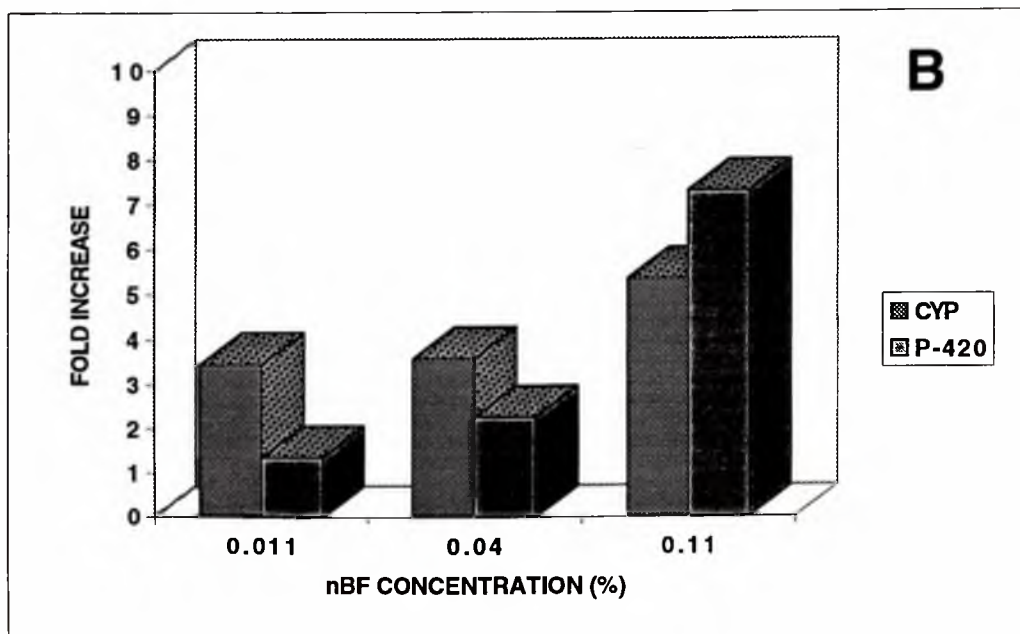
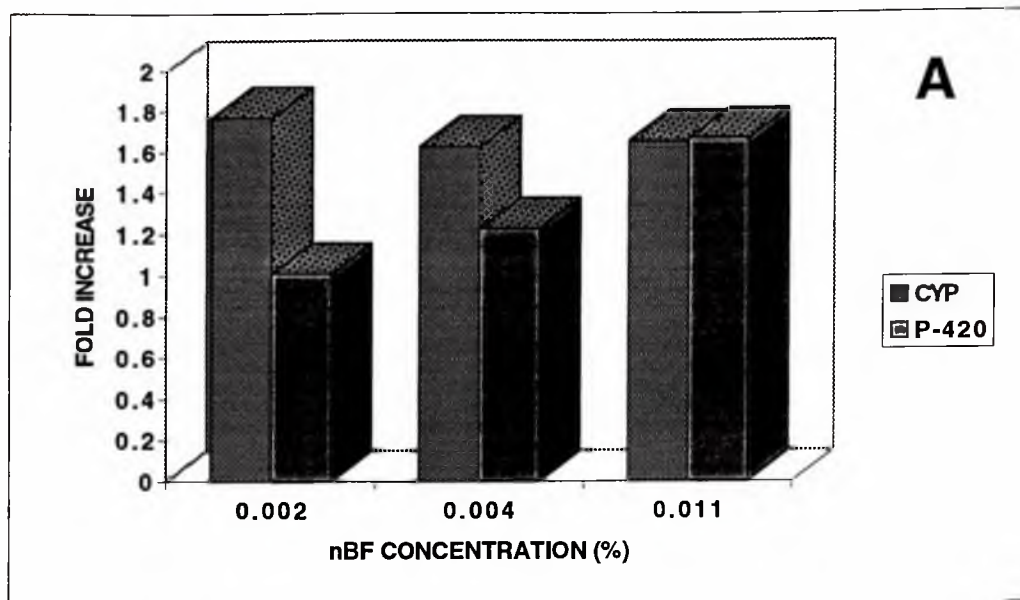
**LEVELS OF (A) CYP AND (B) P-420 IN BIBM LIVER
MICROSOMES BEFORE AND AFTER nBF ADDITION**

The fold increases of CYP and P-420 contents of the liver microsomal preparations after nBF addition are shown in Fig. 3.16. In both the NIEM and BIEM, the levels of CYP increased at lower concentrations of nBF more than P-420. But at a higher concentration, the P-420 level was greater than CYP in BIEM, whereas in NIEM they were almost the same.

Fig. 3.17 shows the difference spectra of the microsomal preparations before and after the addition of 0.57mM salsolinol. The levels of CYP and P-420 before and after addition of salsolinol to the microsomal preparations are shown in Fig. 3.18. CYP levels increased approximately by 2-fold and 4.5-fold, whereas P-420 levels decreased by 0.2-fold and 0.4-fold in NIEM and BIEM respectively. Unlike the nBF effect, the P-420 levels decreased with the addition of salsolinol.

In another set of experiments, the nBF was pre-incubated with the microsomes before NADPH addition. Immediately after the addition of NADPH, the rate of reduction of CYP was followed by recording the change in absorbance of the CYP-CO complex at 450 nm for 25 minutes at 5 minutes interval. The CYP and P-420 contents of the microsomal preparations were determined only once after this period. Table 3.3 shows the CYP and P-420 levels determined for these pre-incubation experiments.

FIG. 3.16

FOLD INCREASES IN CYP AND P-420 LEVELS IN (A) NIBM AND (B) BIBM LIVER MICROSOMES AFTER nBF ADDITION

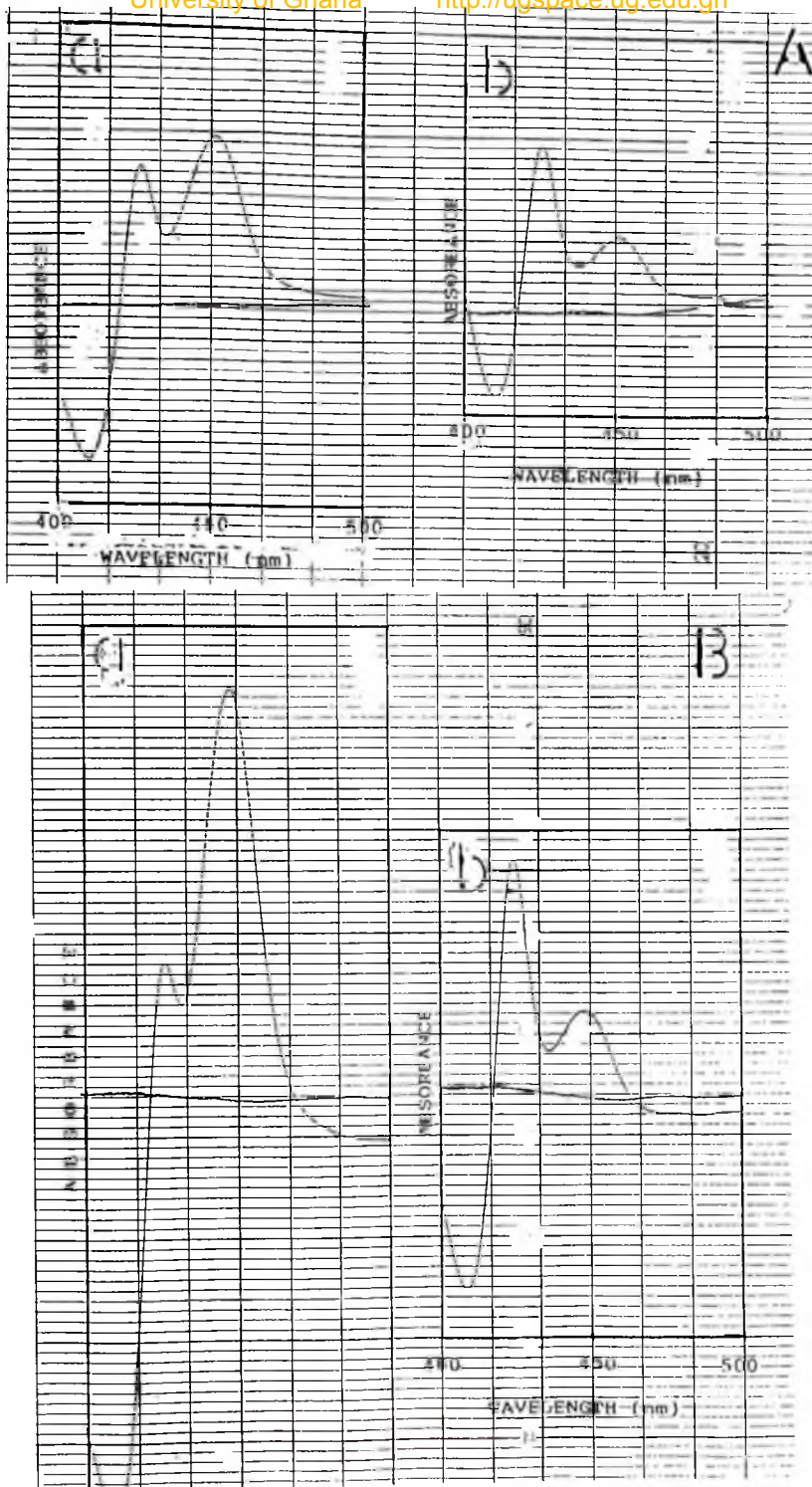


FIG. 3.17 CO-DIFFERENCE SPECTRA OF MICROSOMAL PREPARATIONS FROM A:NIBM AND B:BIBM (a) after AND (b) BEFORE 0.57mM SALSOLINOL ADDITION

FIG. 3.18

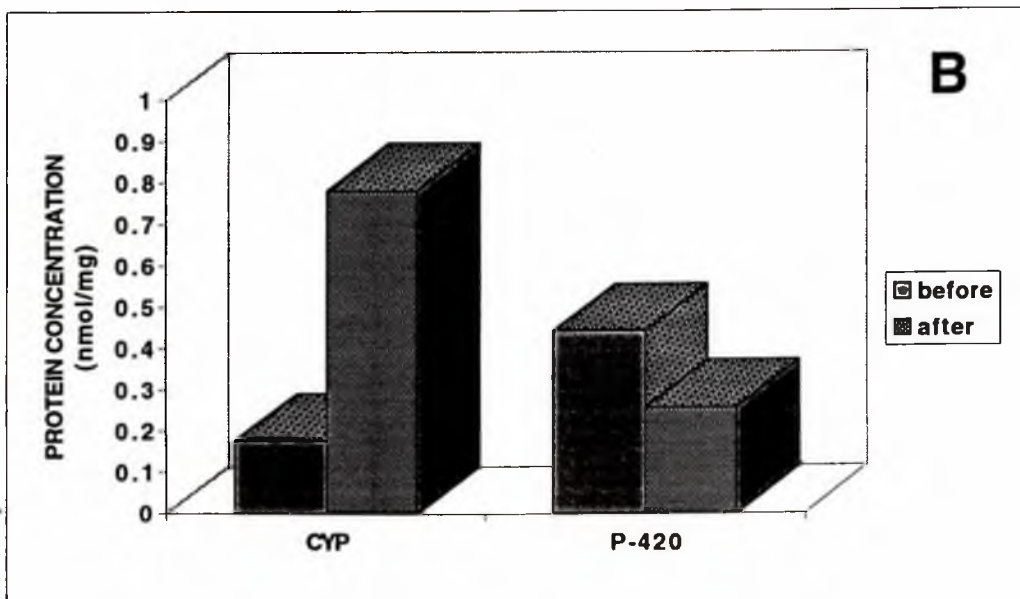
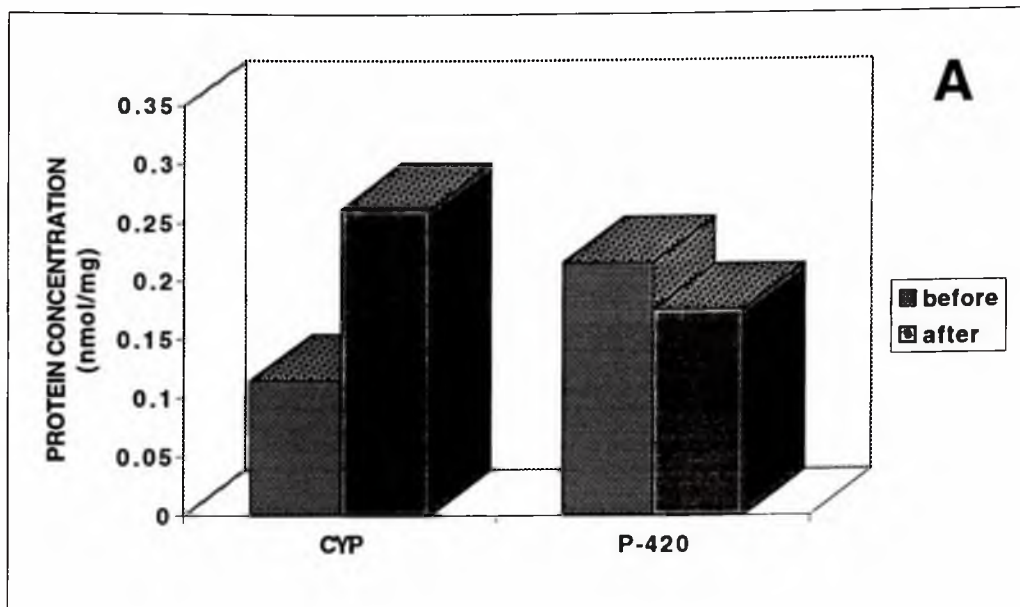
EFFECT OF SALSOLINOL (0.57mM) ON THE LEVELS OF CYP AND P-420 IN (A) NIBM AND (B) BIBM LIVER MICROSOMES

Table 3.3 Effect of nBF on CYP and P-420 levels in mice liver microsomes preincubated with nBF

Sample	Concentration of nBF	CYP (nmol mg ⁻¹ protein)	P-420 (nmol mg ⁻¹ protein)
A:NIEM	0.0%	0.242	0.191
	0.004%	0.247	0.300
	0.008%	0.275	0.295
	0.011%	0.341	0.327
	0.02%	0.450	0.500
B:BIEM	0.0%	0.929	0.295
	0.008%	0.970	0.459
	0.011%	0.857	0.486
	0.20%	1.044	0.573

0.0% nBF implies the addition of only NADPH

Fig. 3.19 shows the time-dependent reduction of CYP in NIEM and BIEM at different concentrations of the nBF. For NIEM, the rate of reduction increased with increasing concentration of nBF using NADPH as the reference level. In the case of BIEM, a similar pattern was observed but these increases were lower than the NADPH (reference level) except at 0.20% which reduced CYP to almost the same level as NADPH.

Fig. 3.20 & 3.21 show that pre-incubation increased the levels of CYP and P-420 in both NIEM and BIEM. Pre-incubation increased the P-420 levels clearly only in microsomes from BIEM.

FIG. 3.19

**TIME-DEPENDENT REDUCTION OF (A) NIBM AND
(B) BIBM LIVER MICROSOMES BY DIFFERENT
CONCENTRATIONS OF nBF (%) AND NADPH**

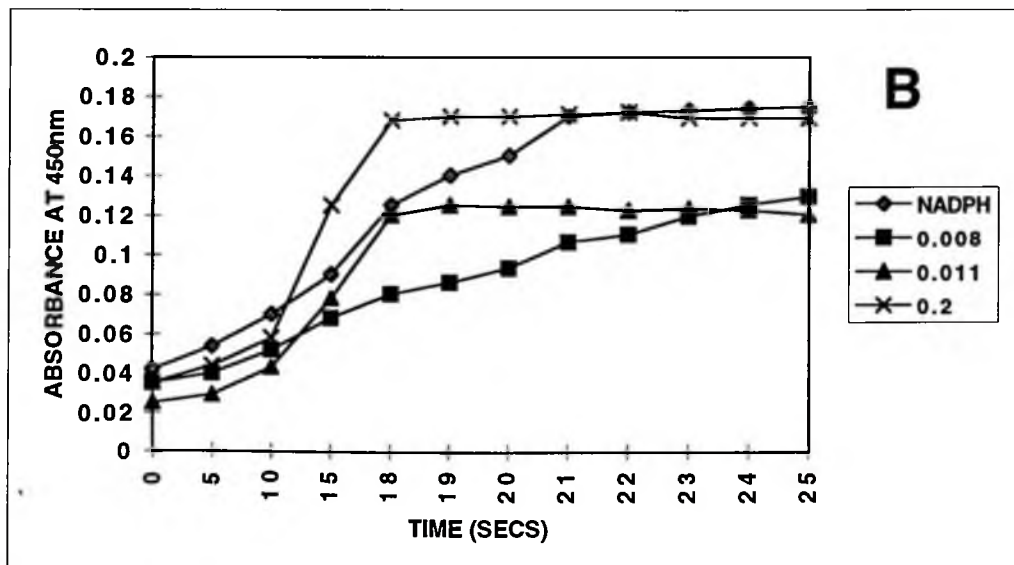
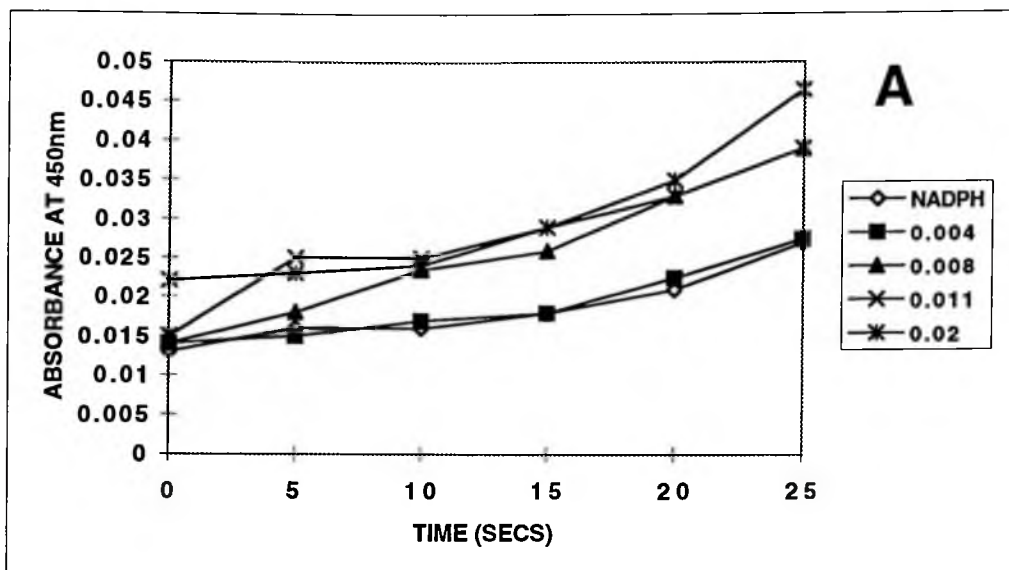
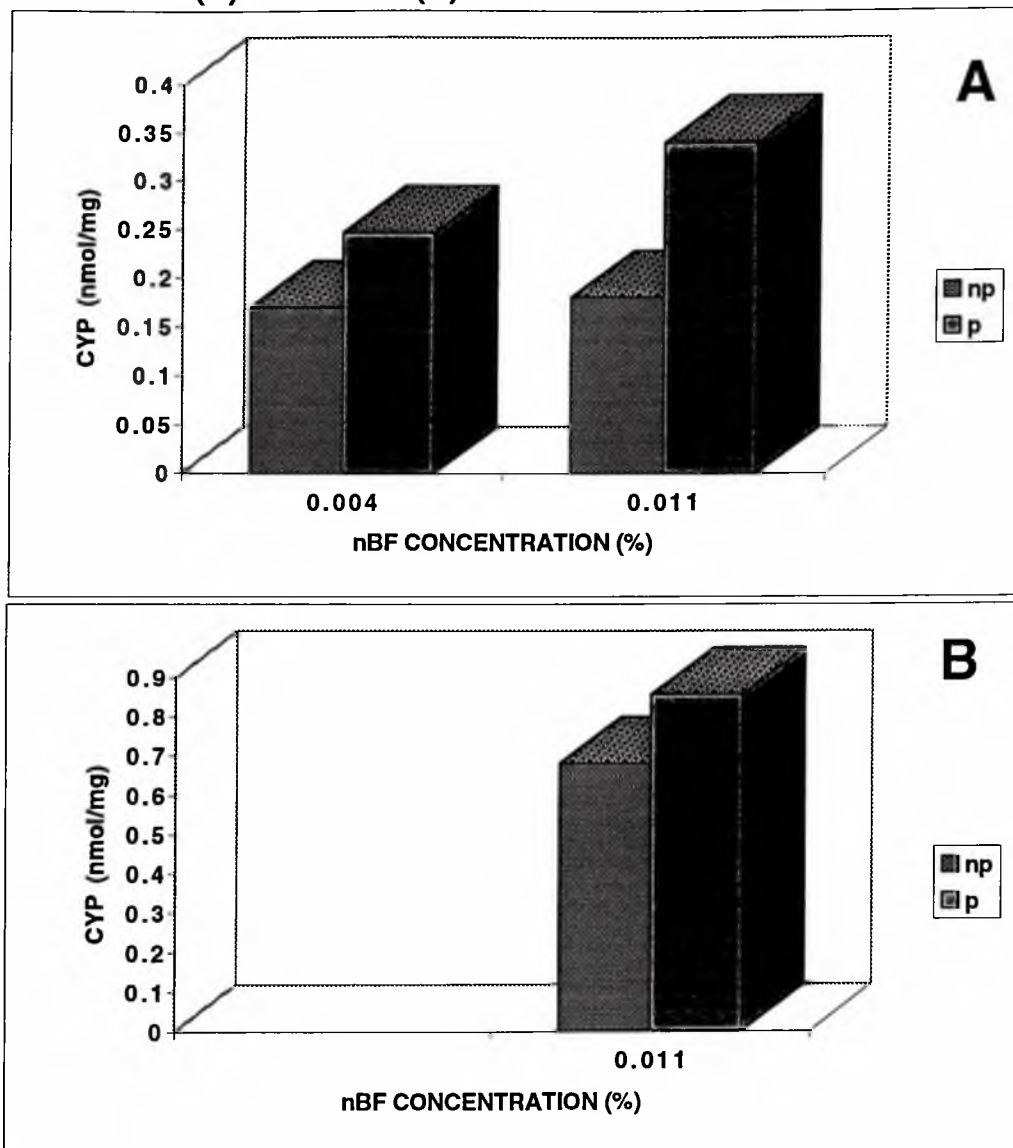
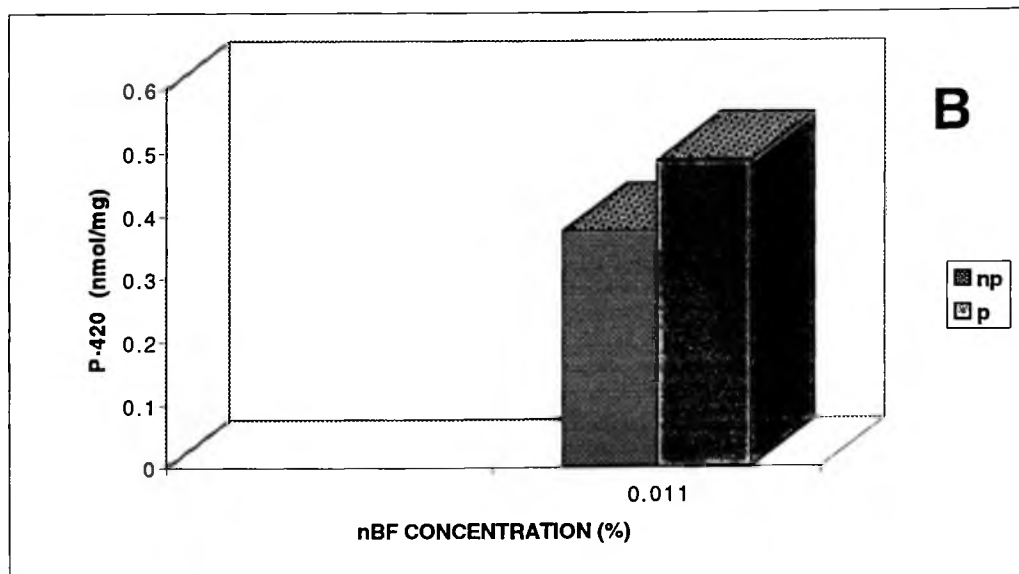
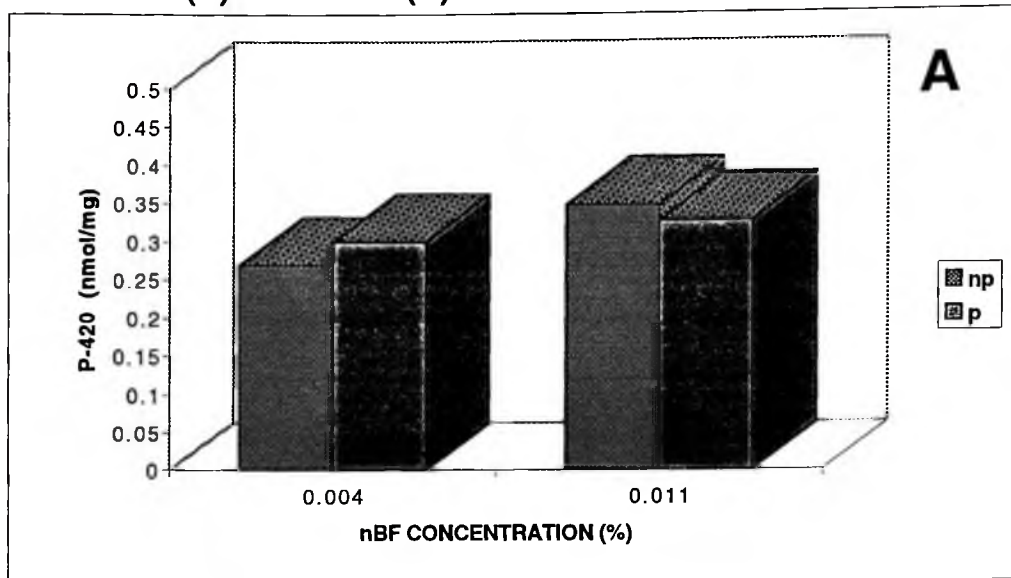


FIG. 3.20

**EFFECT OF PRE-INCUBATION WITH nBF ON CYP LEVELS
IN (A) NIBM AND (B) BIBM LIVER MICROSOMES**

np - no pre-incubation p - pre-incubation

FIG. 3.21

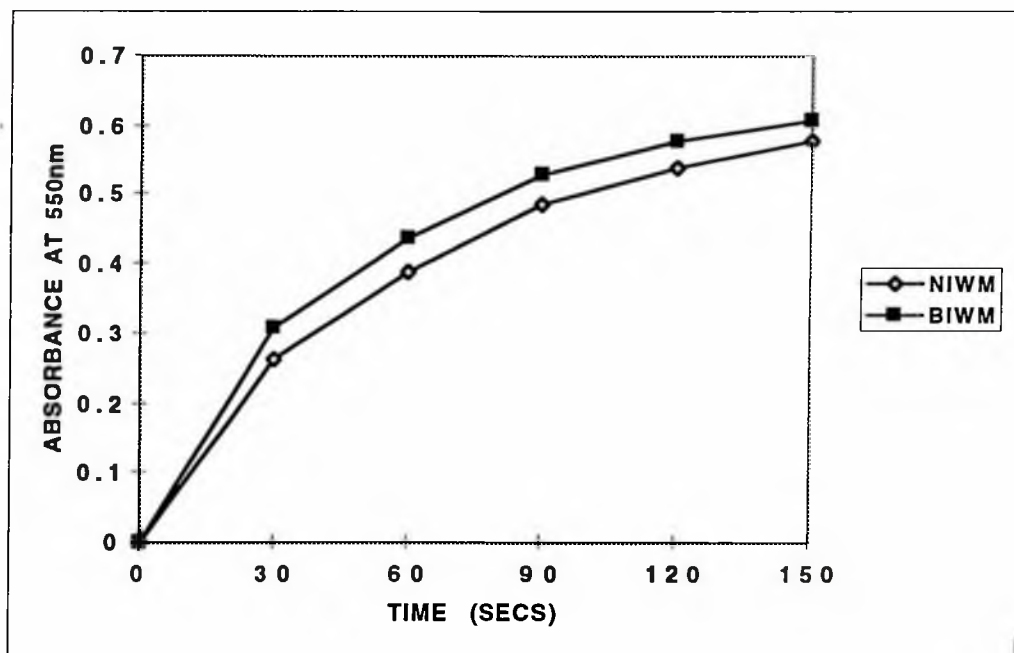
**EFFECT OF PRE-INCUBATION WITH nBF ON P-420 LEVELS
IN (A) NIBM AND (B) BIBM LIVER MICROSOMES**

np - no pre-incubation
p - pre-incubation

3.6 REDUCTASE ACTIVITY

The cytochrome c (P-450) reductase activity of microsomal preparations were calculated using the absorbance change at 550 nm per minute (over a 3 minutes period). Fig. 3.22 shows the change in absorbance with time for the reductase activity of both NIWM (non-induced white mice) and BIWM (BNF-injected white mice).

FIG. 3.22
CYP REDUCTASE ACTIVITIES OF LIVER
MICROSOMAL PREPARATIONS



NIWM - Non-induced white mice
BIWM - BNF-injected white mice

Tables 3.4 shows the specific reductase activities and the effect of nBF on the reductase activities. The closeness of the reductase activities obtained for both NIWM and BIWM as indicated in Fig. 3.22 was expected since ENF and other polycyclic hydrocarbons increase P-448 but not levels of P-450, NADPH cytochrome c reductase, or the rate of P-450 reduction (Smith and Davies, 1980).

Table 3.4 Effect of nBF on NADPH-cytochrome c (P-450) reductase activities of microsomal preparations

Sample	Concentration of nBF	Reductase activity (nmol/min/mg protein)	% Inhibition
A:NIWM	0.00%	26.32	-
	0.004%	25.13	4.50
	0.04%	23.52	10.63
	0.40%	17.61	33.09
B:BIWM	0.00%	27.92	-
	0.004%	27.18	2.70
	0.04%	25.68	8.02
	0.40%	17.45	37.50

3.7 BINDING STUDIES

The UV-VIS spectrum of 7-ER and resorufin are shown in Fig. 3.23. Note the absorption maximum at 482 and 572 nm respectively. Fig. 3.24 shows the spectra of resorufin formed and the effect of varying concentrations of nBF on the resorufin formed. At higher concentration of 0.083%, nBF slightly distorted the resorufin spectrum at wavelengths lower and higher around 572

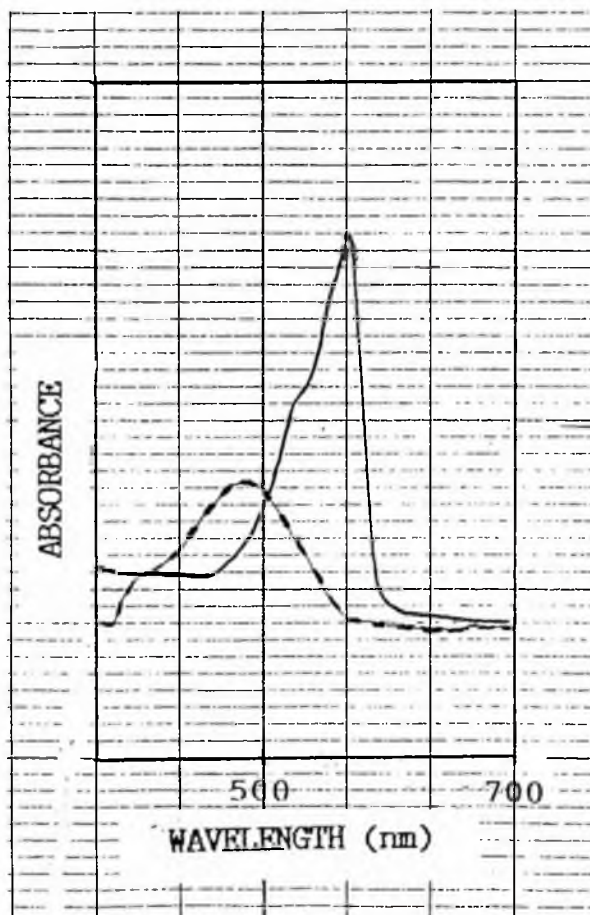


FIG. 3.23 UV-VIS SPECTRA OF RESORUFIN (—) AND 7-ETHOXYRESORUFIN (-----)

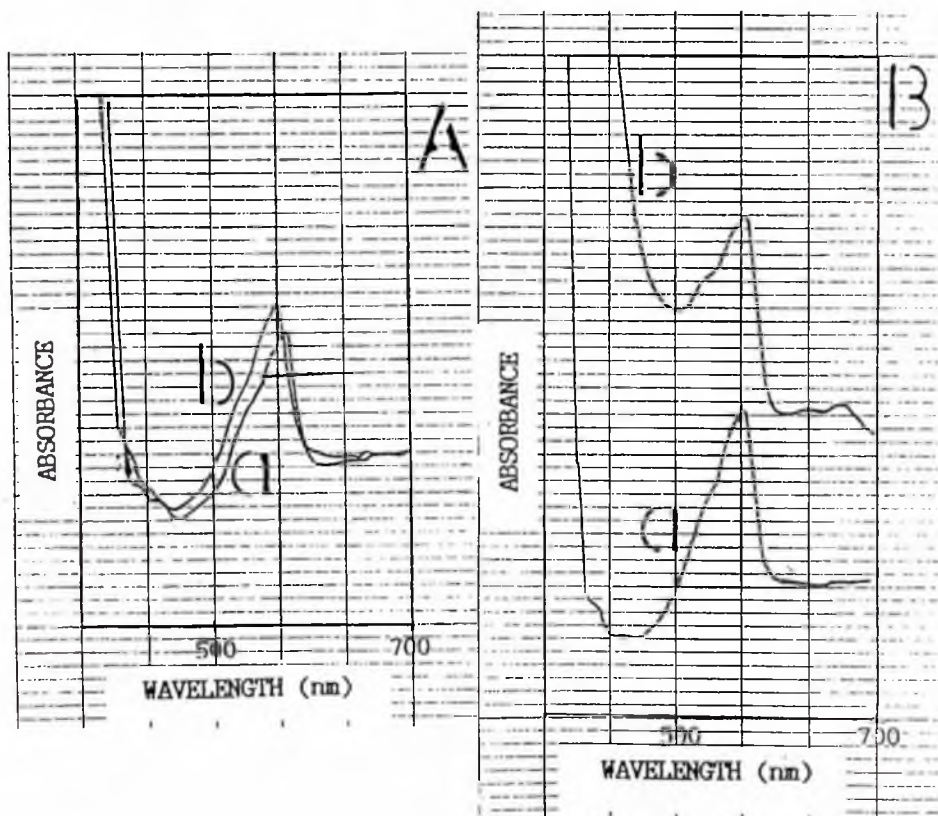


FIG. 3.24 SPECTRA OF (a) RESORUFIN FORMED AND (b) nBF ADDITION TO THE RESORUFIN FORMED [A:0.017%; B:0.083 nBF]

nm, though the 572 nm peak itself was unaffected. At a lower concentration of 0.017% the spectrum was about the same as that without nBF. In both cases, there was an increase in absorbance.

Based on these results, nBF was pre-incubated with 7-ER for 3-5 minutes before NADPH addition. In these experiments the prominent 572 nm peak characteristic of resorufin and indicative of product formation, diminished with increasing concentration of nBF (Fig. 3.25). The spectrum of oxidized CYP solution gave a peak at 409-412 nm. This is not the same as 414-418 nm reported in the literature (Levin *et al.*, 1974). The reported values in the literature are however for partially purified CYP. Increasing nBF concentrations altered the spectrum of the oxidized CYP (Fig. 3.26). The altered spectrum of oxidized CYP in the absence of substrate indicates an interaction between CYP and the plant extract. The addition of substrate (7-ER) altered the spectrum of the oxidized microsomes only slightly (Fig. 3.27a and b). However addition of nBF to the CYP-substrate complex changed the spectrum greatly only at the higher concentration of nBF (Fig. 3.27). The effect of nBF when added before addition of substrate (7-ER) is shown in Fig. 3.28. Addition of nBF completely changed the spectrum of the microsomal preparation as observed before (Fig. 3.26). At a lower concentration of nBF (0.017%), there was only a slight modification in the spectra when nBF was added before and after substrate addition (Fig. 3.27 [Panel B] & 3.28 [Panel A]).

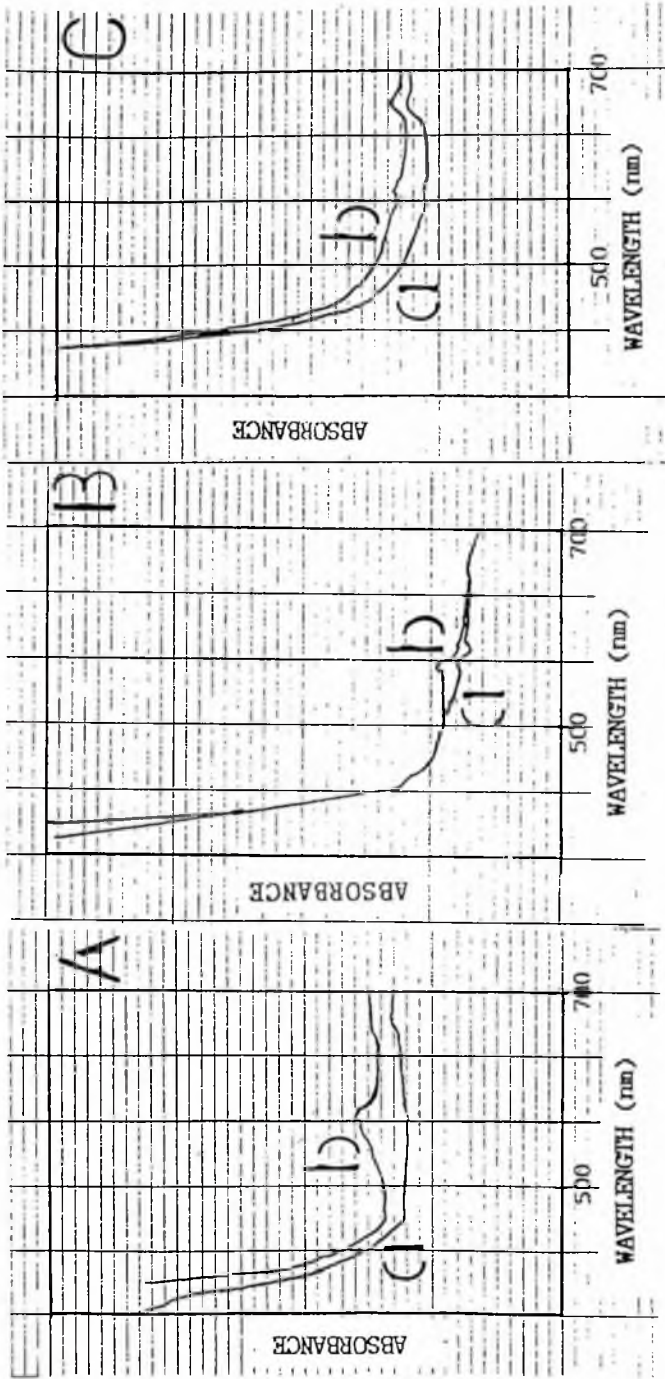


FIG. 3.25 SPECTRA OF MICROSOMAL PREPARATION (25 $\mu\text{g/ml}$), 7-ETHOXYCORSORUFIN (2 μM) AND NBF (a) AFTER AND (b) BEFORE NADPH (0.5mM) ADDITION [A:0.017%; B:0.083%; C: 0.17% NBF]

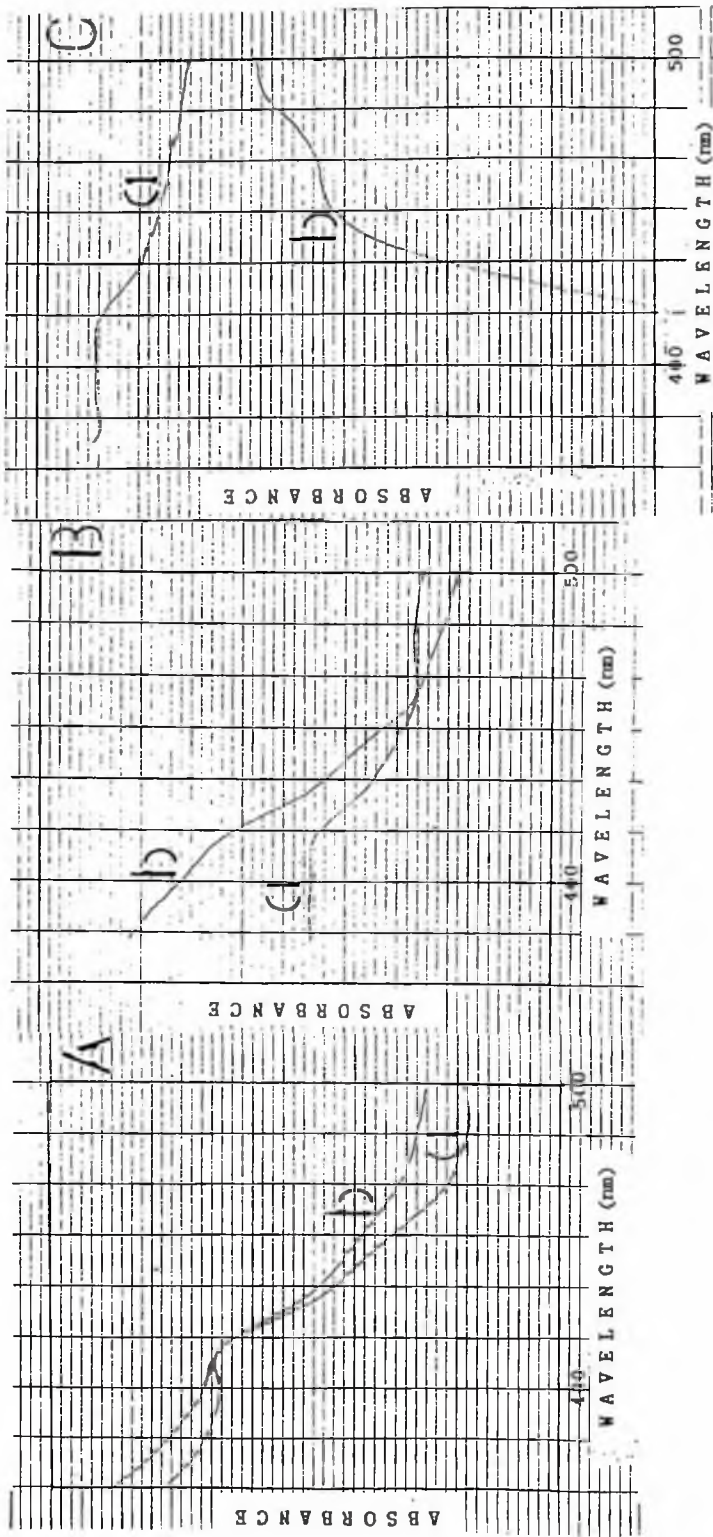


FIG. 3.26 ABSOLUTE SPECTRA OF (a) OXIDIZED MICROSOMES (25 µg/ml) AND (b) OXIDIZED MICROSOMES WITH A:0.017%; B:0.125%; C:0.33% NBF ADDITION

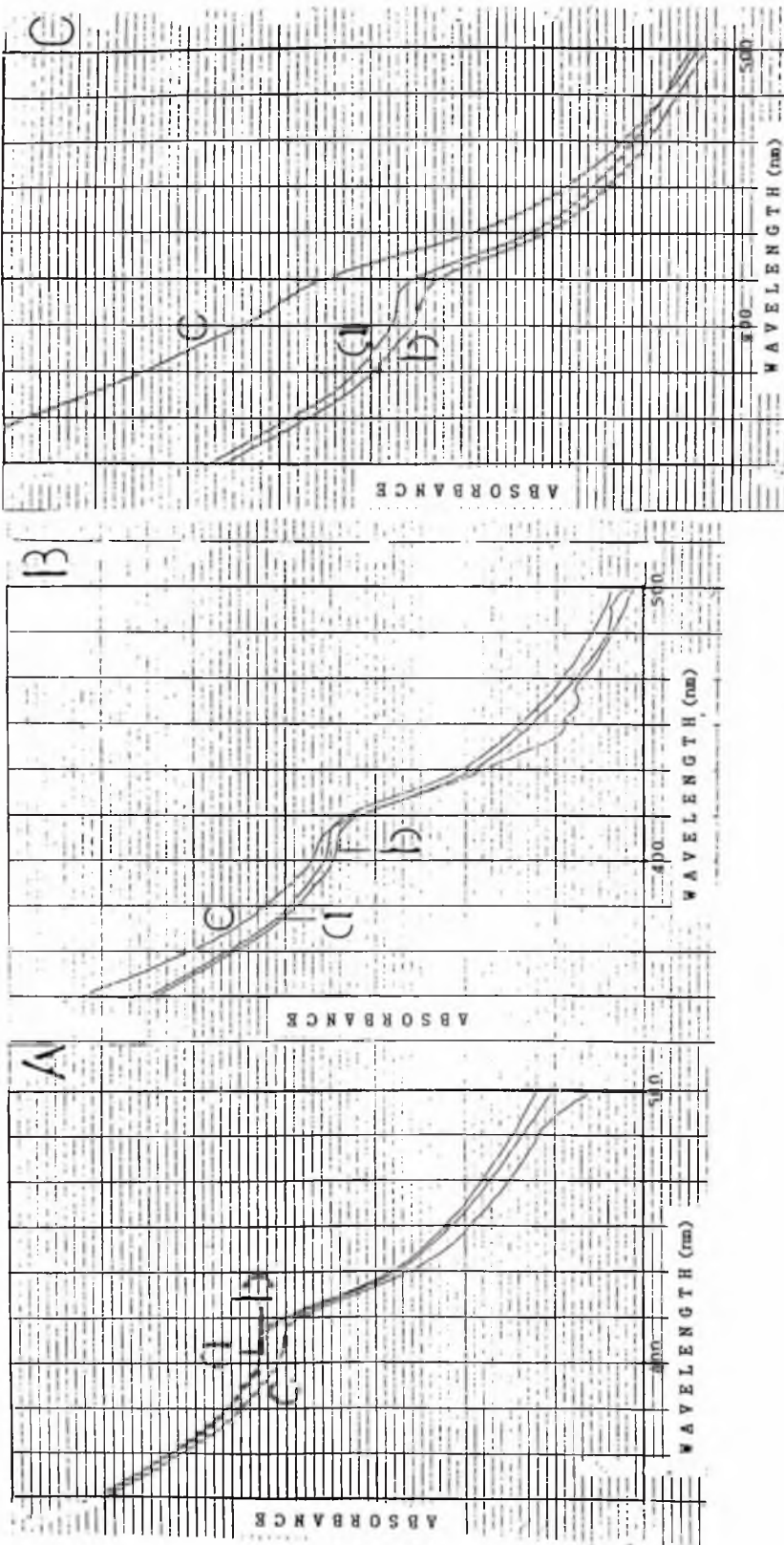


FIG. 3.27 ABSOLUTE SPECTRA OF (a) OXIDIZED MICROSOMES (25 $\mu\text{g}/\text{ml}$) AND (b) OXIDIZED MICROSOMES AND ETHOXYRESORUFIN (2 μM), (c) OXIDIZED MICROSOMES, ETHOXYRESORUFIN, AND WATER OR NBF (A:WATER, B:0.017%; C:0.17%) ADDITION

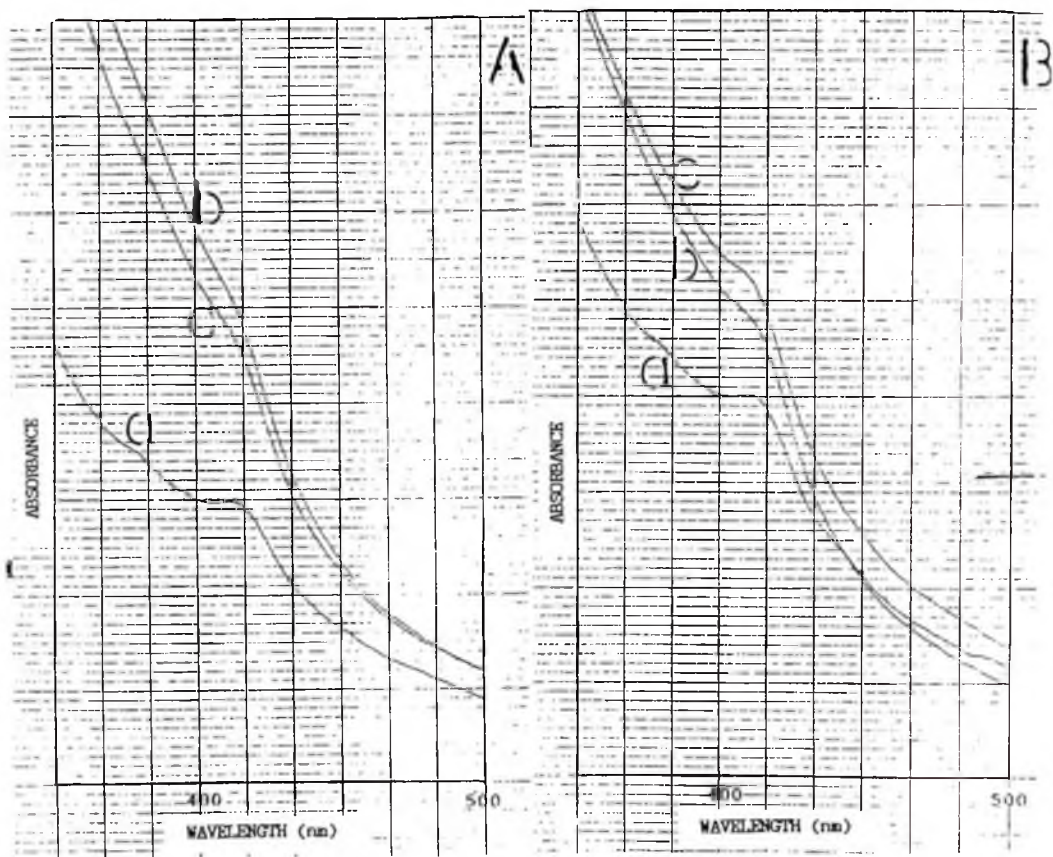


FIG. 3.28 ABSOLUTE SPECTRA OF (a) OXIDIZED MICROSOMES ($25 \mu\text{g}/\text{ml}$) (b) OXIDIZED MICROSOMES ($25 \mu\text{g}/\text{ml}$) WITH nBF AND 7-ETHOXYRESORUFIN ($2 \mu\text{M}$) ADDITION [A:0.017%; B:0.083% nBF]

However at higher concentrations of nBF (Fig. 3.27 [Panel C] & 3.28 [PanelB]) the final spectra were the same, indicating that the effect of nBF is concentration-dependent.

DISCUSSION

The design of the experiments was to obtain both the inducible and constitutive forms of CYP and to investigate the mode of action of nBF in inhibiting the NADPH-dependent cytochrome P-450 reactions. This was to determine whether a particular form of CYP was responsible for the inhibitions.

The protein concentrations and spectral studies show that the two forms of CYP used were different. β -naphthoflavone (BNF) administration increased the total liver protein content. Within 4 days after administration of BNF, there was a 17.8% and 21% increase in liver protein content for the DDY and C57 BL/ksJ respectively. There was also an increase in the amount of protein per gramme of wet weight liver of both strains of mice (Fig. 3.2). The greater than 2 fold elevation of CYP content (Fig. 3.3) in both strains is indicative of the induction of the CYP monooxygenase system by β -naphthoflavone.

Carbon monoxide interacted with reduced microsomal hemoprotein obtained from BNF as expected, but the maximum absorption of this complex was found at 448 nm instead of at 450 nm, which is the maximum for the hemoprotein from microsomes of normal mice (Alvares *et al.*, 1967, Hilderbrandt *et al.*, 1968). When reduced in the presence of CO, the microsomal preparations had

a maximum at 450 nm (448 nm for the BNF-injected mice), with a small peak around 423 nm (Fig. 3.4 [Panels A&B]). Indeed, a peak at 423 nm has been observed in all preparations of partially purified microsomal CYP described to date (Levin *et al.*, 1972; Lu and Coon, 1968; Miyake *et al.*, 1968), and has been the subject of considerable controversy. The shoulder near 423 nm in the CO-reduced spectra is due to a small amount of cytochrome P-420. Cytochrome P-420 (P-420), the solubilized form of CYP was present in all the microsomal preparations, with a proportionate increase in the CYP contents (Fig. 3.3) of the 2 strains of mice (after β -naphthoflavone injection).

Determination of the P-420 concentration in the final microsomal fraction, by the method of Omura and Sato (1964) using the CO-difference spectrum (Fig. 3.4) reveals that 21-29% of the CYP concentration is present as P-420. The CO difference spectrum of P-420 is very similar to that of hemoglobin (Omura and Sato, 1964). Some of the absorbance at 420 nm may be attributed to contamination by blood. Moreover the high P-420 contents could be due to the non-purified nature of the CYP.

In the case of the WWRK, the P-420 content was even greater than the CYP content. This can be seen in the CO-difference spectrum of the dithionite reduced CYP-CO complex in Fig. 3.4 [Panel F]. Unlike the other CO-difference spectra in Fig. 3.4, the 423 nm shoulder is higher than the 450 nm peak. In microsomal fraction from rat kidney cortex, the concentration

of CO-binding hemoprotein estimated on the basis of absorbance of the CO complex is only 10% to 30% (Kato, 1966) of that found in liver microsomes. As shown in Fig. 3.3, the CYP content of WWRK is approximately a third of that found in WURL.

The EROD assays were performed to confirm the inhibition of the NADPH-dependent cytochrome P-450 reactions. β -naphthoflavone (BNF) is known to induce the CYP1A1 isozyme specific for the aromatic ring hydroxylation, and hence the EROD assay. Liver microsomes from mice injected with BNF, deethylated ethoxyresorufin to resorufin.

The non-responsiveness of microsomes from the non-induced mice to increase in the amount of resorufin formed observed with the BNF-injected mice (Fig. 3.6) strongly suggests that different isozymes of CYP are induced under different conditions.

Extract of nBF of *D. adscendens* has been shown to inhibit deethylase activity in EROD assays (Brookman-Amisshah, 1994). The flash chromatography fractions (DAF1, 2 and 3) of *D. adscendens* were found to inhibit deethylase activity in the EROD assays using microsomes from the BNF-injected black and white mice. An interesting pattern of inhibition was observed for the EROD activities when DAF1 and DAF2 were added to the reaction mixtures (Fig. 3.8). The inhibition was biphasic, with this type of inhibition being pronounced in the black mice. DAF3 inhibited the EROD activities of

both the black and white mice with increasing concentration. The % inhibition was greater in BNF-injected white mice than the black mice. The different effects of the flash chromatography fractions on the black and white mice could be due to genetic differences.

The polarity of the solvents used to flash out the nBF of *D. adscendens* increased from DAF1 to DAF5. Thus the varying effects of these fractions on the EROD activities of the black and white mice could also be due to different ratios of the components of the plant extract in a particular fraction. This was the reason why the effects of DAF1, 2 and 3 were studied alongside the crude nBF. DAF4 and 5 were not used because of the low activity reported for them in a work done by Addy in 1989, on the effect of the fractions of *D. adscendens* on the inhibition of smooth muscle contraction.

The investigation of the effect of nBF and its flash chromatography fractions on cytochrome c was carried out based on a previous study in which cytochrome c was found to be directly reduced by salsolinol (Kamassah, 1992). Addition of varying concentrations of DAF2 - 5 to an oxidized solution of cytochrome c did not show any reduction. DAF1 however showed a reduction (Fig. 3.12 [Panels A&B]). From the polarity of the solvents used, DAF1 was the least polar fraction and is likely to contain salsoline and therefore DAF1 could possibly contain the reductant purported to be responsible for the reducing ability of the plant extract.

The NBF did not show any reduction at lower concentrations, but did so at higher concentrations (Fig. 3.11). The inability of the lower concentrations of NBF of the crude extract of *D. adscendens* to reduce cytochrome c directly could be due to a smaller amount of DAF1 present.

The interaction of various compounds with the P-450 enzymes has been shown to result in increased P-450 functions including P-450 reduction, and monooxygenase and oxidase activities (Wang *et al.*, 1993). The NBF of the crude extract of *D. adscendens* under investigation has been known as an inhibitor of P-450 NADPH-dependent AA metabolism (Addy and Schwartzman, 1992). The purpose of the present study was to investigate this, that is, the mechanism(s) underlying the inhibition of the NADPH-dependent CYP reactions.

In an earlier study, cytochrome c was reduced by salsolinol and GSH (Kamassah, 1992). In that study, salsolinol reduced cytochrome c with optimal concentration in the millimolar range. The reduction by GSH and salsolinol were about the same, indicating that, they both have the same redox potentials. It was inferred from these results that the mode of action of salsolinol, and hence salsoline in the plant, was to reduce CYP and keep it in the reduced state, interfering with the first step (Fig. 1.2) where the ferric ion binds the substrate and is reduced to ferrous ion by the electron flow of NADPH.

The present study in which direct reduction of CYP by nBF, salsolinol and GSH was investigated revealed that nBF did not reduce CYP directly. Salsolinol and GSH which reduced cytochrome c, also did not reduce CYP (Fig. 3.13). This is evidenced by the absence of the 450 nm band or peak observed when CO was bubbled through a suspension of microsomes containing these compounds (GSH, salsolinol and nBF). This does not however preclude the presence of a reductant in the nBF of *D. adscendens* since salsolinol and GSH (both known reductants) did not also reduce CYP directly.

In these experiments for the direct reduction of CYP, when nBF was added to microsomal preparation and CO was bubbled through, there was a maximum absorbance at 420 nm (Fig. 3.13 [Panel C]), implying that the cytochrome is being degraded. When GSH and salsolinol were used, the same effect was observed. Considering the fact that GSH naturally occurs in the cell and takes part in oxidation-reduction reactions, the presence of nBF which acted like GSH in this case could also be in the cell and not be harmful to the organism.

nBF could contain a type of reductant which interferes with the flow of electrons from NADPH to CYP. Because of the inability of nBF and salsolinol to reduce CYP directly, the reduction was followed by an indirect method where CYP was reduced in the presence of NADPH (the natural intracellular reducing agent which donates electrons to CYP).

The rationale behind these investigations was to find out how nBF would interact with CYP in the presence of NADPH. The levels of CYP and P-420 were determined after NADPH was added, prior to the addition of nBF. This was to allow for the estimation of the amount of CYP reduced by NADPH. After the 25 minutes period during which the reduction was allowed to proceed, the concentrations of the two cytochromes were determined.

With the exception of CYP from the non-induced mice, increasing concentration of nBF increased both CYP and P-420 levels of the 2 strains of mice (Fig. 3.15A&B). The increased P-420 levels could be due to the modification of the hemoprotein when in the reduced state, leading to the formation of P-420. The conversion of P-450 to P-420 induced by neutral salts has been shown to proceed more rapidly in the reduced form of the hemoprotein than in the oxidized form (Imai and Sato, 1967). The increment was more pronounced in ENF-induced black mice (Fig. 3.15B(B)), suggesting that the CYP1A1 isozyme is more susceptible to nBF reduction in the presence of NADPH. However, the denaturation to P-420 was also greater in ENF-induced black mice. The lesser effect of nBF on the constitutive CYP is understandable, considering the fact that it would be dangerous to have a constitutive enzyme being affected by substances taken into the body.

The absorption spectrum of nBF showed an increase in absorbance from a longer to a shorter wavelength (i.e. 450 nm to 420 nm) (results not shown). Thus, the high P-420 contents observed when higher concentrations of nBF

were added could be as a result of absorbances due to the nBF itself at 420 nm.

The fold increases in CYP and P-420 levels show that CYP levels increased more than those of P-420 in both non-induced black mice and ENF-induced black mice except at high concentrations of nBF (Fig. 3.16). As stated earlier, the high concentrations of nBF could be having deleterious effect on the CYP system, taking into account the fact that reduced CYP is more susceptible to degradation.

For comparison, salsolinol (0.57mM) was used in place of nBF. In both NIEM and BLEM there was an increase in CYP with a corresponding decrease in P-420 levels (Fig. 3.18). Like the nBF, the proportion of increment in CYP was greater for the ENF-induced black mice than the non-induced black mice at the same salsolinol concentration. The action of salsolinol on the fold changes in CYP and P-420 contents suggests a reductive role for this compound with increasing CYP levels. The results, (decreasing P-420 levels) also implies that the CYP is not being reduced to P-420, suggesting that the high P-420 levels with high concentration of nBF is due to the high absorbances of nBF at the lower wavelengths.

In one set of experiments, the NADPH was added before nBF addition. It was therefore possible that NADPH could have reduced most of the CYP before nBF was added, thus rendering the effect of the nBF on the reduction of CYP

negligible. An attempt was therefore made to pre-incubate the microsomal preparations with nBF for 2 minutes before NADPH addition. Fig. 3.19 shows a time-dependent reduction of CYP after NADPH was added, following the 2 minutes incubation with nBF. For both the non-induced black mice and ENF-induced black mice, the rate of reduction was proportionate with increasing concentration of the plant extract (nBF). A higher concentration of nBF was needed to reduce the CYP in the ENF-induced mice more than NADPH alone did. Thus it is possible that, high extract concentrations are needed (during pre-incubation) to reduce the induced CYP of the ENF-induced mice effectively.

Pre-incubation with nBF did, however, have a greater effect on reduction of CYP by NADPH. At the same concentrations of nBF, pre-incubation as opposed to no pre-incubation increased the levels of CYP (Fig. 3.20). The levels of P-420 also increased (except at a higher concentration of 0.011% for non-induced black mice, Fig. 3.21A) but that of CYP was more significant. Thus pre-incubation of microsomal preparations with nBF before NADPH addition enhances positive effect on reduction of CYP by NADPH.

Since nBF did not reduce CYP directly, and also the synergistic effect of nBF and NADPH on the reduction of CYP was not pronounced, the proposal that the plant extract keeps CYP in the reduced state, and thereby interfering with the first step (Fig. 1.2), where the ferric ion binds the substrates, and thus prevents the binding of the substrate to CYP, is not tenable.

The monooxygenase enzyme system comprises CYP, NADPH-CYP reductase and a phospholipid. The NADPH-CYP reductase, which is a flavoprotein transfers the necessary reducing equivalents from NADPH to CYP. In the reaction cycles shown in Figs 1.1 and 1.2 (step 2), the electrons from NADPH through the NADPH-CYP reductase reduce the CYP-substrate complex to allow O₂ binding. With the reducing property of nBF, it was expected that the nBF would enhance CYP reductase activity in the presence of NADPH but this was not so. It rather inhibited the reductase activities (Table 3.4). The inhibition of NADPH-CYP reductase activity could be the possible mode of action of nBF on the NADPH-dependent monooxygenase reactions. The extract could be inhibiting by interfering with the flow of electrons from NADPH to CYP, the rate determining step. This would keep CYP in the oxidized state and therefore O₂ cannot bind to the CYP-substrate complex to form the ternary CYP-substrate-O₂ complex.

In one set of experiments, the reductase reaction was initiated by the addition of nBF in place of NADPH. Cytochrome c (P-450) was reduced but the rate was very minimal. This was expected since the extract has shown reducing properties.

Cytochrome c, the electron acceptor in this assay for the reductase, is an artificial one, not membrane-bound and therefore differs from CYP, the real acceptor. Its direct reduction by nBF was investigated. As indicated by the results, there was a direct reduction of cytochrome c by nBF (Fig.

3.11). This further shows that cytochrome c is different from CYP, which was not reduced directly by NBF.

CYP is different from cytochrome c in that CYP is a b type cytochrome with a protoheme or a related heme (without formyl group) as prosthetic group, not covalently bound to the protein; whereas cytochrome c has covalent linkages between the heme side-chain and the protein.

In the assay for the cytochrome c (P-450) reductase activity, cytochrome c was used as an artificial electron acceptor in the presence of CYP. In the presence of cytochrome c it could be that NBF increases electron flow to CYP (as already indicated by more CYP²⁺ formation when NBF is present), therefore reducing electron flow to cytochrome c. The inhibition of reductase activity therefore is apparent but not real. With this inhibition, the flow of electrons to CYP is not affected.

In the spectrophotometric assay for the EROD activity, the inhibition of the 572 nm peak formation characteristic of resorufin when NBF was pre-incubated with the microsomal preparation and 7-ER, before co-factor (NADPH) addition confirms the inhibition of the EROD assay by NBF and suggests that the extract binds to CYP. However, once 7-ER is deethylated to resorufin, addition of the plant extract (NBF) did not have any interaction which affected the formed product. This is indicated by the presence of the 572 nm peak even after addition of higher concentrations of

nBF (Fig. 3.24).

Addition of nBF completely changed the spectrum of the microsomal preparation and subsequent addition of substrate (7-ER), affected the resultant spectrum only slightly. Comparing this to the spectrum obtained when 7-ER was added first before nBF addition (tracing c, Fig. 3.27 and 3.28), the results show that once nBF binds CYP, the substrate binds to only a small portion of CYP, hence the slight change of spectrum.

In another experiment when nBF and NADPH were added to the microsomal preparation before substrate addition, there was no spectrum of the microsomal preparations, nBF and substrate when scanned between 350 and 700 nm. The absorbance remained negative (results not shown). This supports the proposal that nBF could contain the type of inhibitor acting to prevent donation of electrons from NADPH to CYP.

The plant extract could also form a metabolic-intermediate "MI" complex with CYP in the microsomal preparation. The "MI" complex once formed is stable, preventing further participation in the mixed function oxygenation or dealkylation as in the case of the EROD reactions.

The nBF could also bind to the substrate, thus making it unavailable to participate in the reaction. 7-ER did not show a binding spectrum with

oxidized CYP (Fig. 3.27). This does not however imply that 7-ER does not bind CYP since some substrates have been shown not to have binding spectra but are hydroxylatable (Imai and Sato, 1967a). In the presence of nBF, the spectrum obtained for the oxidized microsomes and the substrate together with nBF changed, indicating an interference with the subsequent steps for product formation.

In the absence of a substrate, nBF enhanced CYP²⁺ formation when NADPH was present. The CYP²⁺ component of CYP was further enhanced if the nBF was pre-incubated with the microsomal preparation before NADPH addition. Thus nBF does not prevent reduction of CYP by NADPH in the absence of a substrate.

In the presence of nBF, the substrate could still bind CYP and form the CYP²⁺-substrate complex. CYP²⁺, once formed can be determined by observing a peak at 450 nm with CO binding. This could however, not be determined due to the unavailability of more CO at this stage of the work.

If this CYP²⁺-substrate is formed and O₂ binds to form the CYP²⁺-substrate-O₂ ternary complex (TC), then nBF could be inhibiting by preventing the breakdown of the TC to form products. Accumulation of this complex leads to its breakdown into substrate, O₂ and CYP³⁺ and the substrate therefore remains unmetabolized.

In the related work by Addy and Schwartzman (1992), during which the formation of products in the monooxygenase pathway was inhibited by nBF, the substrate (AA), which was added to the microsomal preparation containing CYP, remained unmetabolized.

In conclusion, the study indicated that CYP was not reduced directly by nBF, though nBF reduced cytochrome c directly. The NADPH-dependent reduction of CYP, in the absence of a substrate, was however enhanced by nBF. nBF is inhibiting the NADPH-dependent CYP reactions by binding to CYP and preventing either substrate binding or the breakdown of the ternary complex to form products.

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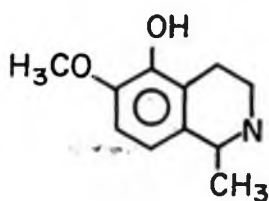
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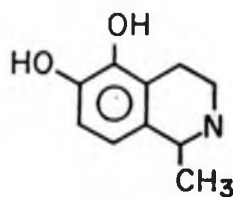
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STRUCTURES

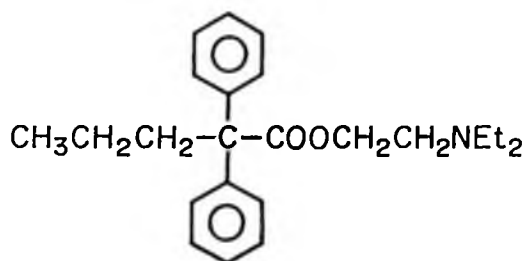
I: Chemical structures of some CYP-dependent monooxygenase inhibitors



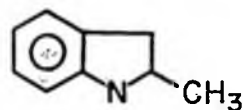
a: salsoline



b: salsolinol

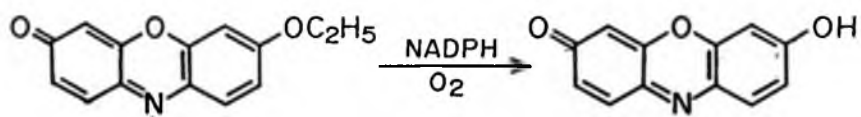


c: SKF 525-A



d: methylindole

II: Deethylation of 7-ethoxyresorufin



ethoxyresorufin

resorufin

PREPARATION OF SOLUTIONS AND REAGENTS**I. Homogenizing Buffer**

13.61g of potassium dihydrogen phosphate and 11.2g of KCl were dissolved in 500ml of sterile double-distilled water (SDDW). The pH of the solution was adjusted to 7.4 with a few drops of concentrated NaOH solution. 0.372g of EDTA, 0.154g of dithiothreitol (DTT), and 115ml of 87% glycerol were added, and the mixture made up to 1 litre with SDDW.

II. Resuspension buffer

13.61g of potassium dihydrogen phosphate and 11.2g of KCl were dissolved in 500ml of SDDW. The pH of the solution was adjusted to 7.4 with a few drops of concentrated NaOH solution. 0.372g of EDTA, 0.154g of dithiothreitol (DTT), and 230ml of 87% glycerol were added, and the mixture made up to 1 litre with SDDW.

III. Alkaline copper reagent

Sodium carbonate - 2% w/v in 0.1M NaOH

Hydrated copper sulphate - 1% w/v in SDDW.

Sodium potassium tartrate - 2% w/v in SDDW. These were mixed in the ratio of 100:1:1 respectively when required.

IV. β -naphthoflavone

0.16g of β -naphthoflavone was measured into 20ml soyabean oil. The mixture was sonicated in an Ultrasonic type 17.202 to ensure an even distribution of the β -naphthoflavone in the oil.

V. EROD Buffer (0.1M $\text{NaH}_2\text{PO}_4 \cdot 2\text{H}_2\text{O}$)

1.5602g Sodium dihydrogen orthophosphate was dissolved in 50ml of SDDW and the pH adjusted to 7.4 with NaOH, and the solution made up to 100ml.

VI. 10mM NADPH

8mg NADPH was dissolved in 1.0ml of SDDW.

VII. Ethoxyresorufin (0.41mM)

1mg 7-ethoxyresorufin was dissolved in 10ml DMSO. 10 μ l of this solution gives 2 μ M substrate in the cuvette.

VIII. Resorufin (0.085mM)

1mg resorufin was dissolved in 50ml DMSO. The working solution was made up of 1 part of the stock solution and 2 parts of DMSO (1+2). 10 μ l of this solution corresponds to 179 pmol resorufin in the cuvette.

IX. 50mM TRIS-HCl buffer, pH 7.4

6.057g Tris-(hydroxymethyl)-amino methane was dissolved in 1 litre of SDDW and the pH adjusted with conc. HCl.

X. Cytochrome c solution

2.2mg of cytochrome c was dissolved in 15.0ml of TRIS-HCl buffer, pH 7.4.

XI. 50mM (\pm) Salsolinol

0.0550g of (\pm) Salsolinol was dissolved in 5.0ml SDDW.

SAMPLE CALCULATIONS

Assume the Absorbance difference (450 - 490)nm = 0.22

Extinction coefficient (450 - 490)nm = 91 mM⁻¹ cm⁻¹

therefore using Beer's Law and assuming a cuvette path length of 1cm, the

$$\begin{aligned} \text{CYP concentration is given by: } & \frac{0.22 \times 1000}{91} \text{ nmol ml}^{-1} \text{ diluted sample} \\ & = 2.4 \text{ nmol ml}^{-1} \text{ diluted sample} \end{aligned}$$

The specific content of CYP in the original sample is then calculated knowing the dilution factor used and the protein concentration of the original sample. For e.g. if the protein concentration was 26mg ml⁻¹, then the CYP specific content is given by :

$$\begin{aligned} & \frac{2.4 \times 10}{26} \text{ nmol mg}^{-1} \text{ protein} \\ & = 0.92 \text{ nmol mg}^{-1} \text{ protein} \end{aligned}$$

For the cyt. P-420 content, the absorbance difference between 420-490nm and a molar extinction coefficient of 110 mM⁻¹ cm⁻¹ were used.