STUDIES ON THE METABOLISM OF SKF 525 A

by

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CONTENTS

ABSTRACT

Spectrophotometric studies have been carried out to determine the pH dependence of binding of SKF 525 A, Brietal sodium and carbon monoxide to cytochrome P-450.

The optimal pH for metabolic conversion of SKF 525 A has been investigated and this agent and its major metabolite, SKF 8742 A, have been metabolised *in vitro* by swine and rat hepatic microsomes.

A suitable gas liquid chromatography assay has been developed and used to analyse metabolic production.

The effects of carbon monoxide, dithiothreitol, n-octylamine and of induction of cytochrome P-450 by phenobarbital on metabolism of SKF 525 A and SKF 8742 A have been investigated.

Attempts have been made to synthesise SKF 525 A N-oxide.

LIST OF ABBREVIATIONS USED

SKF 525 A	2-Diethylaminoethyl 2,2-diphenylvalerate
NADP	Nicotinamide adenine dinucleotide phosphate
E.P.R.	Electron paramagnetic resonance
g.l.c.	Gas liquid chromatography
t.l.c.	Thin layer chromatography
i/p	Intra-peritoneal
R.N.A.	Ribonucleic acid
D.N.A.	Deoxyribonucleic acid
DPEA	2,4-dichloro-6-phenylethoxyethylamine
B S A	Bovine Serum Albumin
I.T.L.C.	Instant thin layer chromatography.
c.	Approximately.

INTRODUCTION

SKF 525 A is widely used experimentally as an inhibitor of cytochrome P-450 but, despite investigation, the mechanism of its action has not been fully elucidated. This agent is thought to act as an alternate substrate for cytochrome P-450, a situation that would explain its competitive kinetics of inhibition. However, the metabolism of some compounds known to be oxidized by cytochrome P-450 is not affected by SKF 525 A. Furthermore, if this agent is extracted into benzene and recrystallized, then the kinetics of inhibition changes, despite there being no detectable changes in the molecule.

Use of this agent in live animals causes fat invasion of the liver and it is thought that this toxic effect may be due to a metabolite.

Anders and co-workers have shown that SKF 525 A is metabolised by rat hepatic microsomes to SKF 8742 A and an unidentified minor metabolite. They were unable to demonstrate metabolism of SKF 8742 A. The same workers investigated the kinetics of SKF 525 A metabolism by normal and phenobarbital induced, rat hepatic microsomes and found that induction greatly increases the rate of de-ethylation of SKF 525 A. They did not, however, evaluate their results by gas liquid chromatography to check for differences in metabolite patterns formed from induced and non-induced systems.

Zeigler *et al*. have shown that amine oxidase, found in hepatic microsomes, is capable of catalysing the formation of N-oxides from tertiary amines. Although SKF 525 A is metabolically and functionally associated with cytochrome P-450, the fact that it is a tertiary amine means that it may act as a substrate for amine oxidase. However, recent evidence indicates

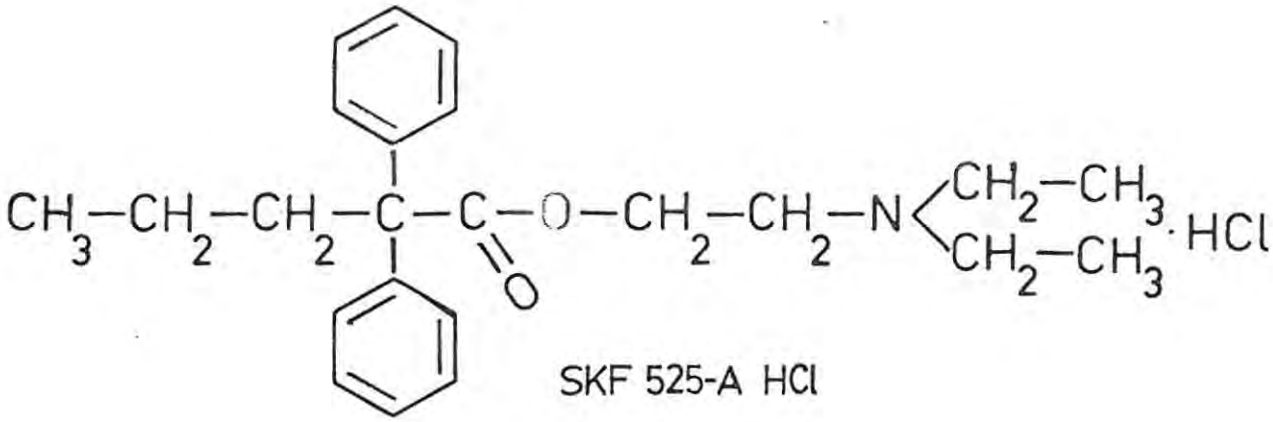
that cytochrome P-450 also participates in the N-oxidation of tertiary amines.

Thus, because of the possible therapeutic applications of SKF 525 A and its wide experimental use, the objective of these studies was to further investigate the metabolism of SKF 525 A and to elucidate the enzyme pathways involved.

CHAPTER 1

LITERATURE REVIEW

SKF 525 A (2-diethylaminoethyl 2,2-diphenylvalerate·HCl) has proved to



SKF 525-A HCl

be of little use as a parasympatholytic agent - its intended purpose - but prolongs the sleeping time caused by hexobarbital in rats. However, Cook et al.[1] showed that it had no sedative properties when administered on its own, and further work[2,3] indicated that SKF 525 A prolongs the duration of action of amphetamine, chloral hydrate, barbiturates and some analgesics. Brodie et al.[4] showed that the prolonging action of this agent is due to inhibition of drug metabolism. In several animal species, the half life times of barbiturates, meperidine, ephedrine and aminopyrine were increased after the administration of SKF 525 A[5]. In vitro studies in which liver microsomes were used showed that SKF 525 A inhibits the side chain oxidation of hexobarbital, pentobarbital and secobarbital; the N-dealkylation of aminopyrine, ephedrine, meperidine and dibenamine; the O-demethylation of codeine; and the deamination of amphetamine[6,7,8]. In spite of its general inhibitory nature, SKF 525 A does not inhibit hepatic microsomal metabolism of all drugs. It does not affect the duration of action of thiopental in rats or mice or the activity of thioethamyl in mice[2]. Similarly, it has slight effect on the N-dealkylation of N-methylaniline and several other N-substituted anilines, monoethyl- and monobutyl-4- aminoantipyrine, and

quinacrine by rabbit hepatic microsomes[8]; the N-demethylation of 3-methyl-4-methylaminoazobenzene by mice hepatic microsomes[10]; the O-dealkylation of phenacetin by rabbit hepatic microsomes[11]; the hydroxylation of acetanilid by rabbit hepatic microsomes[7,12]; the sulphoxidation of chloropromazine by guinea pig hepatic microsomes[13]; the reduction of chloramphenicol, p-nitrobenzoic acid and other aromatic nitro compounds by rabbit hepatic microsomes and the reduction of aminoazo dyes[15] (species not given). It is important to mention the species as inhibition of drug metabolism by SKF 525 A may occur in a given species but not another, e.g. SKF 525 A inhibits the O-dealkylation of phenacetin by rat hepatic microsomes[16] but not by rabbit hepatic microsomes[11]. The effects of SKF 525 A also vary when the microsomal enzymes are induced, as is normally done with phenobarbital or 3-methylcholanthrene, e.g. SKF 525 A has little effect on the metabolism of 3-methyl-4-methylaminoazobenzene in rats that have been induced with 3-methylcholanthrene but it inhibits the N-demethylation of the dye from non-treated rats[17]. Conversely, pre-treatment of rats with phenobarbital causes SKF 525 A to inhibit the N-demethylation of N-methylaniline but the drug has no inhibitory effects in non-induced rats[18].

Anders and Mannering[267] found that microsomes induced with either phenobarbital or SKF 525 A itself increased the rate of N-dealkylation of SKF 525 A. No mention about the formation of the minor metabolite, under these circumstances, was made. Examining the effects of SKF 525 A on hepatic ultrastructure, Rogers and Fouts[269] showed that the agent affected both the rough and smooth endoplasmic reticulum and, furthermore, that it was strongly bound to the protein.

SKF 525 A has been shown to be a widely used experimental tool, and although its pharmacological effects are supposedly minimal, use of this agent *in vivo* and *in vitro* is not without effect. Holmes and Bentz[258] have shown that SKF 525 A inhibits cholesterol biosynthesis *in vitro*, but it is doubtful whether this finding holds *in vivo* for, while serum cholesterol decreases by 75%, total

cholesterol content of the liver increases. Dick et al.[259] found that SKF 525 A reduces total plasma cholesterol and aortic cholesterol in dogs, but chronic administration results in fatty infiltration of the liver. This was rapidly reversible upon withdrawal of the agent. Lena[260] has confirmed these findings.

Goudie et al.[261] have shown that SKF 525 A possesses sedative properties when used at doses of 25-50 mg/kg, and they indicate that this will affect the evaluation of psychotropic drugs. This finding may explain why Takanaka and Ho[268] suggested that "the inhibition of drug metabolising enzymes (by SKF 525 A) can not entirely account for the potentiation of pentobarbital induced narcosis."

Magus et al.[262,263] studied the effect of SKF 525 A on the activation of hydrocortisone-induced tryptophan pyrrolase of rat liver. This agent was shown to enhance tryptophan pyrrolase activity and pituitary involvement was evident. These workers point out that SKF 525 A is not as pharmacologically inert as was initially thought.

Possible Mechanisms of Inhibition by SKF 525 A

SKF 525 A is a very general inhibitor of the mixed function oxidase system and affects oxidative biotransformation, glucuronidation and hydrolysis[27]. Thus, Brodie[4,19] proposed that this inhibitory effect resulted from an interaction between SKF 525 A and the microsomal membrane causing permeability changes which hindered access of the drug to the enzyme. Gillette[20] questioned this postulated mode of action because SKF 525 A blocks the metabolism of some lipid-soluble substrates such as nicotine, codeine and hexobarbital but has no effect on other lipophilic drugs such as monoethyl-4-aminopyrine, methylaniline and acetanilid.

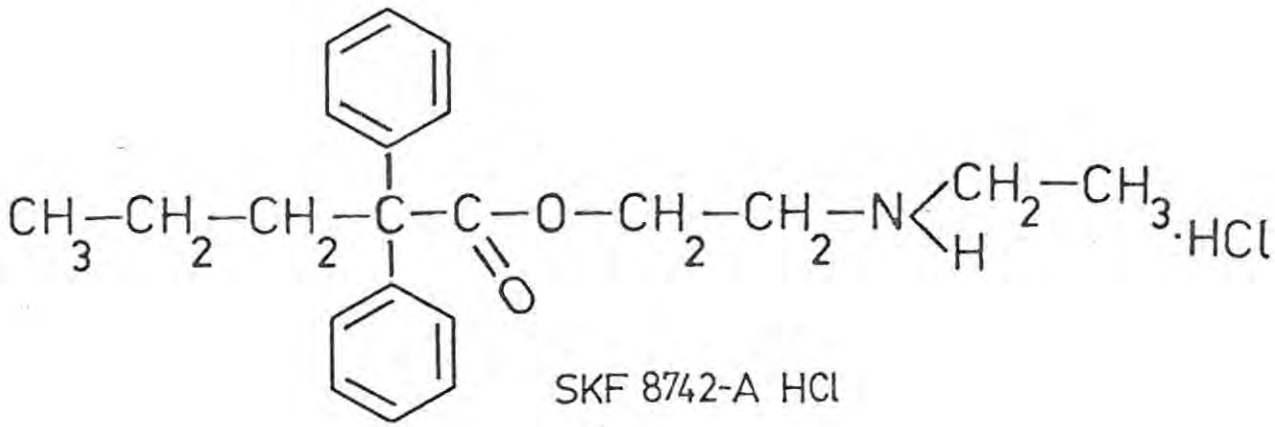
In lower concentrations however, SKF 525 A stabilizes red cell membranes[232], which indicates that the agent may have a non-specific effect on the microsomal membrane as well.

Netter[21] suggested that SKF 525 A uncoupled the microsomal electron transport chain which produced the resulting inhibition. However, SKF 525 A has no effect on NADPH-oxidase, NADPH-cytochrome c reductase, cytochrome oxidase and glucose 6-phosphate dehydrogenase[6,231].

Gillette and Sasame[22] observed that aerobic preincubation of liver microsomes *in vitro* with NADPH and SKF 525 A results in irreversible inhibition of drug metabolism. It was thought that a metabolite of SKF A may be responsible for this inhibition. Anders *et al.*[25] studied the inhibition of ethyl-morphine N-demethylation by SKF 525 A and ten cogeners. The kinetics of inhibition were found to be competitive in all cases which suggests that these compounds produce their inhibitory effects by combining with the active site of the N-demethylase. Schenkman *et al.*[23] proposed that a stable oxygenated complex of ferrous cytochrome P-450 forms when SKF 525 A is actively metabolised *in vivo* and *in vitro*. This species has an absorbance maximum at 455nm and was thought to be responsible for the observed non-competitive inhibition of drug metabolism *in vitro*. Chemical oxidation by potassium ferricyanide causes the 455nm peak to disappear and addition of sodium dithionite (a chemical reductant) does not restore the peak. This was taken to indicate that the peak was not due to CO binding to cytochrome P-450. Jenner and Netter[24] reported that recrystallisation of SKF 525 A from benzene, as opposed to other solvents such as water and methanol alters the inhibition kinetics from non-competitive to competitive. Despite extensive physico-chemical analysis, no structural change of the molecule could be detected. These findings further complicate the issue.

Metabolism of SKF 525 A

Anders et al.[26] studied the metabolism of SKF 525 A in vitro and in vivo using gas-liquid chromatography and thin-layer chromatography to separate and identify products. Metabolism produced two products; the major one was identified as the secondary amine, SKF 8742 A. The other metabolite was not identified. Metabolism of SKF 8742 A in vitro did not produce any products.



SKF 8742-A HCl

In the in vivo studies, neither SKF 525 A nor SKF 8742 A were recovered from the urine, bile or faeces of rats that had received these compounds; nor were metabolites found in these materials. These findings have been misinterpreted to indicate that SKF 525 A is metabolically dideethylated to yield the primary amine SKF 26754 A[233].

Cytochrome P-450

Before further consideration of SKF 525 A is possible, it is necessary to review the work done on cytochrome P-450.

Chance and Williams[51] were studying the kinetics of cytochrome b_5 reduction when they noticed that disproportionately large quantities of NADPH are required to reduce the enzyme. This led them to propose that "unknown intermediate reactions occur in the reduction of cytochrome b_5 by NADH".

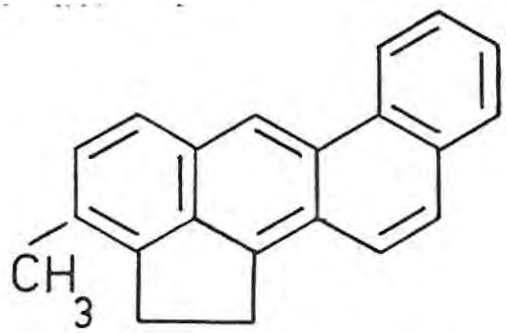
Four years later in 1958, Klingenberg[52], investigating the spectral characteristics of the liver microsomal fraction, found that it reacts with carbon monoxide forming an absorption band maximal at 450 nm which is unstable to treatment with detergents (cholate) and acid pH. In attempting to further characterise the microsomal pigment, Garfinkel[53] suggested that it contained a metal ion in view of its complexing properties but that it was not an iron haemoprotein as there was no photochemical effect on the CO complex[52]. Furthermore, a search failed to reveal α and β peaks in its spectrum, characteristic of an iron haemoprotein. Omura and Sato[34,35,37] confirmed Klingenberg's conclusions however that the pigment contained a haemoprotein. They described its isolation and partial purification and characterised it. Furthermore, they found that the cytochrome, cytochrome P-450, may be converted stoichiometrically to cytochrome P-420 by treatment with heated snake venom or deoxycholate in the absence of oxygen. Work done by Mason et al.[54] showed these reactions to be of a very complex nature.

Omura and Sato[37] also showed that cytochrome P-420 underwent aggregation in media more acid than pH 7.5. This is indicated by an increase in turbidity of the preparation as the pH is lowered. Schoeman et al.[55] confirmed this finding and showed, by the use of electron microscopy, that the aggregates form long tubules. Unlike the inactive, soluble cytochrome P-420, these aggregates combine with drugs to produce spectra characteristic of active cytochrome P-450. The absorption spectrum was also found to vary with pH and these changes were ascribed to splitting of the aggregates at more alkaline pHs. Mason[56] presented an hypothesis for the stoichiometry of mixed function oxidase reactions and this was confirmed by Cooper et al.[57]. Oxygen consumption was measured polarographically and oxidation of NADPH was measured by fluorimetry. It was found that 1 mole of oxygen and 1 mole of NADP are consumed per mole of 17-hydroxyprogesterone hydroxylated.

Using the microsomal fraction from bovine adrenal cortex Estabrook et al.[58] investigated the oxygen activating enzyme for the C21-hydroxylation of 17-hydroxyprogesterone to cortexolone. They found that cytochrome P-450 acts as the "terminal oxidase". Cooper et al.[59] confirmed this result using bovine adrenal cortex microsomes and further, found that cytochrome P-450 fulfills the same function in rat liver microsomes. This led them to conclude that cytochrome P-450 is the final electron acceptor in the mixed function oxidase system of all mammals.

Induction of Cytochrome P-450

The first evidence indicating that foreign compounds could stimulate microsomal enzyme systems was produced in 1954 by Brown et al.[139]. They found that liver microsomes from mice fed different diets are capable of varied demethylase activities. Cramer et al.[140] showed that pretreatment of rats with 3-methylcholanthrene and other polycyclic hydrocarbons stimulate



3-Methylcholanthrene

the liver microsomal enzymes that catalyse the hydroxylation of 2-acetylamino-fluorene, 3,4-benzpyrene[141] and zoxazolamine[142]. Administration of polycyclic hydrocarbons to rats has little effect on the microsomal enzyme systems that catalyse metabolism of the type I substrates, chlorzoxazone, p-methoxyacetanilide, hexobarbital, aminopyrine and chlorpromazine[143]. Griem et al.[144] showed that phenobarbital has a prolonged and delayed action as an inducing agent. It causes an increase in the concentrations of cytochrome P-450, NADPH-cytochrome

c reductase and cytochrome b_5. When the level of haemoproteins reaches a steady state induced level then the rate of haeme destruction is elevated to balance the increased rate of synthesis. Conney and Gilman[164] demonstrated that puromycin blocks the ability of 3-methylcholanthrene or phenobarbital to increase the activity of the hepatic mixed function oxidase system. Jick and Shuster[165] have shown that inducing agents tend to reduce protein break down. In a similar study, using 3-methylcholanthrene, Levin and Kuntzman[145] showed that cytochrome P-450 exists in two forms, each with a different turn-over rate. Treatment with the inducer leads to a three-to four-fold increase in the slow phase component. More recently, Stonard[177,178] has studied the inductive effects of hexachlorobenzene and concluded that this compound represents a new class of inducers which exhibits the properties of both the polycyclic hydrocarbons and phenobarbital. Fouts and Rogers[146] examined the morphological changes in the liver which accompany induction by different agents. They found that phenobarbital and chlordane stimulate a wide range of microsomal drug oxidations and also caused a marked proliferation of the smooth endoplasmic reticulum. Benzpyrene and 3-methylcholanthrene only stimulate a few enzymes and do not cause proliferation of the smooth endoplasmic reticulum. Studying the chemical and enzymatic composition of microsomal subfractions from rat liver after induction with phenobarbital and 3-methylcholanthrene, Glauman[175] noted that phenobarbital increases levels of protein, RNA, phospholipid and cholesterol, in what he termed smooth I microsomes while smooth II microsomes remain unchanged. Enzymes associated with the electron transport chain are also found to be induced. 3-Methylcholanthrene has no effect on the quantities of protein, RNA, phospholipid or cholesterol in the microsomal subfractions nor did it increase levels of NADPH cytochrome c reductase.

Sladek and Mannering[17] have shown that administration of phenobarbital to rats increases absorption in the "absolute" spectrum at 455nm and 430nm

proportionately, while treatment with 3-methylcholanthrene increases the absorption at 455nm preferentially. This led them to suggest that 3-methylcholanthrene pretreatment causes the formation of a new haemoprotein. Conney et al.[177,180] investigated the hydroxylation of testosterone at the 6β, 7α and 16α positions. They found that the ratios of CO : O_2 required for 50% inhibition of each reaction differs significantly. This indicates the existence of more than one form of cytochrome P-450. Imai and Mason[168] studied the binding of ethyl isocyanide to cytochrome P-450 which was obtained from phenobarbital induced animals. They concluded that liver cytochrome P-450 was a single molecule capable of existing in more than one form. Further studies[83] supported the view of Conney et al.[180]. It was shown that SKF 525 A inhibits the N-demethylation of 3-methyl-4-methyl-aminoazobenzene (3-MMAB) in microsomes from untreated or phenobarbital pretreated rats but not in 3-methylcholanthrene pretreated rats. Thioacetamide blocks phenobarbital induction but has no effect on 3-methylcholanthrene induction. It appears that the new haemoprotein formed - termed cytochrome P_1-450 or P-448 - differs from cytochrome P-450 in that it only possesses the type II binding site[68]. The absorption maximum of cytochrome P_1-450 appears at slightly lower wavelength and the absolute spectrum of the two haemoproteins differ[61]. Parli and Mannering[149] studied the relative abilities of polycyclic hydrocarbons to increase levels of 3-MMAB N-demethylase and cytochrome P_1-450. They found that each of the agents is only capable of producing a certain inductive effect regardless of the size of the dose used. Arcos and co-workers[142] pointed out that many polycyclic hydrocarbons which are potent carcinogens are poor inducing agents and vice versa. Schenkman et al.[91] proposed that cytochrome P_1-450 was formed when 3-methylcholanthrene or one of its metabolites combined irreversibly with the type I binding site of cytochrome P-450. Conversely, Gnosspelius et al.[151] postulated that cytochrome P_1-450 is formed independently of cytochrome P-450 and does not contain 3-methylcholanthrene or any of its metabolites.

Bidleman and Mannering[150] supported the latter concept and showed that cytochromes P-450 and P_i-450 are formed simultaneously in rats treated with phenobarbital and 3-methylcholanthrene. Further work on the differences in cytochromes P-450 and P_i-450 was done by Fujita et al.[167]. The spectral characteristics of these two haemoproteins exhibit small qualitative and quantitative differences. Whereas cytochrome P-450 gives rise to both type I and type II spectra, cytochrome P_i-450 only gives rise to type II spectra. Using tritiated 3-methylcholanthrene, they showed that cytochrome P_i-450 is not complexed with 3-methylcholanthrene or any of its metabolites. Recently, Fischer and Spencer[234] using female rats, have presented evidence for a direct effect of polycyclic hydrocarbons on the formation of cytochrome P_i-45 Pretreatment of female rats with benzanthracene causes an increase in cytochrome P_i-450. In mature, but not immature, animals actinomycin D and puromycin prevent this increase. The results suggest that binding of benzanthracene to cytochrome P-450 depends on the level of the sex hormones. Thus, young female rats have low estrogen levels and benzanthracene may bind to existing cytochrome P-450 converting it to cytochrome P_i-450. Sex hormones stabilize cytochrome P-450 and, thus, this change cannot take place in older animals: therefore, conversion occurs during or soon after synthesis of cytochrome P-450. Alvares and Mannering[152] investigated the mixed-function-oxidase system of hepatic microsomes and found that in preparations from untreated rats different N-demethylase systems metabolise ethylmorphine and morphine. In microsomes from rats pretreated with phenobarbital, the same enzyme system is involved for the oxidation of both drugs. In a recent study, Mailman et al.[186] have shown that subfractionation of hepatic microsomes from untreated rats yields cytochromes P-450 that are qualitatively different from each other. In spite of the low concentrations of cytochrome P-450 found in uninduced livers, Stanton and Khan[188] have developed a high yield procedure suitable for the subfractionation.

Nebert and Gelboin[153] studied the induction of aryl hydrocarbon hydroxylase in cell cultures and found that it appears to require two phases. Firstly, synthesis of an induction specific RNA which is translation independent, i.e. does not require formation of protein from RNA and, secondly, translation related to an inducible specific RNA which is transcription independent, i.e. does not require the formation of RNA from DNA. They proposed that the increased enzyme activity may be due to an increase in the amount of either enzyme protein or other protein involved in enzyme activation. Nebert[154] also showed that the appearance of cytochrome P_i-450 is dependent upon protein synthesis rather than the presence of intracellular hydrocarbon as such. Studying the fate of inducers in cell cultures, Nebert and Bausserman[155] found that these agents diffuse passively into the cells and less than 1% of the total agent that enters is enzymatically bound to cellular macromolecules. This process may be inhibited by SKF 525 A. Half of the physically bound hydrocarbon is located on the nuclear or microsomal fractions. It was shown that the level of intracellular hydrocarbon decreases after 30 minutes of exposure of the cells to benz(a)anthracene. This decrease was thought to be due to induction of the microsomal oxygenase. Gielen and Nebert[157] showed that phenobarbital, DDT and polycyclic hydrocarbons all induce aryl hydrocarbon hydroxylase (aryl hydrocarbon hydroxylase is a microsomal mixed function oxygenase (cytochrome P_i-450) which is induced by polycyclic hydrocarbons). An additive effect is obtained when either phenobarbital or DDT is present with a polycyclic hydrocarbon in the medium but not when the hepatocytes are treated with phenobarbital plus DDT or with the combination of benz(a)anthracene plus 3-methylcholanthrene. The induction of aryl hydrocarbon hydroxylase activity by either phenobarbital or benz(a)anthracene was shown by Nebert and Gielen[158,163] to require RNA and protein synthesis initially and protein synthesis continuously. They proposed that the action of either inducer is at the level of transcription and that with either agent

there is also a secondary effect at the post-translational level, in which the normal rate of decay of the induced enzyme is delayed. More recently, similar work has been done by Hook et al.[176]. Using β-naphthoflavone and phenobarbital to induce cytochromes P_i-450 and P-450 respectively, Haughen et al.[184] concluded that this phenomenon is largely the result of an increased rate of de novo protein synthesis rather than a conversion of pre-existing polypeptides or a decreased rate of degradation. In other studies[17,159,160,161,162], the genetic differences in the expression of aryl hydrocarbon hydroxylase have been described.

Pelkonen and Karki[166] examined the physicochemical and pharmacokinetic properties of barbiturates on the induction of the hepatic mono-oxygenase system. They concluded that the ability of a compound to cause induction is related to its lipid solubility, pharmacokinetic properties and chemical groups present in the molecule.

Lu et al.[169] prepared cytochromes P_i-450 and P-450 from animals pretreated with 3-methylcholanthrene and phenobarbital respectively. Cytochrome P_i-450 was much more active in catalysing the hydroxylation of 3,4-benzpyrene and cytochrome P-450 was much more active in the demethylation of benzphetamine. The reductase fractions from the 3-methylcholanthrene induced liver and the phenobarbital induced liver were equally active in the hydroxylation of 3,4-benzpyrene. After studying the effect of piperonyl butoxide on the formation of cytochrome P-450 difference spectra, Philpot and Hodgson[170] concluded that the synergist binds to one form of cytochrome P-450 at a single site. They further proposed that an additional form of cytochrome is present in hepatic microsomes obtained from non-induced mice which does not bind type I substrates or interact with piperonyl butoxide. Wagstaff and Short[193] have shown that piperonyl butoxide and some analogues are capable of inducing cytochrome P-450. This finding has been confirmed by Yu and Terriere[194],

who showed that the stimulatory effect could be blocked by actinomycin D or cycloheximide.

Schoeman et al.[171] examined the differences between cytochromes P_i-420 and P-420. The electrophoretic and spectral characteristics of these two preparations differed and this supports the concept that these two cytochromes are separate entities. Three spectrally distinguishable forms of cytochrome P-450 were found in rat liver microsomes by Comai and Gaylor[172]. The relative amounts of the three forms are altered by pretreatment of rats. Form III is increased by pretreatment with 3-methylcholanthrene, phenobarbital preferentially induces form II and dietary ethanol preferentially induces form I. The authors did not imply any structural difference between these three forms.

Recently, Guenther and Mannering[173] found that the foetal liver is refractory to induction by phenobarbital but not to induction by 3-methylcholanthrene. After parturition, induction of neonatal cytochrome P-450 is possible and the authors suggested that a control mechanism exists in the foetal rat which suppresses induction of cytochrome P-450 but allows induction of cytochrome P_i-450. Simultaneous administration of 3-methylcholanthrene and SKF 525 A partially reversed the supression of phenobarbital induction. Working on induction in pregnant rats, they showed that cytochrome P-450 is induced 4-fold in non-pregnant rats but only 2-fold in pregnant rats. This impairment of phenobarbital induction lasted from 3 days before conception to five days after parturition. The ability to induce cytochrome P_i-450 was not impaired during pregnancy. Again, co-administration of 3-methylcholanthrene partially reversed phenobarbital induction. The similarities in the response between the foetus and the mother led the authors to propose the existence of a common regulatory mechanism.

Imai and Sato[196] were the first workers to produce a cytochrome P-450 preparation that is homogenous on examination by gel electrophoresis. Their preparation of the oxidised form is stable in 20% glycerol and, if stored at -70°C, loses no activity within 30 days. The reduced form undergoes slow conversion to cytochrome P-420 even in the presence of glycerol. Van der Hoeven et al.[70] partially purified cytochrome P-450 and NADPH cytochrome P-450 reductase from rabbit liver microsomes and investigated their properties. Further work[71] led to the purification and characterisation of four distinct forms of cytochrome P-450. Using immunologic techniques, Welton et al.[148] prepared an antibody to the haemoprotein induced by phenobarbital. They showed that the antibody binds preferentially to liver microsomes isolated from phenobarbital pretreated rats and they proposed that there are radical structural and catalytic differences among the different P-450 haemoproteins. Levin et al.[72] partially purified cytochromes P-450 and P_i-450 from animals induced with phenobarbital and 3-methylcholanthrene respectively. (As the ratio of cytochrome P-450 to cytochrome b_5 is greater than 200 : 1 in the active reconstituted system, cytochrome b_5 is not thought to be an obligatory component of the mixed function oxidase.) Comparing the activities of rat and rabbit cytochromes P_i-450 in the hydroxylation of benzo(a)pyrene, Kwalek and Lu[182] concluded that these two haemoproteins are catalytically different from one another. Further work by Kwalek et al.[73] strengthened this opinion. They purified cytochrome P-450 from rabbits induced with 3-methylcholanthrene and allowed it to react with rat antibody produced against cytochrome P_i-450. The poor resultant reaction and the enzymatic and molecular size differences led them to conclude that these two haemoproteins differed from one another. By purifying cytochromes P_i-450 and P-450, Ryan et al.[195] showed that these cytochromes have molecular weights of 48 000 and 53 000 respectively. Thomas et al.[183] prepared an antibody against purified rat cytochrome P_i-450. This showed immunological differences between purified rat cytochromes P-450 and P_i-450 and confirmed

the presence of multiple forms of cytochrome P-450 in purified extracts obtained from phenobarbital pretreated rats. Huang et al.[135] separated and purified four different cytochromes P-450 from liver microsomes of phenobarbital treated mice. All these fractions differ in their spectral and catalytic properties. Using a reconstituted liver microsomal hydroxylase system, Kwalek et al.[74] confirmed spectrally that different forms of cytochrome P-450 exist.

Arinc and Philpot[190] and Jernstrom et al.[189] have both worked on the isolation and purification of cytochrome P-450 from rabbit and rat lung respectively, and the latter group have shown that this haemoprotein is spectrally different from its hepatic counterpart. Cytochrome P-450 has been partially purified from other sources including mice[188,191] and houseflies[192]. Johnson and Muller-Eberhard[197] found three forms of cytochrome P-450 present in hepatic microsomes after pre-treatment of rats with 2,3,7,8-tetrachlorodi-benzo-p-dioxin. Forms a, b and c have molecular weights from 48 000, 54 500 and 60 000 respectively and forms a and b display spectra typical of low spin ferric haeme. Benz(a)pyrene activity is catalysed primarily by the b form and acetanilid hydroxylation by the c form.

Using SDS-polyacrylamide gel electrophoresis, Philpot and Arinc[200] have demonstrated separation of two forms of liver microsomal cytochrome P-450 with identical molecular weights. Ingleman-Sundberg and Gustaffson[201] have used electrofocusing to resolve the P-450 LM_2 fraction of liver microsomes into four forms. They propose that this technique may be used to resolve different types of cytochrome P-450 with very similar physical characteristics.

Anders and Mannering[187] have shown that SKF 525 A induces hepatic microsomal

cytochrome P-450. This results in enhanced N-dealkylation of both itself and ethylmorphine. This activity however does not nullify the microsomal inhibitory activity of SKF 525 A because further doses of the agent inhibit the newly synthesised microsomal enzymes as well as those that were originally present. Furthermore, the major metabolite of SKF 525 A is also a potent inhibitor of the mixed function oxidase system[26]. Recently, Beuning and Franklin[199] have shown that administration of SKF 525 A for several days increases the total cytochrome P-450 content up to five fold. Thus it appears to be an even more potent inducer than phenobarbital.

It has been well established that induction of animals changes the nature of cytochrome P-450[17,172,183] and thus it is important to investigate the metabolism of SKF 525 A under both sets of conditions.

Drug Interaction with Cytochrome P-450

Addition of drugs to adrenal or hepatic microsomal systems results in a variety of spectral changes which are dependent on the existing conditions. Narasimhulu *et al.*[60] working with adrenal cortex microsomes noted a significant spectral change on addition of 17-hydroxyprogesterone.

Cooper *et al.*[79] and Oldham *et al.*[80] have both shown that solubilized adrenal preparations, produced by sonication, still display the same spectral characteristics as found by Narasimhulu *et al.*[60]. Further work by Cooper *et al.*[62] showed that both the concentration of the drug and cytochrome P-450 affects the magnitude of the spectral change. This suggests a direct binding between the active site of the enzyme and the substrate. Remmer *et al.*[63] noted that hexobarbital and SKF 525 A give rise to the type I spectral change while aniline and 2,4-dichloro-6-phenyl-phenoxyethylamine HCl (DPEA) addition to liver microsomes results in a type II spectral change. This is

characterised by a trough at 390nm and an absorption band at ± 430nm. Two hypotheses were formulated to explain these phenomena: I) addition of substrate resulted in the conversion of an "activated oxygen form" of cytochrome P-450 to its oxidized state II) the different type of substrates either binds to the haeme of cytochrome P-450 or results in different conformational changes. It was thought that the two types of spectral changes resulted from substrate interaction on different sides of the haeme in cytochrome P-450. Schenkman et al.[64] confirmed findings by Cooper et al.[62] that the magnitude of the spectral change is dependent on the substrate and cytochrome P-450 concentrations. Furthermore, Type II substrate binding was shown to displace CO from its complex with reduced haemoprotein iron. In addition to the two hypotheses advanced by Remmer et al.[63], they proposed the existence of two different enzymes of the microsomal mixed function oxidase, which accounted for the two types of spectral change. As a result of experimental data, they questioned the first hypothesis of Remmer et al.[63] and from confirmatory data obtained by electron paramagnetic resonance they supported the second hypothesis of ligand modification. Using ferriprotohaeme as a model system, Schenkman and Sato[67] suggested that the spectral changes were due to "the displacement of the sixth ligand from a hydrophobic region of the apoenzyme (possibly the active site) by the substrate". They considered that cytochrome P-450 may exist in two forms i) free enzyme and ii) substrate bound enzyme. During substrate metabolism the first form is converted to the second, accompanied by the type I spectral change. Hildebrandt et al.[61] studied the absolute spectra of cytochromes P-450 which were induced by pretreatment of the animals with phenobarbital or 3-methylcholanthrene. The results indicated that cytochrome P-450 existed in two spectrally distinct forms which they termed high and low spin. Further[81], it was shown that these two forms of the enzyme were interconvertible and were capable of interacting with different types of substrates to produce different spectra. The presence of more

than one molecular species of cytochrome P-450, however, has been suggested by the different product patterns formed during microsomal oxidation of substrates, variations in relative rates of metabolism with different animal species, and the preferential influence of inducing agents for the metabolism of some substrates but not others[82,83,84]. The results of work done by Leibman et al.[85] led them to conclude that cytochrome P-450 was a single enzyme capable of interacting in two different ways with the two classes of compounds. In 1970, Schenkman[65] re-examined the type I and II spectral changes and found that the type I spectrum was also present in the type II spectral change. It was the former component which was responsible for the asymmetric trough seen in the type II difference spectrum. If this spectrum was corrected for the type I component, the resulting spectrum was symmetrical and much larger. In 3,4-benzpyrene treated rats, the failure of type I substrates to give rise to a type I spectral change was due to an inability of type I substrate to interact, rather then the lack of the type I binding site.

Estabrook et al.[89] proposed that type II spectral changes resulted from an interaction of the substrate with the sixth ferric ligand of the haeme. They suggested that this binding probably does not lead to hydroxylation of the substrate. Gorrod and Temple[88] confirmed Schenkman's findings of a type I component in type II spectral changes and suggested that this phenomenon may explain observed metabolism of type II substrates.

Jefcoate et al.[86] showed that pre-treatment of animals with 3-methylcholanthrene increased the content of high spin (type a) cytochrome P-450 in liver microsomes while similar treatment using phenobarbital increased both forms of cytochrome P-450 but favoured the low spin (b) form. Schenkman[65] pointed out that the type a form mentioned above is due to the overlapping of the type I and II difference spectra and the type b form is due to the type II spectrum alone.

Gorrod, Disley and Temple[87] studied the microsomal binding of the two structurally related compounds quinoline and isoquinoline. These compounds gave rise to a type I and type II spectral change respectively. The authors concluded that spectral binding phenomena are "a result of both the steric and basic features of the nitrogen and may be influenced by the lipophilic nature of the molecule." It is of interest to note that type I substrates tend to increase the rate of cytochrome P-450 reduction[66,90], while type II substrates tend to have the opposite effect[90].

A third type of spectral change was noted by Schenkman et al.[64] and was called the modified type II spectrum or reverse type I. This is characterised by a peak at about 420nm and a trough at about 392nm in the difference spectrum and was thought to be due to the displacement of some pre-existing substance, which was bound in vivo, from the enzyme[91]. Wilson et al.[92] found that at low concentrations, agroclavine interaction with liver microsomes produced a type I spectral change. As the agroclavine concentration increased, so the reverse type I spectrum manifested itself. It was thought that the type I binding was responsible for the metabolic conversion of agroclavine as the K_s value for the type I spectral change was very similar to the K_m value. In a recent study, Misselwitz et al.[96] studied the effect of temperature on substrate binding to cytochrome P-450. It was found that type I substrates binding was increased by higher temperatures and the reverse applied to the type II substrates. They reaffirmed the idea of separate binding sites for the two classes of substrates and their results indicated that the type I site was lipophilic in nature.

Electron Paramagnetic Resonance (E.P.R.) Studies

Before Narasimhulu et al.[60] had documented the substrate induced spectral changes, Hashimoto et al.[93] undertook a study of microsomal electron

transport pathways using electron spin resonance spectroscopy. They found present a compound, designated Fex, which they considered was probably a haeme protein. Reduction of Fex was catalysed *via* enzyme systems involving NADH and NADPH and in this reduced state Fex or a component of the system to which it transported electrons, was capable of binding CO reversibly. Mason *et al.*[54] proposed that Fex was the sulphide of cytochrome P-420 and that cytochrome P-450 in its reduced or oxidized state was the phospholipid complex of the sulphide of P-420. In the CO complex, CO was thought to displace -S but continued to interact both with it and the phospholipid. In the functional enzyme, substrate, oxygen and -S were thought to interact at the protohaeme group with the -S acting as a terminus of the electron transport chain which supplied the two electrons required for metabolism.

Crammer, Schenkman and Estabrook[94] studied the manner in which substrate interaction with cytochrome P-450 affected the E.P.R. spectra. They suggested that a correlation may exist between the two types of optical and E.P.R. spectra. They went further, on the basis of their E.P.R. data, to support the idea that substrates interact in association with the haeme of cytochrome P-450 resulting in modification of the haeme iron ligands. Hildebrandt *et al.*[61] concluded from difference absorption spectroscopy that different spin states of liver microsomal cytochrome P-450 exist which change state upon substrate interaction. Whysner *et al.*[95], working with steroids and adrenal microsomes, reached the same conclusions.

Using rats that had been induced with 3-methylcholanthrene, Jefcoate and Gaylor[97] produced evidence for a second type of cytochrome P-450 and a scheme for protein binding to cytochrome P-450 in the two oxidation and spin states of iron. Similar studies were carried out by Peisach and Blumberg[98], who suggested that cytochrome P-450 could exist in two forms in which the haeme iron may be in either the high or low spin state depending upon the

nature of the non-porphyrin ligands of the metal. They did not consider it necessary to propose the existence of two different cytochrome P-450 proteins. Further work by Jefcoate and Gaylor[99] in 1970 reinforced their opinion that two forms of cytochrome P-450 were thought to exist. They showed that 11-deoxycorticosterone and 11-dehydrocorticosterone occupy distinct binding sites on cytochrome P-450 and concluded from their spectral data that these molecules may alter the spin state of the forms of cytochrome P-450 to which they are bound. As a result of their studies, Reichman et al.[100,101] proposed that the haeme of cytochrome P-450 exists in a hydrophobic pocket formed by adjacent amino acid residues. Conversion of cytochrome P-450 to cytochrome P-420 is thought to result in an increase in the distance between the haeme and the protein surroundings. Type I binding supposedly results in removal from the haeme of some of the protein ligands. This decreases the electron density on the iron, converts it from low to high spin and renders cytochrome P-450 more succeptible to reduction.

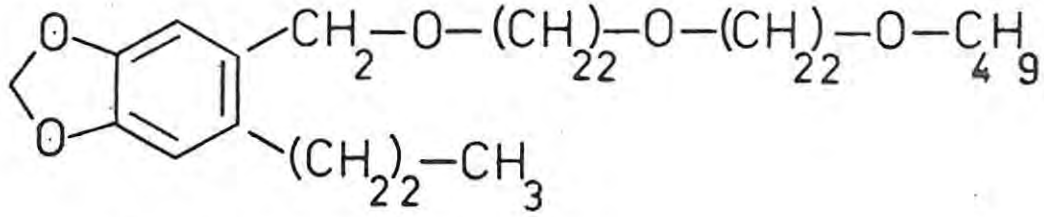
In 1964, it was shown that cytochrome c reductase was responsible for the transfer of an electron from NADPH to cytochrome P-450[77]. Estabrook et al.[75] proposed the necessity of a component to act as a bridge between the flavoprotein reductase and cytochrome P-450. These workers also studied the enzymatic reduction of cytochrome P-450 and found that two electrons were required for every molecule of substrate metabolised. Further investigation by Waterman and Mason[76] showed that only one electron is required for reduction of the oxidised form of cytochrome P-450. Currently, the electron transport system is thought to be divided into two branches; an NADPH dependent flavoprotein[264] coupled with cytochrome P-450[72] and an NADH dependent flavoprotein[62] linked to cytochrome b_5[265]. These four components are thought to be linked to one another by an unidentified "factor x"[266]. Controversy over the electron accepting

properties of cytochrome P-450 still persists, and recent results indicate that only one electron is required for its reduction[198].

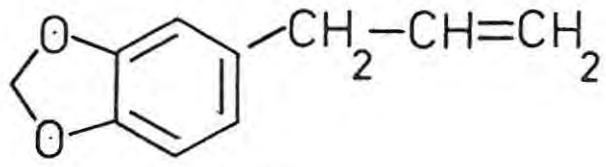
In vitro activity requires an NADPH-generating system and the presence of Mg^{++} [78,235,236]. Initially, nicotinamide was thought to be essential to prevent breakdown of NADP by nucleotidases[236,11], but more recently it has been shown to inhibit drug metabolism[237].

Spectral Studies with SKF 525 A

As mentioned previously, Schenkman et al.[23] demonstrated the formation of the 455nm peak which appears on metabolism of SKF 525 A in vitro and this peak was ascribed to the formation of an oxygenated complex. Production of this 455nm peak is not peculiar to SKF 525 A however and the methylene dioxyphenyl compounds such as piperonyl butoxide and safrole also give rise to similar



Piperonyl Butoxide



Safrole

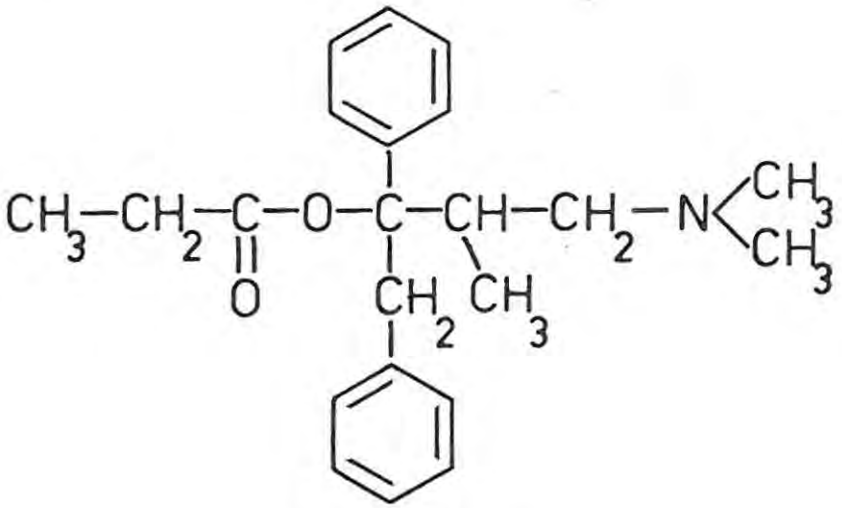
peaks when incubated with liver microsomes, NADPH and oxygen in vitro[28] and in vivo[29]. This 455nm peak was thought to be due to the formation of an "active intermediate", possibly a carbene or a carbanion[30,33], which then binds to the haeme of cytochrome P-450. The complex was thought to inhibit oxygen activation and further metabolism of substrate[31].

Elcombe et al.[32] found that the 455nm peak disappeared after removal of reducing conditions but reappeared immediately on readdition of a reducing agent.

Omura and Sato[34,35] noted that ethyl isocyanide also gave rise to the 455nm peak when added to reduced microsomes. Studies carried out on this spectrum indicate that both of the two bands in the Soret region (430 and 455nm) are caused by the binding of ethyl isocyanide to the reduced form of cytochrome P-450[34,35,36,37,38]. Imai and Sato[39] found that pH exerts a profound effect on the relative heights of the 430- and 455nm peaks such that, as the pH becomes more alkaline, so the 455nm peak increases in magnitude and the 430nm peak decreases. This behaviour suggests an interrelationship between the two peaks and Imai et al.[39] proposed that reduced cytochrome P-450 is split into interconvertible states, which are in a pH-dependent equilibrium. They further reported[40] that dithionite reduced protohaeme interacted with ethyl isocyanide to produce three spectrally different compounds. The 414nm and the 428nm peaks were assigned to the monoisocyanide and the normal di-isocyanide compound ("n" form) respectively. The third species ("a" form) was anomalous in that it showed a Soret peak at 455nm and it was thought to correspond to a polymerised state of the di-isocyanide compound of protohaeme. Further work by Imai and Sato[41] indicates that ethyl isocyanide exerts both a stimulatory and inhibitory effect on the microsomal hydroxylase system. The competition between ethyl isocyanide and oxygen for the haeme of reduced cytochrome P-450 is responsible for the inhibitory effect, whereas the stimulatory effect is thought to result from the change of sensitivity of oxidized cytochrome P-450 caused by its combination with ethyl isocyanide.

Franklin[42,43] showed that amphetamines and some sympathomimetic amines produced the 455nm peak when they underwent mixed function oxidation.

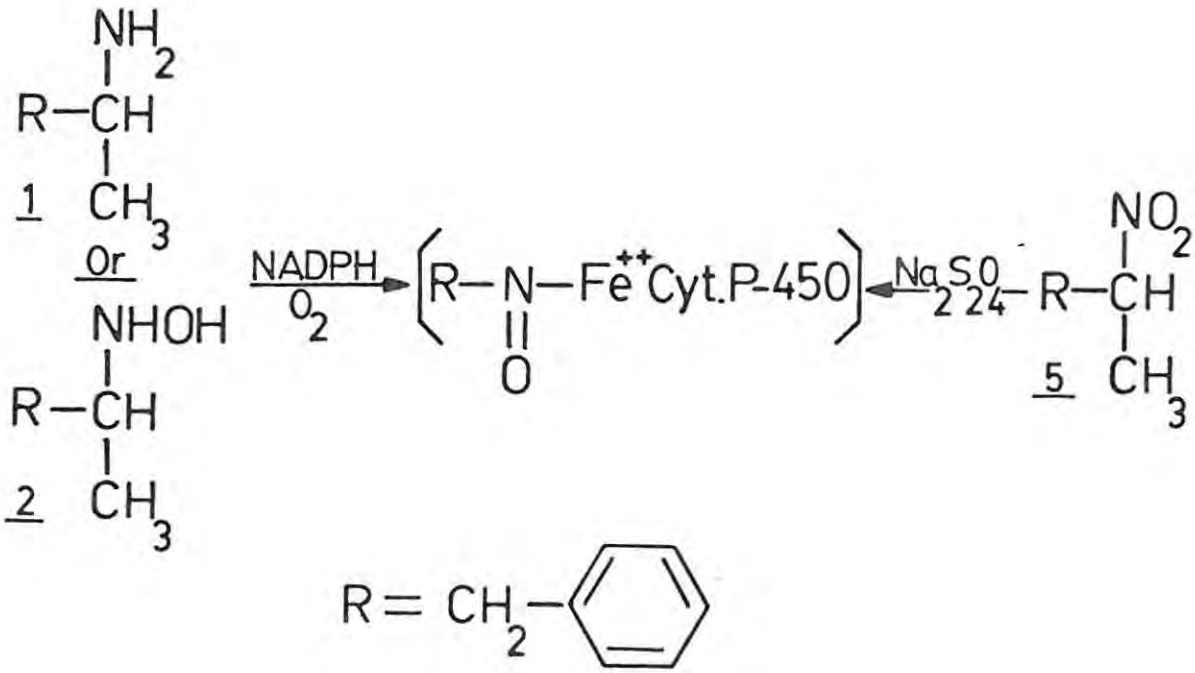
Phenobarbital pretreatment of rats drastically increases the rate of peak formation as the levels of cytochrome P-450 and the activity of NADPH cytochrome-c-reductase are increased[44]. Induction also increases the rate of the deamination reaction which tends to suggest that the 455nm complex is formed from an intermediate of this reaction. James and Franklin[45] compared 455nm complex formation between rat and rabbit microsomes and found that the latter showed a stereo-chemical preference for the l-isomer of amphetamine. With SKF 525 A and propoxyphene, much lower rates of metabolism were observed with rabbit microsomes, which is not surprising as the major metabolites differ between the two species. Again, induction with phenobarbital was shown to increase the rate of deamination and also the rate of 455nm complex formation. This finding adds substance to the suggestion that oxidative metabolism at or around the nitrogen is implicated in the 455nm complex formation. Buening and Franklin[46] demonstrated that compounds structurally related to SKF 525 A (adiphenine, benactyzine, propoxyphene, diphenhydramine, desipramine and nortriptylene) are also capable of forming the 455nm complex when they undergo mixed function oxidation.



Propoxyphene

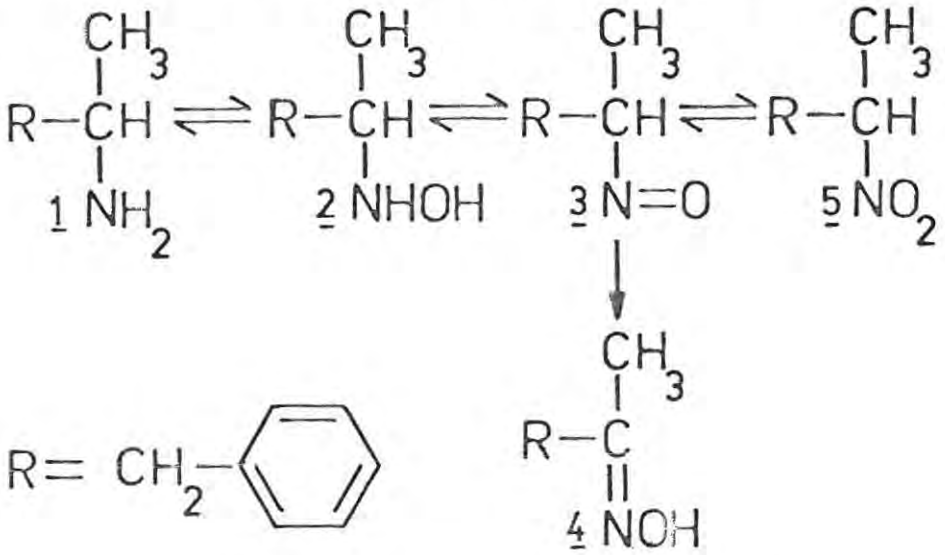
Again phenobarbital pre-treatment enhances 455nm peak formation. It is interesting to note that, contrary to findings of Schenkman et al.[23], Buening and Franklin were unable to produce the 455nm complex on metabolism of SKF 26754 A. Franklin[47] reported that metabolism of p-chloroaniline, sulfanilamide, 2-methyl-indoline and dapsone produced complexes, which absorbed maximally at 448, 450, 451 and 452nm respectively. These complexes resemble those produced by SKF 525 A but differ in that they are unstable in the presence of sodium dithionite. It is thought that these compounds represent a new class which form complexes during microsomal oxidative metabolism.

Mansuy et al.[48] proposed the following reaction scheme to account for the 455nm complex formed during amphetamine metabolism. The 455nm complex

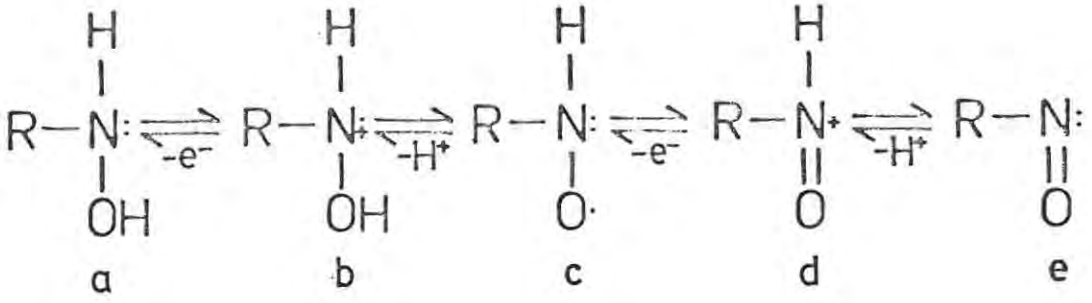


appeared to be formed after reduction of 5 and, as Franklin[49] showed that the complex is formed after oxidation of 2 , the metabolite bound to reduced cytochrome P-450 should have the same oxidation level as 3 or its tautomer 4 .

The following diagram shows the possible redox intermediates between amphetamine 1 and its corresponding nitro compound 5 .



Jonsson and Lindeke[50] investigated the metabolism of phenylalkylamines and found that, on metabolism, they produced a peak at 455nm. This was dependent on one oxygen atom being attached to the nitrogen. This is illustrated in the following reaction scheme.



They suggest that c may represent the intermediate between N-hydroxyamphetamine and the nitroso compound which binds to the iron in cytochrome P-450.

Possible Metabolic Routes for SKF 525 A

Anders *et al.*[26] studied metabolism of SKF 525 A showing it to be dealkylated to the secondary amine, SKF 8742 A, and an unidentified minor metabolite. Much work has been done concerning the metabolic fate of nitrogen containing compounds, similar in structure, to a greater or lesser degree, to SKF 525 A. The following review facilitates prediction of the possible pathways of SKF 525 A metabolism.

N-oxidation, N-hydroxylation and N-dealkylation

Due to the complexity and interdependence of these reactions they shall be considered together.

Norris and Benoit[105] showed that trimethylamine is converted to its N-oxide when injected into the rat. This result was confirmed by Baker and Chaykin[103] using pig liver microsomes *in vitro*. There has been considerable difference in opinion on the reaction pathways involved. Mueller and Miller[109] presented indirect evidence, which indicated that the *in vitro* demethylation of dimethylaminoazobenzene occurred through an N-hydroxymethyl intermediate. McMahon and Sullivan[106] also suggested that direct oxidative attack occurred on the methyl group of N-methyl tertiary amines to form the N-hydroxymethyl derivative. This would readily have decomposed to the secondary amine and formaldehyde. Zeigler and Pettit[107] considered that the oxidative attack on the nitrogen atom of the tertiary amine gives rise to an intermediate N-oxide. This intermediate is then dealkylated by a second enzyme system. Cholate treatment of microsomes did not destroy the N-oxidizing capacity of the enzyme. This indicates that the N-oxidizing ability of microsomes may reside in a separate system to that required for N-dealkylation. Further work[102] strengthened this view and it was shown that SKF 525 A and carbon monoxide inhibit the N-dealkylation of

N,N-dimethylaniline (D.M.A.) but permit N-oxide formation. Axelrod et al[5]. showed that both these agents inhibit the N-dealkylation of the parent tertiary amine.

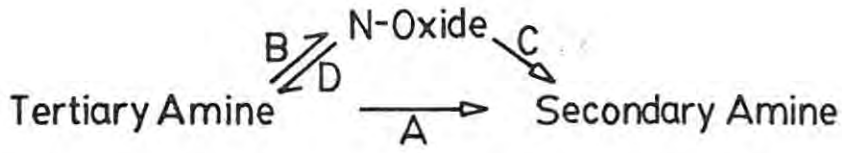
McMahon[222] found that tertiary amines are generally N-dealkylated to a greater degree than secondary amines, this being due to a correlation which exists between lipid solubility and rate of N-dealkylation. Approximately 80% of a dose of the diethyl substituted tertiary amine, diethylpropion, undergoes de-ethylation to produce the secondary amine. Only 50% of a dose of the secondary amine metabolite is converted to the primary amine[223]. Anders et al.[26] have established that the unidentified metabolite of SKF 525 A is indeed present in reduced quantities as compared to the secondary amine and the situation outlined above may apply.

Gorrod and Temple[137] identified N-hydroxymethyl-carbazole as an intermediate in the mammalian metabolism of N-methylcarbazole both in vitro and in vivo. In this report, similar intermediates are shown for a number of compounds. However, all of these have a carbonyl group adjacent to the nitrogen which may stabilize this intermediate. The carbonyl group in SKF 525 A is well removed from the nitrogen atom and is thus unlikely to exert a stabilizing influence.

Zeigler and Pettit[104] proposed that the lipid soluble tertiary amine could penetrate to the dimethylaniline (D.M.A.) oxidase where it is oxidized to the polar N-oxide. Because of its increased polarity, the N-oxide is unable to escape from the lipoidal compartment surrounding the enzyme and is thus N-dealkylated to the less polar secondary amine. They showed that sonication disrupts the membrane and this allows the N-oxide to leave its site of synthesis. Bickel[124] and Jenner[202] pointed out that because the N-oxide is far more polar than the parent amine extraction techniques must be modified accordingly.

Both Baker and Chaykin[103] and Zeigler and Pettit[104] agreed that the enzymes catalysing N-oxidation and N-dealkylation are very similar. Dehner et al.[108] showed that SKF 525 A only inhibits the dealkylation of the N-oxide intermediate but does not directly affect the oxidation of the tertiary amine to the N-oxide. Using the aliphatic amine N, N-dimethyl-n-octylamine (D.M.O.A), they found that increased excretion of N-oxide results when SKF 525 A was injected into the test animals. They concluded that the N-oxide may also be an intermediate in the oxidative N-dealkylation of aliphatic tertiary amines. Hodgson et al.[111] studied the metabolism of N-dimethylcarbamates. It was not possible to identify the products but they were expected to be either the N-oxide or the N-hydroxymethyl derivatives. Battersby[110] has proposed that the N-hydroxymethyl intermediate may arise from the tertiary amine N-oxide intermediate.

Masters et al.[119] showed that microsomal NADPH cytochrome c reductase antibody does not inhibit the N-oxidation of N,N-dimethylaniline and other tertiary amines. Moreover, Reiner and Uehleke[120] found that an NADPH cytochrome c reductase preparation from microsomes does not catalyse any N-oxidation or N-hydroxylation reactions. Bickel et al.[114] studied the metabolism of imipramine. Their findings indicated that reactions C and D are dependent on both haeme catalysis and enzymatic processes. Furthermore, they found that reactions A and B were inhibited by heat pre-treatment, N_2, CO and SKF 525 A. In a later series of experiments, Bickel[115] found that CO, SKF 525 A and excess substrate inhibit tertiary amine demethylation



but not N-oxidation. This observation is in accord with the findings of Gigon et al.[116], who concluded that N-oxidation is catalysed by an alternative microsomal electron transport system, also dependent on NADPH but not cytochrome P-450. Gorrod[133] showed that N-oxidation and N-dealkylation of N-ethyl-n-methylaniline are different metabolic routes and N-oxidation does not involve cytochrome P-450. Bickel[115] demonstrated that tertiary amine demethylation and N-oxidation occur in the 9 000 g supernatant fraction and in hepatic microsomes plus NADPH. These two reactions did not occur in liver homogenate. N-Oxide reduction and N-oxide demethylation occur in the latter system but not the former and all four reactions occur simultaneously in liver homogenate plus an NADPH generating system. It was also shown that imipramine N-oxide formation is far more sensitive to a decrease in PO_2 than the corresponding N-demethylation or aromatic hydroxylation[125]. As a result of the determination of reaction constants, Bickel[115] did not consider it possible for the N-oxide to act as an intermediate in the N-demethylation of imipramine.

Nicotinamide N-oxide reduction has been investigated by Chaykin et al.[130], who showed that the enzyme responsible is a metalloflavoprotein dependent on NADH and other cofactors. This compound was also reduced by xanthine oxidase from liver or milk with xanthine acting as the electron donor[131].

Uehleke[118] studied the effects of phenobarbital, 3-methylcholanthrene and DDT pretreatment on rats in evaluating the N-oxidation reaction. In all cases, it was found that induction does not stimulate the reaction. The converse applies to N-hydroxylation. This tends to confirm the lack of involvement of cytochrome P-450 in some N-oxidation reactions. Further work[117] confirmed that cytochrome P-450 participates in the N-hydroxylation of primary arylamines but not in the N-oxidation of N-alkylanilines and tertiary amines. Pre-incubation of microsomes at 37°C in the absence

of NADPH rapidly destroys enzyme activity for the N-oxidation of secondary and tertiary amines and precautions against this must be taken in experimental work.

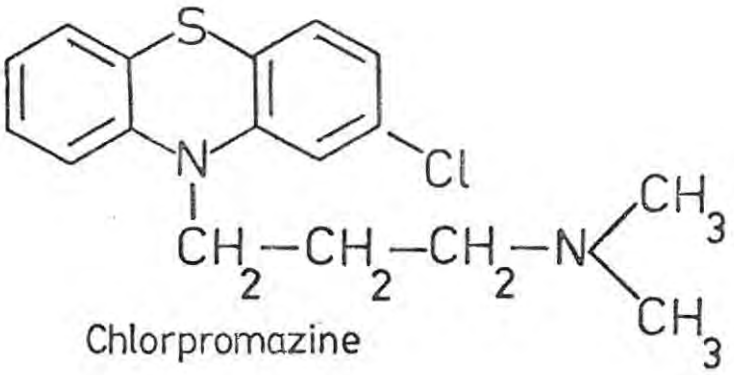
Working with amphetamines, Beckett et al.[121] found that SKF 525 A and CO block α-carbon oxidation on either side of the nitrogen while dithiothreitol and cysteamine inhibit N-oxidation.

While Beckett et al.[121] claim that dithiothreitol and cysteamine are specific inhibitors of the N-oxidase system, Mull et al.[204] have recently investigated the inhibitory effects of cysteamine and have found that this agent inhibits type I activity (polycyclic hydrocarbon substrates) in the mixed function oxidase system. Evidence indicates that cysteamine interacts with an intermediate electron carrier of non-haeme iron or glycoprotein character thus inhibiting mixed function oxidase activity. Furthermore, Gorrod[132] has suggested that two N-oxidase systems exist, one dependent on cytochrome P-450 and the other independent. The ability of a substrate to utilize these enzymes is dependent upon its pKa. Thus, a basic amine such as SKF 525 A should be N-oxidised by a non-cytochrome P-450-dependent system. Non-basic nitrogen containing compounds, e.g. urethane[205] and 4-acetyl-aminobiphenyl[206], utilize a cytochrome P-450 dependent system while compounds of intermediate pKa, e.g. dimethylaniline[207], may be substrates for both enzymes and will thus yield the same products but by different pathways. Weisburger and co-workers[208,209] were uncertain whether the N-oxidation of some N-acetylarylamines was mediated by cytochrome P-450 or by a non-cytochrome P-450-dependent flavoprotein. Investigating the N-hydroxylation of 2-acetylaminofluorene Thorgeirsson et al.[210] showed the involvement of NADPH cytochrome c reductase in the reaction. Immunochemical procedures were used.

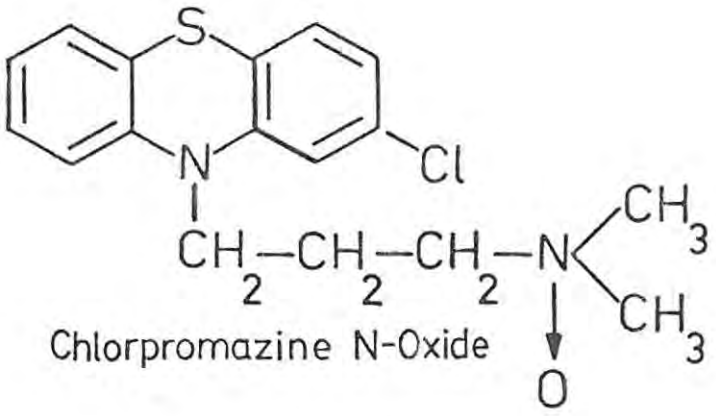
There is still uncertainty as to whether the cytochrome P-450-dependent N-oxidase system is identical with that involved in other cytochrome P-450-mediated processes. Evidence from some studies suggest that this is not the case. While pretreatment of hamsters with 3-methylcholanthrene distinctly increased microsomal acetylaminofluorene N-hydroxylation, there was little difference in ring hydroxylation[211,212]. Similarly, pretreatment of rats with 3-methylcholanthrene resulted in an increase in ring hydroxylation of acetylaminofluorene but little change in the N-hydroxylation pattern[213]. Zeigler et al.[112] succeeded in purifying a microsomal N-oxidase from pork liver microsomes. They determined that it was an NADPH dependent, but cytochrome P-450 independent flavoprotein which catalyses N-oxidation. Use of SKF 525 A as an inhibitor greatly increases N-oxide production while use of CO/O_2 mixtures have less effect[113]. This could indicate that SKF 525 A is either a more effective inhibitor of further oxidation or that it was metabolised by the amine oxidase, as it is itself a tertiary amine, and contributed to the amine oxide formation. This enzyme appears to possess an activator site as well as a catalytic site[214,215,216]. Substrates such as primary alkylamines, which are not N-oxidized by this system, can interact with the regulatory site and enhance the rate of N-oxidation of other amines[216,241]. The pH optimum for this enzyme has been shown to be above pH 8[215]. Burke and Mayer[239] have shown that phenobarbital pretreatment of animals does not induce amine oxidase.

Beckett and Al Sarraj[123] showed that there is considerable variation in species dependent metabolism of amphetamines and Gorrod[138] has reported that hepatic microsomes prepared from rats have very poor N-oxidizing capacity, while those prepared from the guinea pig are far more active. Bickel[124] has pointed out that N-oxides are usually minor metabolites and it is possible for them to have a more potent activity than the parent amine. Thus, metabolite 1 of Anders et al.[26] may well fit into this catetory and

possibly account for the toxicity of SKF 525 A. Exceptions to this rule of thumb exist however, and Beckett and Hewick[126] have shown that N-oxide is a major metabolite of chlorpromazine biotransformation in vitro. Demonstration



Chlorpromazine



Chlorpromazine N-Oxide

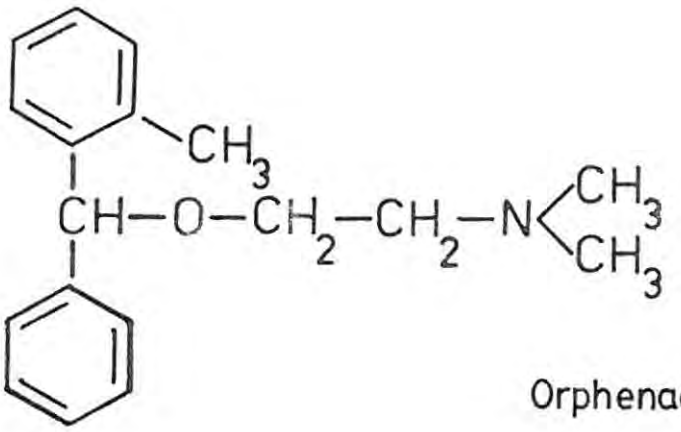
of this fact is dependent on the correct extraction procedure. Beckett and Eissen[127] showed that chlorpromazine is N-dealkylated and the resulting compounds are then N-oxidized to form hydroxylamines. Primary and secondary amines may also be N-oxidized to produce hydroxylamines. Tertiary amines may be dealkylated in vivo and in vitro to produce secondary amines, which may be N-oxidized to form hydroxylamines[128]. These pathways may be pertinent to SKF 525 A metabolism and are known to apply to dimethylamphetamine[128] and phendimetrazine[129] metabolism.

Beckett and Belanger[134], studying the metabolism of phentermine and chlorphentermine, proposed a mechanism whereby a complex of the oxygen

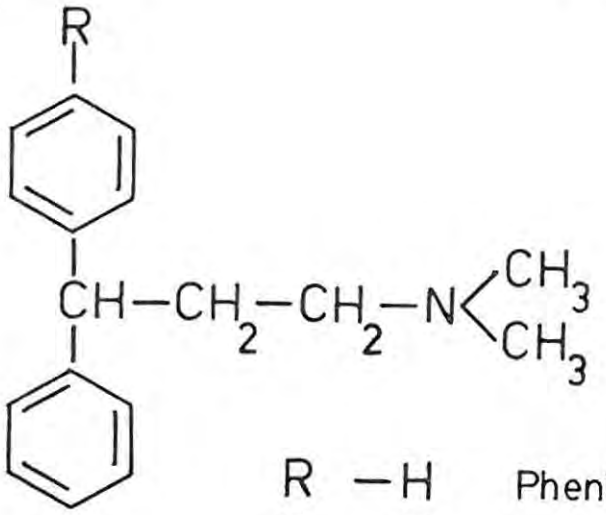
radical/flavoprotein with the nitrogen radical cation give rise to both the nitroso and hydroxylamine derivatives. Separate mechanisms for the formation of each of these compounds are involved. An extension of this work[135] outlined general pathways for the metabolism of primary aliphatic amines to yield oximes, hydroxylamines, C-nitroso products, ketones or aldehydes and secondary amines to yield primary and secondary hydroxylamines, nitrones and the dealkylated and deaminated products. They concluded that there is a common metabolic N-oxidative pathway for primary and secondary amines which involves different enzymes from those responsible for the N-oxidation of tertiary aliphatic amines. Extrapolation of these findings[136] provided analogous pathways for the metabolism of primary and secondary aromatic amines.

Stereochemical features around the nitrogen atom also affect the nature of metabolic conversion. Hewick and Beckett[203], studying metabolism of phenothiazine derivatives with different side chains, found that, generally, a dimethylamine group attached to a straight chain with 2, 3 or 4 methylene groups is N-oxidized by rat liver microsomes. However, N-oxidation is abolished when a diethylamino is substituted in place of the dimethylamino group and steric factors are thought to be responsible. This is the state of affairs existing for SKF 525 A and thus it is unlikely that this agent should form the N-oxide. However, as a rule, N-oxidation of tertiary aliphatic amines occurs in large molecules containing two aromatic rings[220] and SKF 525 A falls into this catetory. Nevertheless, it is likely that steric factors should override this latter consideration.

Antihistamines are also capable of forming N-oxides[221]. Using liver preparations from guinea pigs, the N-oxide metabolite represented about 15% of the incubated amount of orphenadrine, whereas pheniramine, chlorpheniramine and brompheniramine produced 15%, 15-30% and 40% N-oxide respectively.



Orphenadrine



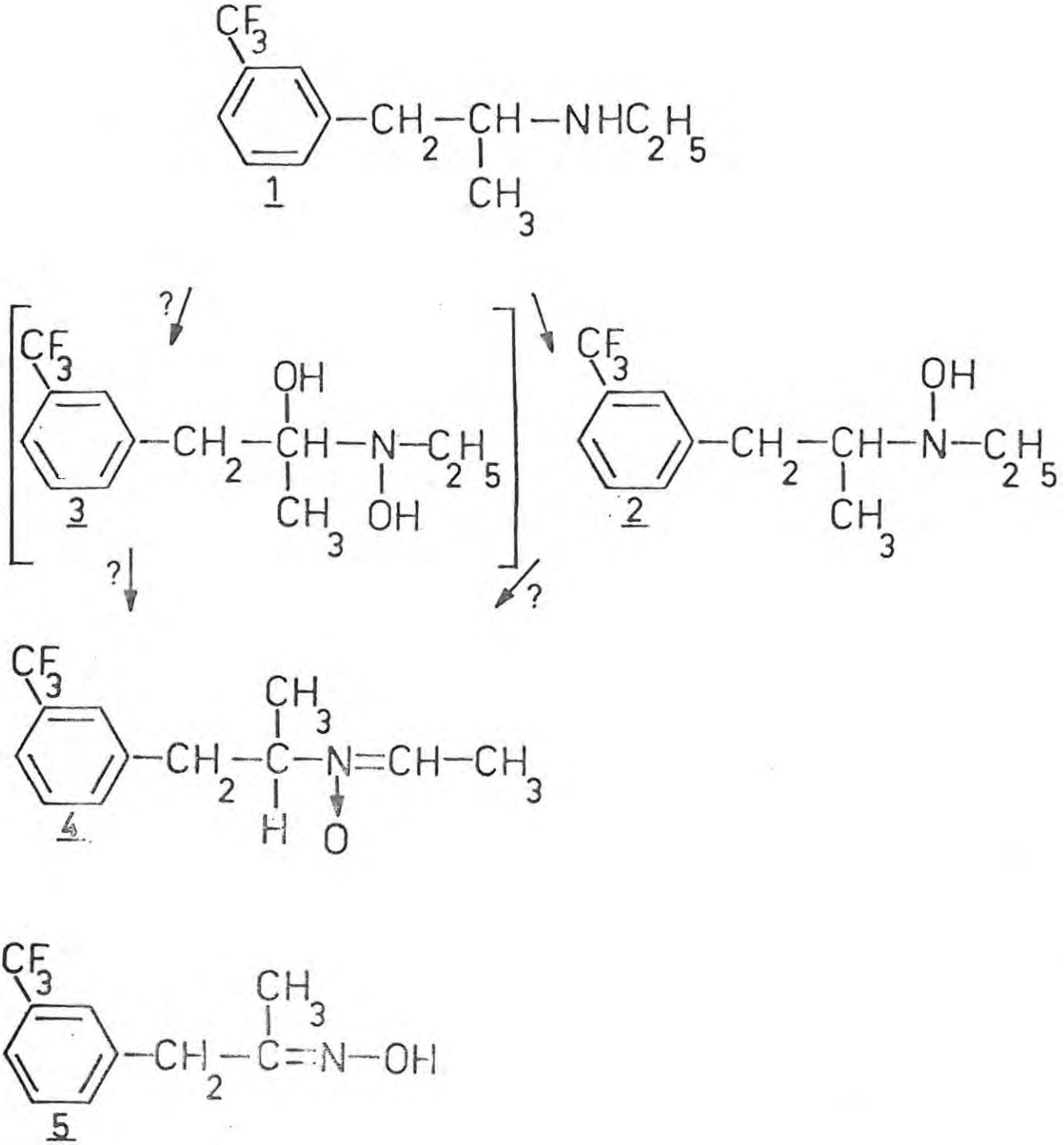
R —H Pheniramine
R —Cl Chlorpheniramine
R —Br Bromopheniramine

It is interesting to note that, in spite of the structural similarity of the latter three antihistamines mentioned, N-oxide production varies considerably. Thus, it appears that long range steric effects exert more influence than is apparent at first sight. These factors make it more difficult to predict the metabolites of a molecule as complex as SKF 525 A.

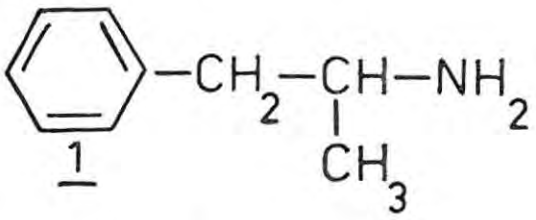
Fenfluramine is structurally related to SKF 8742 A. Beckett et al.[217,218] showed that fenfluramine is metabolically converted to the hydroxylamine (2),

the ketoxime (5) and the nitrone (4).

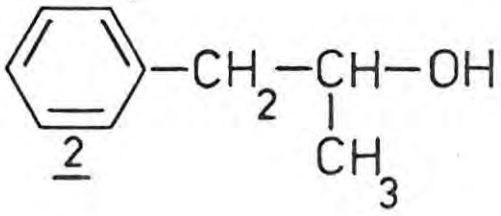
Metabolic Conversion Of Fenfluramine



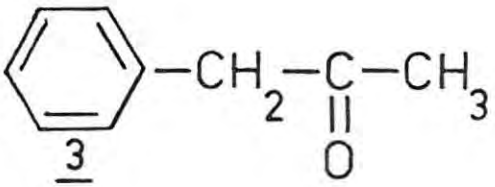
Metabolic Conversion Of Ethylamphetamine



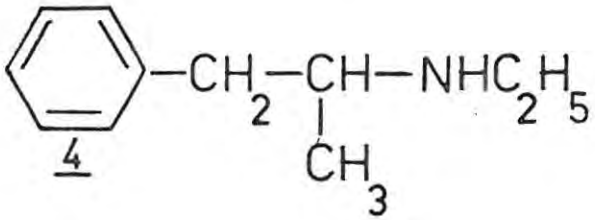
1



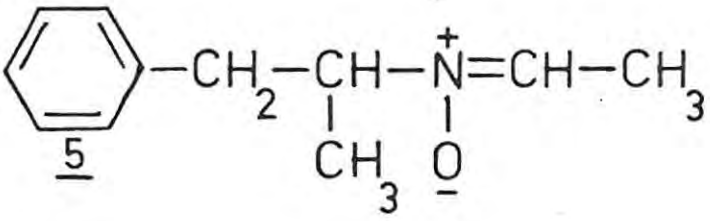
2



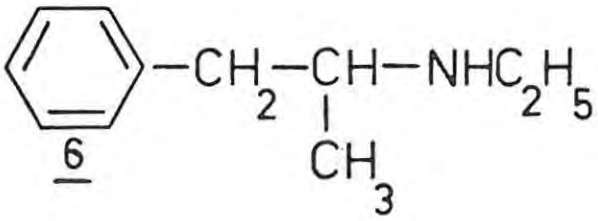
3



4

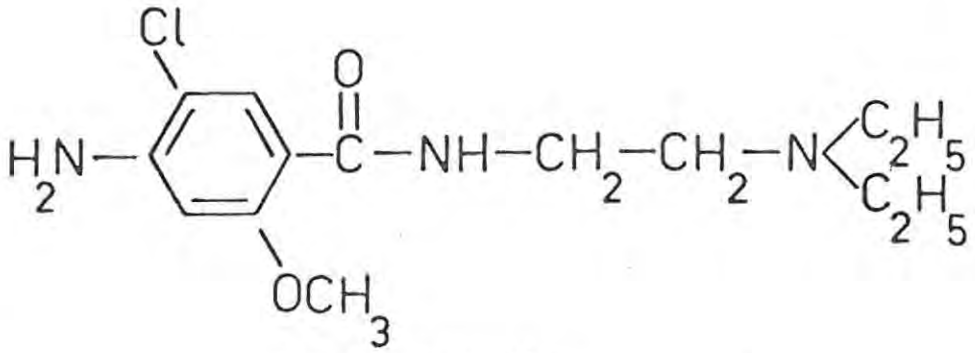


5



6

It is not definitely known whether the nitrone is an actual metabolite or if it is formed by non-enzymic reactions. The same workers have investigated the metabolism of metoclopramide *in vitro* and have

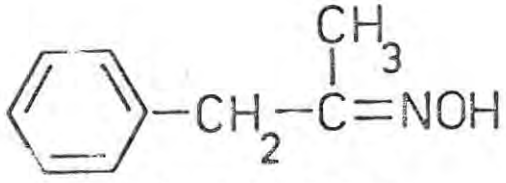


Metoclopramide

shown this molecule to be di-de-ethylated[230]. The metabolism of ethylamphetamine (6) gave rise to the following metabolic products[219]: 1. amphetamine, 2. 1-phenyl-propan-2-ol, 3. phenylacetone, 4. N-hydroxyethylamphetamine and 5. α-methyl-N- (1'-phenylprop-2'-yl). nitrone. (See page 37).

Deamination

Hucker and co-worker[224,225] have studied the metabolism of amphetamine using rabbit liver microsomes and claimed that, under their conditions,



Phenylacetone Oxime

phenylacetone oxime was the key intermediate in the oxidative deamination of the drug. The results of Parli et al.[226,227] under the same conditions differed, and they demonstrated the incorporation of molecular oxygen into phenylacetone, whereas the pathway of Hucker et al.[229,225] would involve the incorporation of oxygen from water. The work of Hes and Sternson[228] confirms these findings. Deamination of aliphatic amines substituted with large groups may also occur. In the metabolism of propranolol[229], it was found that isopropylamine is present as a metabolite. Beckett et al.[122] have shown that oximes may be involved in the deamination pathway and that N-oxide formation preceeds oxime formation[123].

Physiological Factors Affecting Drug Metabolism

The pharmacological effect of a drug may vary according to a number of different physiological factors which are often interrelated. Thus, it is important to consider the species, strain, sex, age etc. of the test animals used to evaluate drug metabolism.

Williams[270] has reviewed species differences in drug metabolism. Parke[271] has shown that the in vivo hydroxylation of aniline varies according to the species used, and Creaven et al.[272] have confirmed these results by monitoring the species variation in the in vitro hydroxylation of biphenyl.

Strain variation within a species also causes differences in drug metabolism, and a study of imipramine and aminopyrine N-demethylase, p-nitroanisole O-demethylase and aniline 4-hydroxylase in three different strains of rat indicated differences in metabolism[273]. Jori and Pescador[274] have revealed that strain differences exist in the induction of rat microsomal enzymes by 3-methylcholanthrene and phenobarbital.

The sex of the test animal also affects metabolic patterns and one of the factors involved is the balance between the male and female sex hormones[275]. Irradiation of male rats decreases aminopyrine metabolism but it has no effect on female rats. This is thought to be due to the inhibition of the synthesis or release of testosterone[276].

Age affects development of drug metabolising enzymes, and after birth there is an initial rapid increase for 2-4 days and, then, a slower rise in activity for about 30 days. This pattern holds for a number of species[277]. After reaching a maximum, activity may decrease to a constant lower level, e.g. biphenyl-4-hydroxylase, it may slowly increase, it may remain constant or it may decrease to negligible levels[278].

Jori et al.[138] found that diurnal variation also affects drug metabolism with maximal activity occurring at night. Variations range from 10-50% of average values and reversal of light and dark sequences leads to reversal of metabolic patterns.

Pregnancy decreases drug metabolising activity in the rat and rabbit[147,279] while rabbits at full term pregnancy show no reduction in liver weight or cytochrome P-450, glucuronyl transferase activity decreases by 20% and coumarin 7-hydroxylase activity by 60%. These changes are prevented by pretreatment of animals with phenobarbital[279].

As the liver is the main site for drug metabolism in the body, hepatic disease will affect metabolic patterns, e.g. malarial infection causes interference of metabolism[280].

CHAPTER 2

OPTIMISATION OF SKF 525 A METABOLISM IN VITRO

Objectives

Buffers with a pH of 7,4 are most commonly used in experiments dealing with hepatic microsomes. The rationale behind their use is unclear and, to the author's knowledge, little investigation in this field has been performed. When dealing with *in vitro* metabolism, the metabolic system as a whole should be investigated and not merely the terminal oxidase. Thus, the properties of other enzymes involved should be taken into consideration as their efficient function is essential for optimal metabolic conversion. Isocitrate dehydrogenase forms part of the NADPH generating system required for reducing equivalents. The optimal pH for activity of this enzyme is 7,8[249] and, hence, its use in a medium at pH 7,4 will not be ideal.

It is, therefore, the objective of these studies to investigate the following:

i. The effect of pH on drug binding to cytochrome P-450. Schenkman *et al.*[64] have shown that the type 1 difference spectrum results from interaction between cytochrome P-450 and the substrate and furthermore the magnitude of the type 1 spectrum has been shown to parallel enzyme activity[253]. It is on the basis of this rationale that the effect of pH on the magnitude of the type 1 spectral change will be investigated.

ii. Procedures required for metabolism of SKF 525 A and SKF 8742 A.

iii. The effect of pH on metabolism of SKF 525 A and SKF 8742 A.

iv. The stability of the metabolites.

1. The Effect of pH on the Magnitude of the Type I Binding Spectra

i) Materials and Methods

a) Preparation of Microsomes

Microsomes were prepared according to the method of Cinti et al.[244]. The sex of a rat was noted, it was then killed by cervical dislocation and weighed. The liver was removed, immersed in ice cold 0,9% NaCl and weighed. All subsequent operations were performed at c. 4°C. The liver was perfused with 0,9% NaCl to remove haemoglobin and ensure a rapid fall in tissue temperature. It was then sliced up and a 10% mixture was prepared with 0,25 M sucrose. This was homogenised with 6-8 excursions of the Thomas tissue grinder, and the resulting mixture was centrifuged at 600g and 12 000g for 5 and 10 minutes respectively in a refrigerated M.S.E.18 high speed centrifuge. This removed the cell debris, nuclei and mitochondria. The supernatant was treated with 8mM Ca^{++} and centrifuged at 27 000g for 15 min which allowed complete sedimentation of the microsomes into a compact pellet. The pellet was washed (to remove any remaining haemoglobin) by resuspension in an equal volume of 1,15% KCl and divided equally between six centrifuge tubes. These were spun for a further 15 minutes at 27 000g, the clear supernatant was discarded and the microsomal pellet immersed in buffers of varying pHs. The pellets were stored on ice and homogenised as required. All animals used in these studies were male, 300-350g, Rhodes University Tick Research rats and they were sacrificed at 09h00 for each experiment.

All glassware used was soaked in chromic acid, well rinsed with distilled water and dried in an oven.

b) Protein Determination

Two methods were used.

i) Goa's Modified Microbiuret Protein Determination

Method. 0,2ml protein solution was added to 3,8ml 3% NaOH followed by 0,2ml Benedict's reagent. After 15 min the absorbance was read at 550nm in a 1cm path length cell.

Benedicts Reagent. 17,3g trisodium citrate and 10g sodium carbonate were dissolved in about 80ml of hot water. This was filtered into a graduated vessel and made up to 85ml with water. 1,73g of copper sulphate was dissolved in about 10ml water and this was added to the citrate-carbonate solution with constant stirring and made up to 100ml with water.

Calibration curve: Bovine serum albumin (B S A) of known water content was dissolved in 0,9% saline to a standard concentration (octanol was used to prevent foaming) and diluted with saline to produce protein concentrations in the range desired. A calibration graph was constructed.

Blank: The alkaline Benedict's solution was prepared as above but 0,2ml saline was used instead of the protein solution. Determinations were performed in duplicate. (Using this method, the Beer-Lambert Law should be obeyed up to 10 mg/ml of protein.)

ii) Miller's Modified Lowry Protein Determination[247]

The coloured complex formed in this assay is only proportional to the protein concentration in the range of 50 to 200 µg/ml. Thus the microsomal suspension had to be diluted accordingly.

Method. B S A of known water content was used as the standard protein and a calibration curve was constructed using standard solutions of protein in place of the protein test solution. All determinations were performed in duplicate. One ml of the protein solution (or 1ml water for the blank) was added to 1ml of the copper reagent, mixed and allowed to stand for 10 min at room temperature. Three ml of diluted Folin-Phenol reagent was added and immediately mixed well. The tubes were heated for 10 min at 50 ^{o}C in a water bath and the optical density was read at 540nm. The values obtained from the calibration curve were corrected for the dilution factor. All reagents were freshly prepared from stock solutions.

Folin-Phenol reagent: Dilute 5 ml of Folin Phenol Reagent with 50ml of distilled water.

Copper reagent: (from the following stock solutions) 1ml 1% copper sulphate; 1ml 2% sodium tartrate; 20ml 10% sodium carbonate in 0,5 M NaOH. The precision of these two methods was tested and compared using the same protein solution. A Beckman D.B.25 UV spectrophotometer was used to measure absorbance.

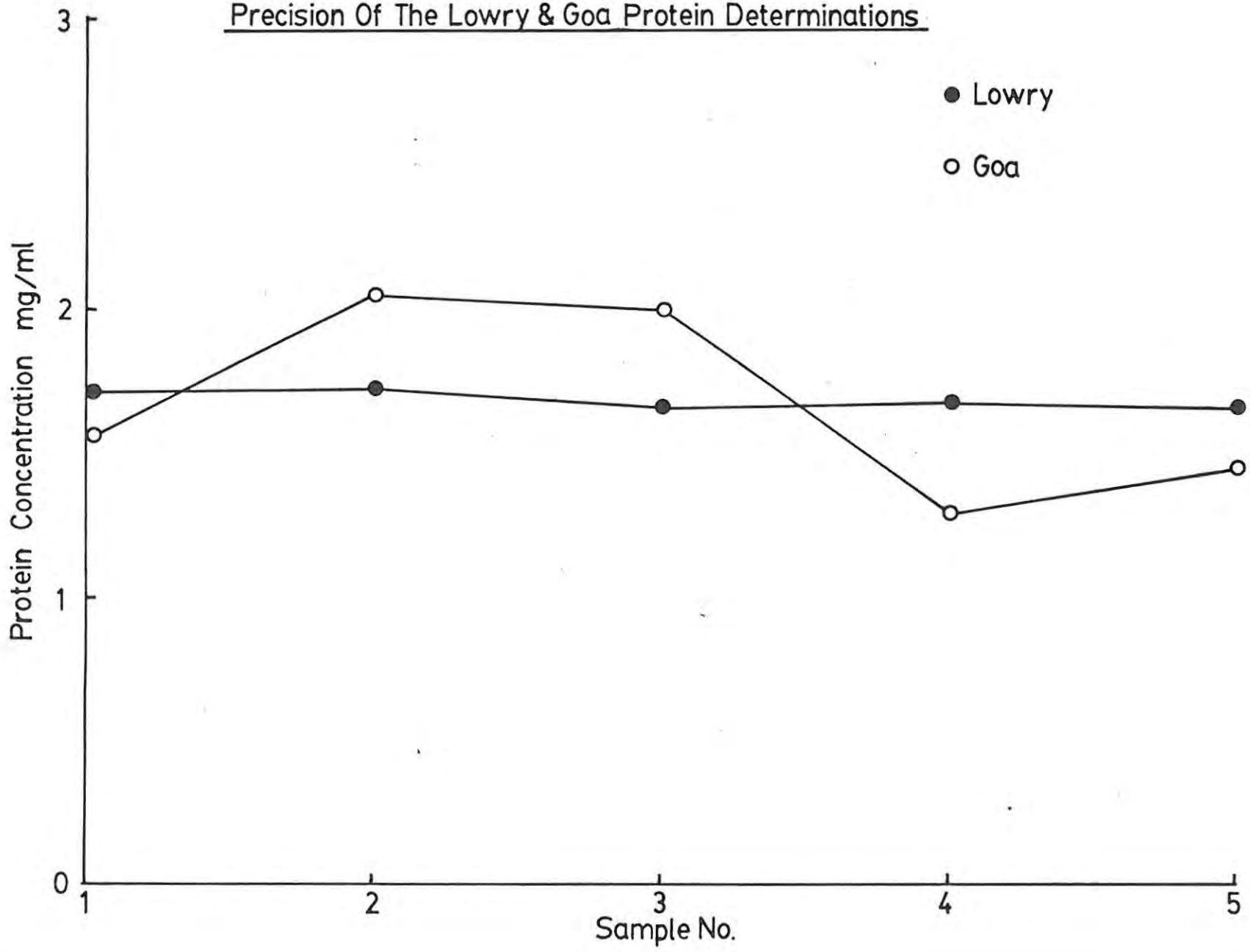
Results

See Figure 1.

Discussion

Miller's modified Lowry protein assay was found to be far more accurate than the biuret method and was used in preference. These results concur with the findings of Albro[283] who investigated various methods for microsomal protein determination.

Fig 1


Precision Of The Lowry & Goa Protein Determinations
Lowry
Goa
Protein Concentration mg/ml
0
1
2
3
1
2
3
4
5
Sample No.

Buffers

Two types of buffers are normally used for microsome preparations, viz. phosphate and Tris. Neither of these are ideal, for, above pH 7,5, phosphate has poor buffering capacity and below pH 7,5, the same applies to Tris[246]. Ideally, the buffer used should approach the ionic strength and composition of the hepatic intracellular fluid. Attempts were made to produce such a buffer but they were not successful and thus it was decided to use the conventional buffers. Sørensen's phosphate buffer[248] was used for these studies.

A Beckman Acta M VI ultraviolet spectrophotometer was set up as detailed by the operating booklet.
Wavelength scanned: 500-360nm (Tungsten source)
Period selector switch: 2
Span control: 0,02
Mode switch set at DB Servo slit position.
Slit program selector set in program position.

3ml of the microsomal suspension were pipetted into each cuvette and placed in the sample compartment of the spectrophotometer. Absorbance was scanned between 500 and 360nm and the instrument was adjusted to obtain a straight baseline. It was found that dilute microsomal suspensions (ca.1mg/ml) gave a more satisfactory baseline.

The type I difference spectrum was observed by performing a titration with both SKF 525 A and Brietal sodium. These drugs were added in increasing titres using a Hamilton syringe. Where large volume additions were involved an equal volume of distilled water was added to the reference cuvette. The type I difference spectrum was run four times for each addition of drug and the average of these four readings was used.

As this was a lengthy procedure, experiments were repeated starting from either end of the pH range to ensure that enzyme degradation did not affect the results. The final results were obtained by using a single addition of 10 µl each of 4×10^{-4} M SKF 525 A and Brietal sodium.

Results were evaluated by measuring the difference in height between the four peaks at c.390nm and the four troughs at 420nm. These values were averaged, corrected for baseline anomalies, protein concentration and finally expressed as a percentage of the maximal difference obtained at each pH. Carbon monoxide binding was studied in a similar fashion. After a suitable baseline had been obtained, the microsomes were pooled and the iron in cytochrome P-450 reduced with c.5mg sodium dithionite. Three ml of the suspension was pipetted back into the reference cuvette and the remainder was gassed with carbon monoxide for 1 min. This suspension was protected from light to prevent the CO-cytochrome P-450 complex dissociating. The spectrum was run and results were evaluated as described above.

Male, 350-400g rats were used in this study.

Results

See Figures 2-4. The different lines on the graphs refer to duplicate experiments.

Discussion

It appears that the optimal pH for formation of the type I difference spectrum and hence binding of the drug to cytochrome P-450 is c. pH 7,8. Binding of carbon monoxide to reduced cytochrome P-450 appears to have a slightly lower optimum at c. pH 7,7. The pH optimum for type I binding and carbon monoxide binding should not be expected to be the same as

Fig 2

pH Dependent CO-Cytochrome P-450 Binding

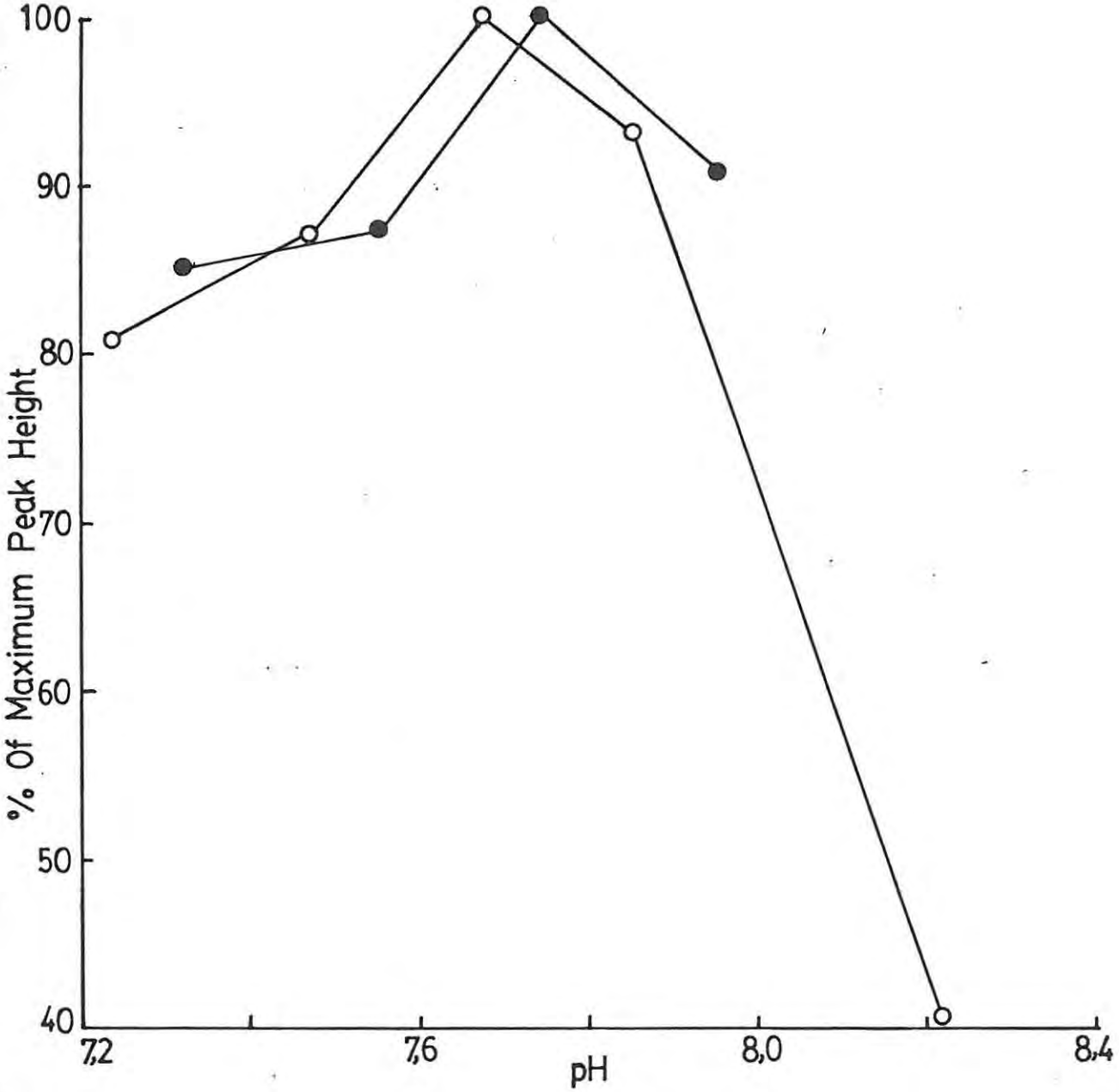


Fig 3

pH Dependent Type1 Difference Spectrum
(SKF 525-A)

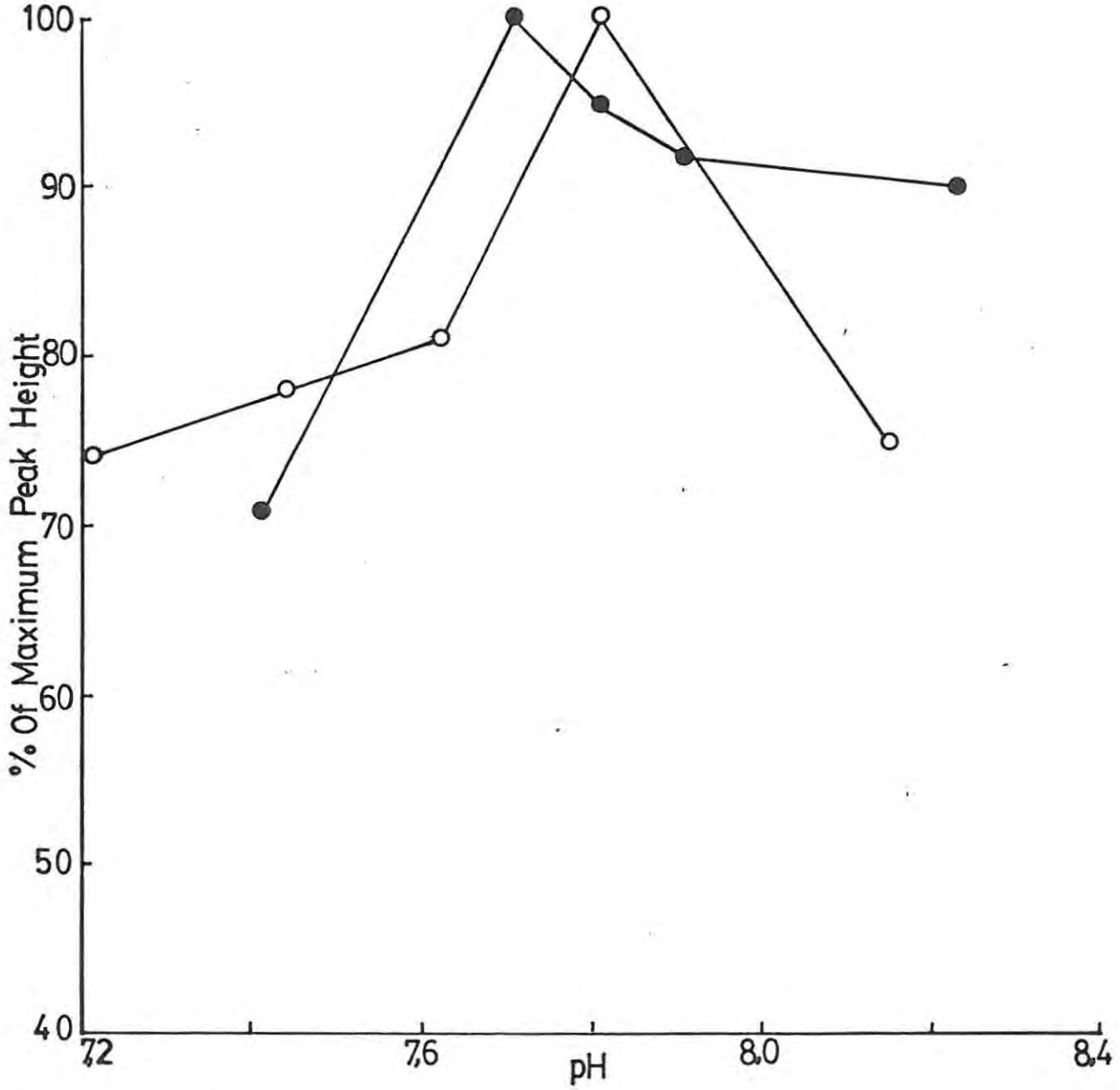
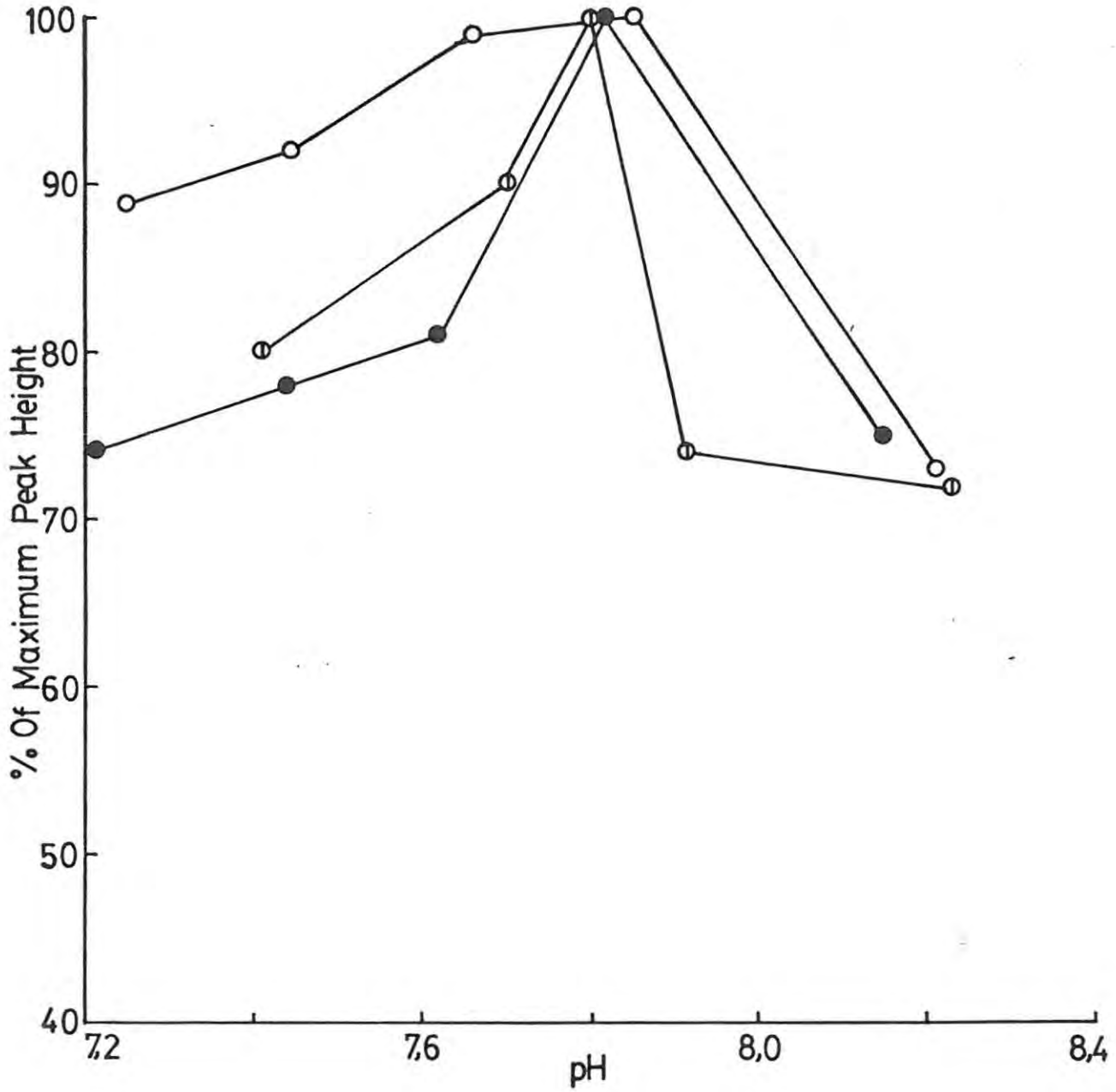


Fig 4

pH Dependent Type1 Difference Spectrum
(Brietal Sodium)



different binding sites are involved. As type I binding is a prerequisite for SKF 525 A metabolism, it was expected that pH 7,8 should also be optimal for metabolism.

2. Metabolism of SKF 525 A and SKF 8742 A

Materials and Methods

a) Preparation of microsomes. The microsomes were prepared as described earlier.

b) Buffer Tris-HCl

A Tris-HCl buffer[248] (pH measured at 38 °C) was used in all metabolic studies. The pH of the buffer used in the initial studies to establish metabolism was 7,8. This figure agreed with the results of the pH dependent drug binding experiments. Furthermore the pH optimum of isocitrate dehydrogenase used in the NADPH generating system is 7,8[249].

c) Metabolic incubations

10ml incubation flasks, complete with rubber stoppers, were used. The following components were added to each flask and the order of additions was kept constant to prevent variation.

1.	Mn^{++}/Mg^{++} solution (5mM $MgCl_2$ + 0,005mM $MnCl_2$/0,2ml)	0,2ml	[249]
2.	Distilled water	0,5ml	
3.	N A D P	1,4mg	[250]
4.	Isocitric acid	2,5mg	[250]
5.	SKF 525 A/SKF 8742 A	1,0mg	[26]
6.	Microsomal suspension (c.6mg protein/ml)	1,0ml	
7.	Isocitrate dehydrogenase	0,2mg	[249]

The final volume of each incubation mixture was 2,0ml.

The NADPH generating system functioned in the following manner[250]:

$$\text{D,L Isocitric acid} + \text{NADP} \xrightarrow[\text{Isocitrate Dehydrogenase}]{Mg^{++}\ /\ Mn^{++}} \alpha\text{-Ketoglutaric Acid} + \text{NADPH} + H^{+} + CO_2.$$

A glass marble was added to each incubation flask to facilitate mixing and increase the surface area exposed to the atmosphere. Before incubation, each flask was gassed with oxygen and sealed. Incubation was performed for 30 min at 38 ^{o}C (body temperature of the rat) and the rate of oscillation of the metabolic shaker was kept constant at 120 cycles/min.

To detect metabolism the following controls were used:

1. Complete incubation mixture (Test)
2. Incubation mixture minus isocitrate dehydrogenase
3. Incubation mixture minus drug
4. Incubation mixture minus microsomes.

It was expected that control No. 2 may give rise to some metabolism due to the presence of reducing equivalents in the microsomal suspension. Control No. 3 would indicate adventitious g.l.c. peaks not due to the drug or its metabolites, and control No. 4 would unmask non-microsomal conversion of the drug.

Enzymatic processes were stopped using 1ml 10% trichloroacetic acid, which precipitated the protein. The mixture was filtered and the filtrate was brought to pH 9,5 using concentrated ammonia solution (c.12 drops). As the physical properties of the minor metabolite were not known, five different extraction procedures were investigated to find the most efficient process.

i. Before alkalinising the filtrate, petroleum ether was used to extract any lipid that may have been present. This procedure was discontinued when the extractant was found to contain SKF 525 A.

ii. Before the addition of ammonia, the filtrate was freeze dried in ampoules, which were then sealed under nitrogen and stored. When required, the contents were alkalinised with ammonia vapour and extracted with chloroform. This procedure was successful but too slow and cumbersome for the large number of samples used.

iii. The alkalinised filtrate was extracted with 4 x 3ml chloroform, which was passed through anhydrous sodium sulphate to remove the water. The chloroform was evaporated in a water bath.

iv. As the minor metabolite was possibly a polar N-oxide, 2 x 3ml diethyl ether and 2 x 3ml n-butanol were used sequentially. The extract was evaporated at 70 ^{o}C under vacuum.

v. SKF 525 A and its metabolites were tested for stability in the presence of diethyl ether. Metabolic extracts were analysed by g.l.c. and the chromatogram was retained to check against a chromatogram of the same metabolic extract which had been incubated with diethyl ether. No difference between the two chromatograms was detected and so 3 x 3ml aliquots of diethyl ether were used to extract the alkalinised filtrate.

While the latter three of the methods tested were all practical, the use of ether was preferred as it was very easy to evaporate and three 3ml aliquots extracted SKF 525 A quantitatively from the filtrate.

All evaporated extracts were gassed with nitrogen in a boiling water bath to remove any traces of remaining water and, as required, they were made up with a specific volume of analytical grade methanol for injection into the g.l.c.

Results

(For details on g.l.c. techniques see chapter 3.)

After eliminating various sources of metabolite contamination (plastic funnels etc.), clean g.l.c. traces were obtained. As the recorder was run at the same chart speed, detection of metabolites was simplified by superimposing the test chromatogram over each of the control chromatograms in turn. Peaks which appeared on the test chromatogram but not on the controls were shown to be metabolites. SKF 525 A was shown to form SKF 8742 A with a retention time of 8,97 min and a minor metabolite with a retention time of 7,31 min.

SKF 8742 A was shown to form a metabolite with a retention time of 7,29 min.

Discussion

These results on the metabolism of SKF 525 A agree with the findings of Anders *et al.*[26]. However these workers did not *detect* any metabolite of SKF 8742 A. Judging by the almost identical retention times, it appears that the metabolite of SKF 8742 A and the minor metabolite of SKF 525 A are the same compound.

3. pH Dependent Metabolism of SKF 525 A and SKF 8742 A

Materials and Methods

These studies were carried out as described with the following adjustments.

1. After the final washing with 1,15% KCl the microsomal pellets were homogenised in Tris-HCl buffers of pH ranging from c.7,2 - 8,6.

2. After the metabolic extracts had been prepared, metabolites which may have adhered to the walls of the sample tube were washed down with ether. This helped ensure accurate quantitation. To visualize quantitative peaks in the g.l.c., a concentrated injection of 10 µl was required. Thus, the volume of methanol used to dissolve the metabolites was necessarily small (100 µl) and, hence, it was not possible to ensure dissolution of metabolites remaining high up the walls of the sample tube.

3. A Chaney adaptor was used to deliver reproducible injection volumes.

4. Male 300-350g rats were used.

Results

Four flasks were used for each pH studied, these results were averaged and expressed as a percentage of the maximal percentage conversion of SKF 525 A to its metabolites.

See Figures 5 and 6. The different graphs refer to duplicate experiments.

Discussion

It appears that the pH optimum for formation of metabolite 1 from SKF 525 A differs somewhat to that for the formation of SKF 8742 A. It is difficult

Fig 5 pH Dependent Metabolic Production Of SKF 8742-A From SKF 525-A

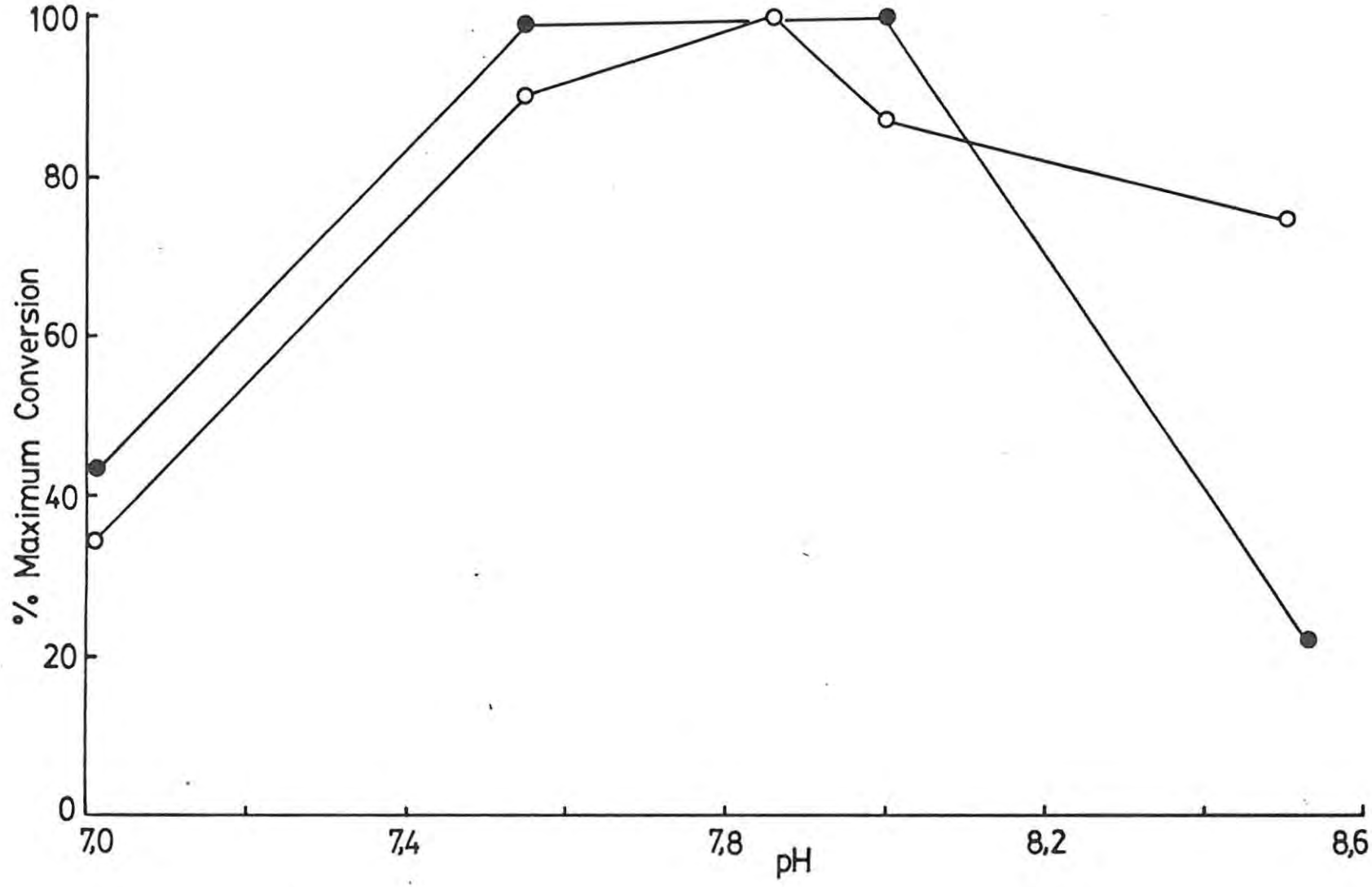
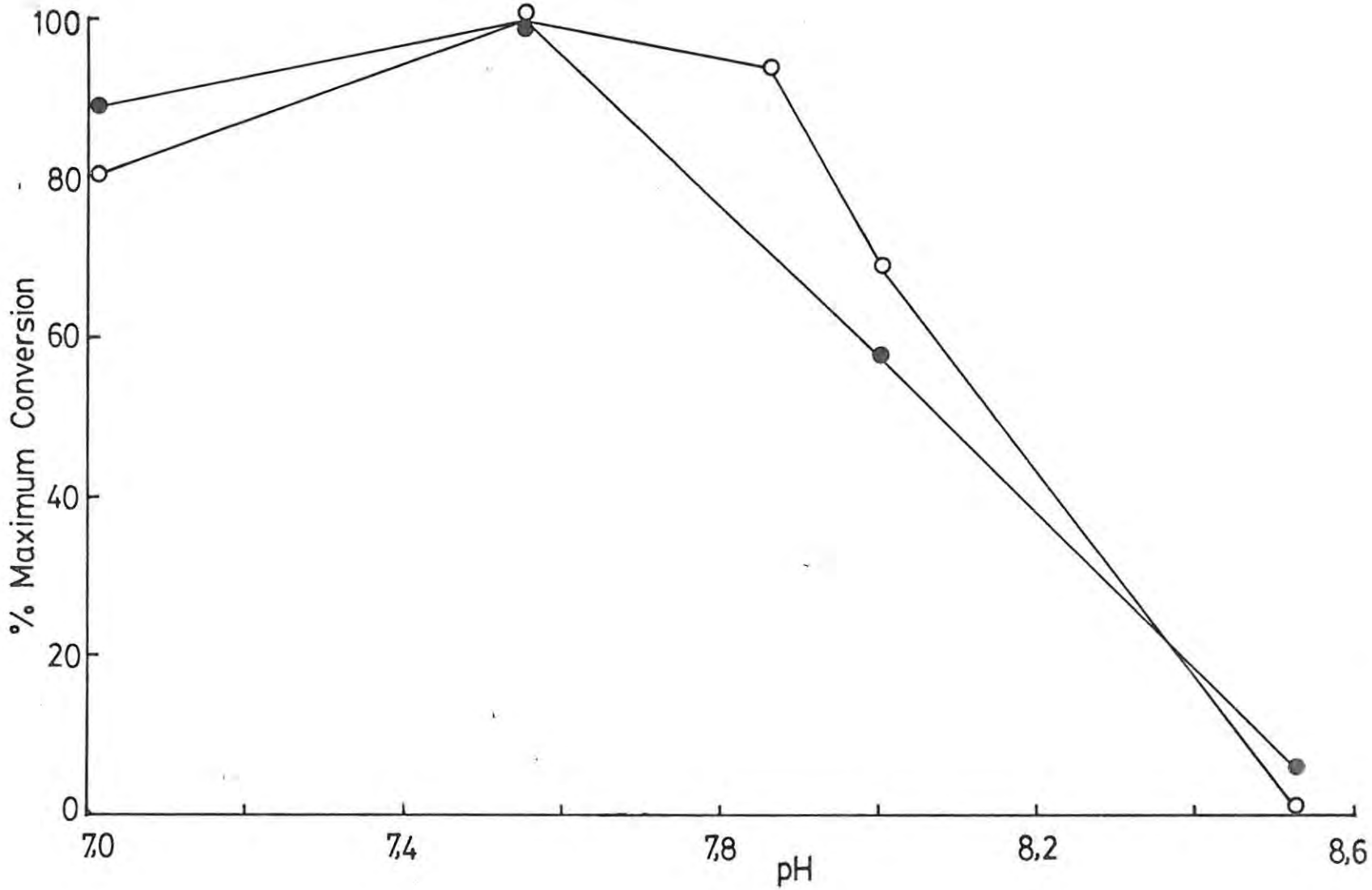


Fig 6 pH Dependent Metabolic Production Of Metabolite 1 From SKF 525-A



to reach a definite conclusion with results from a study such as this, as Gorrod[132] has pointed out. A change in the pH may affect the stability of one of the metabolites. Furthermore if different enzyme systems are involved in producing the metabolites, then pH changes may affect the relative abundance of one or the other.

It appears from this study that the production of both SKF 8742 A and metabolite 1 are mediated by cytochrome P-450. It is difficult to reconcile the differences in pH optima for the production of both metabolites but were amine oxidase involved, one would expect metabolite formation to be enhanced at more alkaline pHs (c.8,6).

4. Stability of Metabolites

Materials and Methods

Metabolites were prepared as described and samples were analysed by g.l.c. These samples were allowed to stand unsealed under normal room conditions for three weeks after which time they were re-analysed by g.l.c. The chromatograms were compared.

Results

No detectable change took place during exposure to air and light.

CHAPTER 3

GAS LIQUID CHROMATOGRAPHY (g.l.c.)

Objectives

Anders et al.[26] in their studies of the metabolism of SKF 525 A nd SKF 8742 A were able to resolve two metabolites of SKF 525 A and to identify one of them by g.l.c. on A/W DMCS treated chromosorb w coated with 2% SE 30 packed into 2m 6,5mm/O.D. glass columns. They were, however, unable to identify the second metabolite or to detect metabolism of SKF 8742 A.

It is, therefore, the objective of these studies to develop a suitable method to detect the metabolites, optimise conditions to enable the columns to be used for g.l.c./mass spectrometry and develop a quantitative procedure for analysis of the metabolites.

Materials and Methods

Throughout these studies a Hewlett Packard 5830 A chromatograph fitted with dual flame ionisation detectors (F.I.D.) was used. The following gas pressures were kept constant during operation. High purity nitrogen carrier gas 60 p.s.i., F.I.D. hydrogen 30 p.s.i., F.I.D. air 28 p.s.i.

The following columns were prepared and tested.

i. 2m 6,5mm/O.D. glass column packed with A/W (acid washed) DMCS (dimethylchlorosilane) treated Chromosorb W coated with 2% S.E. 30.

ii. 2m 3mm/O.D. stainless steel column packed with A/W DMCS treated Chromosorb W coated with 7.5% Carbowax 20M.

iii. 2m 3mm/O.D. stainless steel column packed with A/W DMCS treated Chromosorb W coated with 10% Apiezon L.

iv. 2m 3mm/O.D. stainless steel column packed with A/W DMCS treated Chromosorb W coated with 15% Apiezon L.

v. 2 x 2m 3mm/O.D. stainless steel columns packed with A/W DMCS treated Chromosorb W coated with 15% Apiezon L.

vi. 2 x 4m 3mm/O.D. stainless steel columns packed with A/W DMCS treated Chromosorb W coated with 2% S.E.30.

vii. 2 x 4m 3mm/O.D. stainless steel columns packed with A/W DMCS treated Chromosorb W coated with 15% Apiezon L.

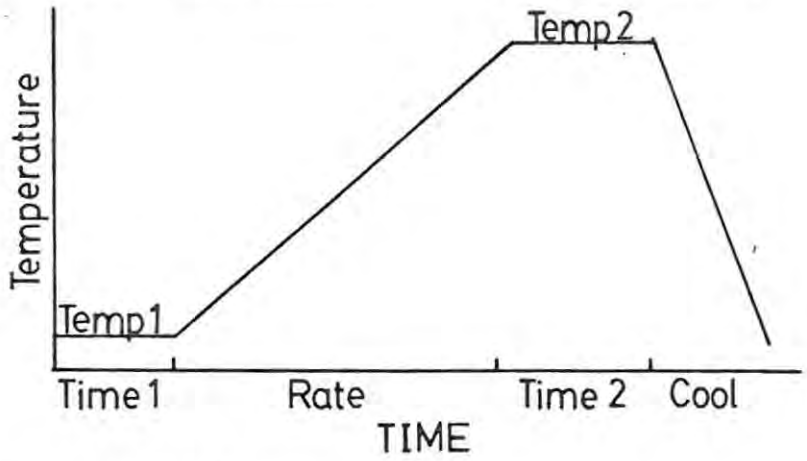
i. A/W DMCS treated Chromosorb W was suspended in a 2% solution of S.E.30 in toluene and degassed. This slurry was evaporated in a rotary evaporator and the packing heated in an oven at 100 ^{o}C for an hour to remove any remaining toluene. A 2m 6,5mm/O.D. glass column was washed three times with toluene, dried with compressed nitrogen and set up in a cushioned retort stand. A funnel was fitted to one end and the other was connected to a vacuum tap. Fine nylon gauze was used to prevent the support from being sucked through into the vacuum tap. Negative pressure was applied to the column and this, in conjunction with vibration from the rotary chuck of a drill, ensured even column packing. Glass wool was inserted into either end of the column to prevent the support escaping under carrier gas pressure. Front and back teflon ferrules were fitted to the column and one end was connected to the injection port inlet on the chromatograph. The column was conditioned overnight at 275 ^{o}C with a carrier flow rate of 80ml/min.

Using authentic samples of SKF 525 A and SKF 8742 A, the oven temperature and carrier gas flow rate were varied in efforts to improve separation. Temperature programming was also used.

Results and Discussion

The following conditions were optimal for this column.

Temp 1	Time 1	Rate	Temp 2	Time 2	F.I.D.Temp	Inj.Temp	Flow rate
130 °C	6 min	30 °C/min	170 °C	15,0 min	255 °C	255 °C	120ml/min



Temp 1 = initial oven temperature

Time 1 = time at temp 1

Rate = rate of increase in temperature

Temp 2 = second oven temperature

F.I.D.temp = flame ionization detector temperature

Inj.temp = injection port temperature

Flow rate = flow rate of carrier gas.

The separation achieved with this column was not adequate and this may have been due to slight differences in materials to those used by Anders et al.[26]. Alternative systems were sought.

ii. A/W DMCS treated Chromosorb W was coated with 7.5% Carbowax 20 M. Chloroform was used as the solvent. Previously, it was found that use of the rotary evaporator did not lead to even coating; consequently the slurry was evaporated at 100 ^{o}C in an oven. Frequent stirring led to a homogenous product. In view of the fragility of the glass column, a 2m 3mm/O.D. stainless steel column was used. Due to its dimension and its non-transparency, this column was more difficult to pack. The volume of packing required was pre-determined with a fluid and it was necessary to vibrate the packing, in a measuring cylinder, down to this volume to obtain the amount required to closely pack the column. More vibration than before was required to bed down the support and care was taken not to further reduce the particle size of the diatomaceous earth.

Results and Discussion

The following conditions were optimal for this column.

Temp 1	Time 1	Rate	Temp 2	Time 2	F.I.D.temp	Inj.Temp	Flow rate
215 ^{o}C					230 ^{o}C	230 ^{o}C	45ml/min

This column was unsuitable as the retention times were excessively long and peaks were assymetrical.

iii. A/W DMCS treated Chromosorb W was coated with 10% Apiezon L, packed into a 2m 3mm/O.D. stainless steel column and conditioned overnight at 290 ^{o}C.

Results and Discussion

The following conditions were optimal for this column.

Temp 1	Time 1	Rate	Temp 2	Time 2	F.I.D.temp	Inj.temp	Flow rate
220 °C					240 °C	240 °C	90ml/min

Separation using this column was not adequate and it was decided to increase the percentage of the liquid phase. This would increase the retention time but would also increase efficiency.

iv. A/W DMCS treated Chromosorb W was coated with 15% Apiezon L, packed into a 2m 3mm/O.D. stainless steel column and conditioned overnight at 290 °C.

Results and Discussion

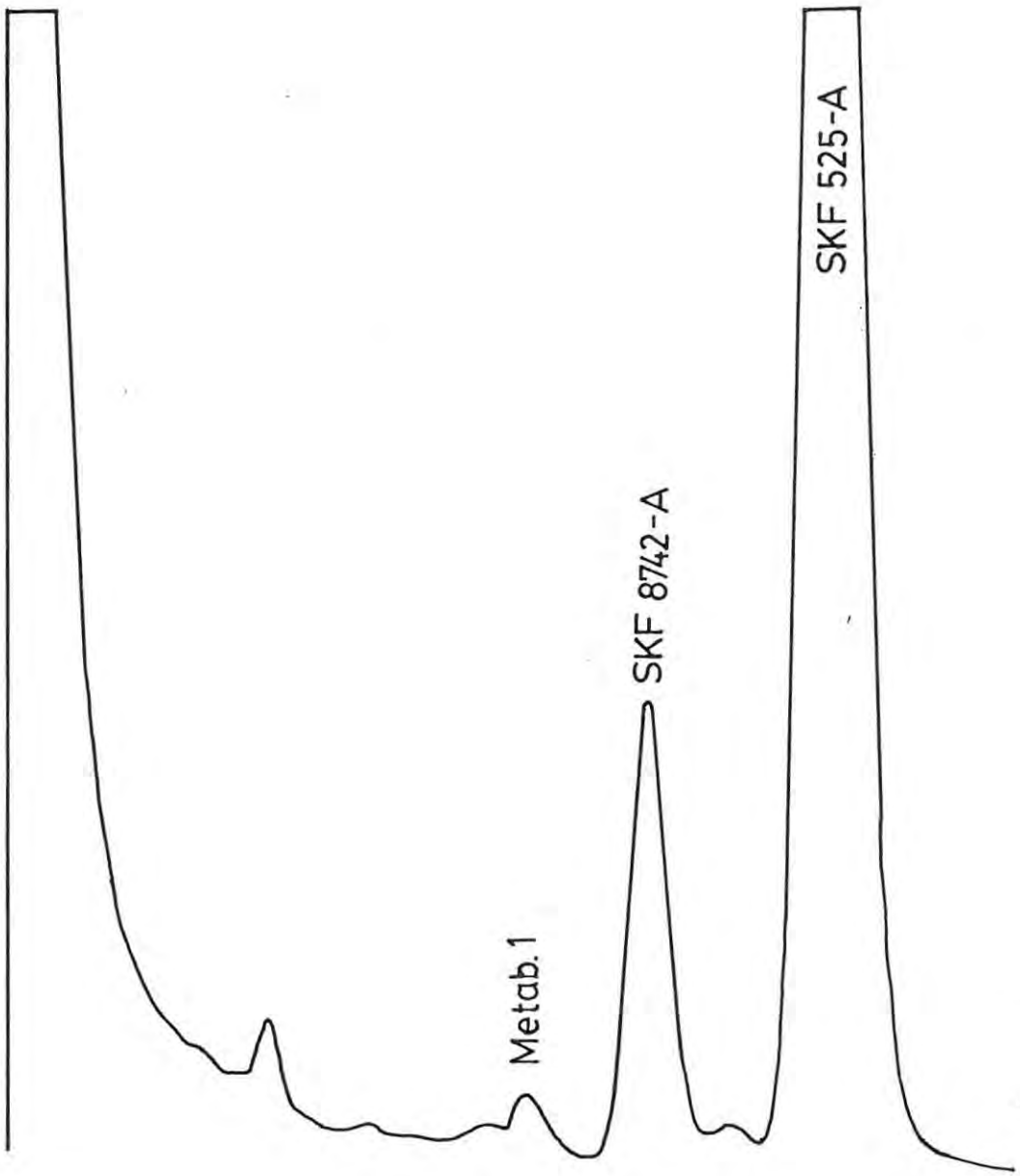
Optimal conditions for this column involved a temperature programme which reduced the retention time.

Temp 1	Time 1	Rate	Temp 2	Time 2	F.I.D.temp	Inj.temp	Flow rate
160 °C	3,0 min	30°C/min	290 °C	20,0 min	320 °C	300 °C	25ml /min

The temperature programme caused column bleeding and so a second identical column was prepared and the chromatograph was used in the dual column mode. Use of a temperature programme over a long term period was not ideal for, on continual removal and replacement of columns from the chromatograph, leaks developed which were difficult to eliminate. Isothermal conditions were investigated and the following conditions produced an adequate chromatogram (Fig. 7).

Temp 1	Time 1	Rate	Temp 2	Time 2	F.I.D.temp	Inj.temp	Flow rate
275 °C					320 °C	300 °C	24ml/min

Fig 7 Chromatogram Of SKF 525-A And Its Metabolites



While these columns appeared to meet requirements for analytical work, it was necessary to further improve separation to enable g.l.c./mass spectrometry to be performed with the metabolites. Thus, further systems were investigated.

v. A/W DMCS treated Chromosorb G was coated with 15% Apiezon L, packed into 2 x 2m 3mm/O.D. stainless steel columns and conditioned overnight at 290 ^{o}C.

Results and Discussion

The following conditions were optimal.

Temp 1	Time 1	Rate	Temp 2	Time 2	F.I.D.temp	Inj.temp	Flow rate
275 ^{o}C					320 ^{o}C	300 ^{o}C	24ml/min

These columns were not at all successful as the peaks were asymmetrical and had very long retention times.

vi. A/W DMCS treated Chromosorb W coated with 2% S.E.30 was packed into 2 x 4m 3mm/O.D. stainless steel columns and conditioned overnight.

Results and Discussion

The following conditions were optimal.

Temp 1	Time 1	Rate	Temp 2	Time 2	F.I.D.temp	Inj.temp	Flow rate
175 ^{o}C					320 ^{o}C	300 ^{o}C	20ml/min

This resulted in good separation of the tertiary and secondary amines but the minor metabolite peak continued to overlap the secondary amine peak at high sensitivity.

vii. A/W DMCS treated Chromosorb W coated with 15% Apiezon L was packed into 2 x 4m 3mm/O.D. stainless steel columns and conditioned overnight.

Results and Discussion

Optimal conditions were as follows.

Temp 1	Time 1	Rate	Temp 2	Time 2	F.I.D.temp	Inj.temp	Flow rate
275 ^{o}C					320 ^{o}C	300 ^{o}C	20ml/min

Excellent separation of the tertiary and secondary amines was obtained but a contaminating product interfered with the minor metabolite peak. After investigation and purity checks of the authentic amine samples it appeared that the newly purchased support differed to that originally used and caused degradation of the secondary amine. The columns as described in (v) were used for future work.

F.I.D. Sensitivity Test

As the production of the metabolites was expected to be small, a sensitivity test was performed to ensure their detection.

Method and Results

80 µl of a 4 x 10^{-4} M solution of SKF 525 A was used for each metabolic incubation. A stock solution of 4 x 10^{-5} M SKF 525 A in methanol was prepared and ultimately 1 µl of this was diluted 1:200. This dilute solution was injected into the chromatograph and appeared as a small peak on the chromatogram.

Conclusion

The F.I.D. is capable of detecting 1:160 000 of each aliquot of SKF 525 A used in a metabolic incubation. Thus, the metabolites produced should be easily detectable.

Quantitative Gas Liquid Chromatography

It was necessary to develop a quantitative g.l.c. procedure to analyse results from the metabolic studies envisaged. The types of procedure available were as follows[242]:

1. Area % This method does not require calibration. (To calibrate, a set volume containing known weights of different compounds is injected into the g.l.c. The integrator assigns percentage area to each peak, and the operator then enters into the computer the weight of each compound giving rise to its peak. In future runs, the computer will convert the area under each peak to the weight of compound present.) It reports all peaks that meet with the slope sensitivity and area reject requirements but it does not correct for detector response. The simplicity of this method allows accurate determination of the percentage composition, e.g. metabolic extracts. As the composition of the extracts does not change the detector response factor is insignificant for comparative purposes.

2. Normalize Method This method requires calibration, it only reports the calibrated peaks and will correct for detector response.

3. External Standard This method requires calibration and it only reports the calibrated peaks. It corrects for detector response but requires a highly reproducible injection procedure. Automatic sample introduction is a prerequisite.

4. Internal Standard This method requires calibration; it only reports the calibrated peaks and corrects for detector response. This is accepted as being the most accurate quantitative method and requires a known amount of internal standard to be present in each sample.

The most suitable of the methods tested were the area % and the internal standard methods. The former procedure allows comparison of percentage components in a mixture while the latter will indicate the mass of each component.

Area Per Cent Method

Unless the Hewlett Packard integrator adheres to certain parameters it is not possible to obtain accurate results using any of these methods. The integrator is termed "software" and it is capable of reviewing stored data at the end of each run. (A "hardware" integrator cannot review stored information and may only process it as it is received.) The integrator will automatically perform the following functions[242]:- distinguish peaks from detector noise, determine the beginning and end of peaks, measure retention times of peaks, correct for baseline drift, correct for asymmetric peaks, separate partially resolved peaks and tangent skim peaks on the tail of solvent peaks. Use of the area reject and slope sensitivity functions enables the integrator to reject peaks, ignore specific portions of the chromatogram, sum groups of adjacent peaks, tangent skim designated peaks and analyse the detector signal and determine the maximum utilisable sensitivity.

The slope sensitivity determines the gradient of the slope of the chromatogram at which the integrator will start recording the area of the peak. A slope sensitivity setting of 0,5 was generally found to be suitable.

Use of the area reject facility in conjunction with a time programme enabled the integrator to ignore the large solvent peak which accounted for the majority of area. This led to more accurate designation of percentage areas to the relevant peaks.

Internal Standard Method

The area reject and slope sensitivity parameters as described, were also used for this method. An internal standard with the following properties was required. It should have a similar structure to the compounds being analysed, it should elute from the column in an area of the chromatogram not occupied by the sample peaks and it should be used in equivalent concentrations to the sample. This latter stipulation was difficult to meet due to the large variation in concentration between SKF 525 A and its metabolites.

Tests for an Internal Standard

1. The following compounds were tested for suitability as an internal standard. They all produced suitable peaks but these overlapped or interfered with SKF 525 A or its metabolites on the chromatogram. Temperature programming could not eliminate this problem.

Compounds tested: imipramine, diphenhydramine, ephedrine, chlorpheniramine, mepyramine maleate, chloramine T, caffeine, scopolamine, scopolamine bromide, methyl nicotinate, dihydrolysergic acid.

2. The following compounds did not produce a peak at all: Phenobarbitone, neostigmine bromide, brucine, gramine, guvacine, strophanthin, veratrine, l-dihydroxyphenylalanine, 4-methylaminophenolsulphate and acetylcholine.

3. Hydrastine and berberine gave peaks that were not reproducible, testosterone tailed badly and theophylline would not have been suitable as it reacts with amines.

4. The most suitable compounds included: ethyl morphine, ethyl morphine HCl, and codeine phosphate.

Codeine phosphate was finally chosen as internal standard but its dissimilarity in structure to SKF 525 A, its long retention time and slight tailing detracted from its value. See chromatogram, Fig. 8a.

Method

The internal standard stock solution contained 0,12207g codeine phosphate/ 100ml methanol.

The internal standard calibration mixture contained 0,00650g SKF 525 A and 0,00298g SKF 8742 A/7,5ml of internal standard stock solution. One µl of this calibration solution was injected into the g.l.c. for calibration. After calibration each metabolic extract was analysed with a specific volume of internal standard stock solution added.

The calibration solution was packed into 2ml amber ampoules, sealed under nitrogen and kept in the refrigerator. The internal standard stock solution was packed in an amber bottle and was also refrigerated between runs.

A very recent publication[283] describes the use of SKF 525 A as an internal standard for the determination of trimethoprim in urine. Similar conditions were used and it appears that trimethoprim would be a more suitable internal standard to codeine phosphate.

Peak Shift Technique Studies

Anders and Mannering[282,26] described a method whereby derivatives were formed on the g.l.c. column by injecting volumes of acetone, acetic or propionic anhydrides. They used this method to facilitate identification of the metabolites of SKF 525 A. It is expected that anhydrides will form esters with phenolic and alcoholic hydroxyl groups on the column and amides with primary or secondary amines. Acetone reacts with primary amines to form Schiff base derivatives.

Materials and Methods

The following types and volumes of analytical grade derivative forming agents were injected into the column 5 seconds after the metabolic extract had been injected.

Acetic and propionic anhydride	5 µl
Acetone	5 µl

Results

It was not possible to duplicate the results of Anders et al.[26] and while acetic and propionic anhydrides shifted the SKF 8742 A peak, at no stage did this reappear as a well defined peak with a reproducible retention time.

Preparation of SKF 525 A N-oxide

As the minor metabolite of SKF 525 A may possibly be an N-oxide, synthesis of SKF 525 A N-oxide as a reference material was undertaken[284] and the reaction products were evaluated using g.l.c.

Fig 8a Chromatogram of the Internal Standard Calibration Mixture

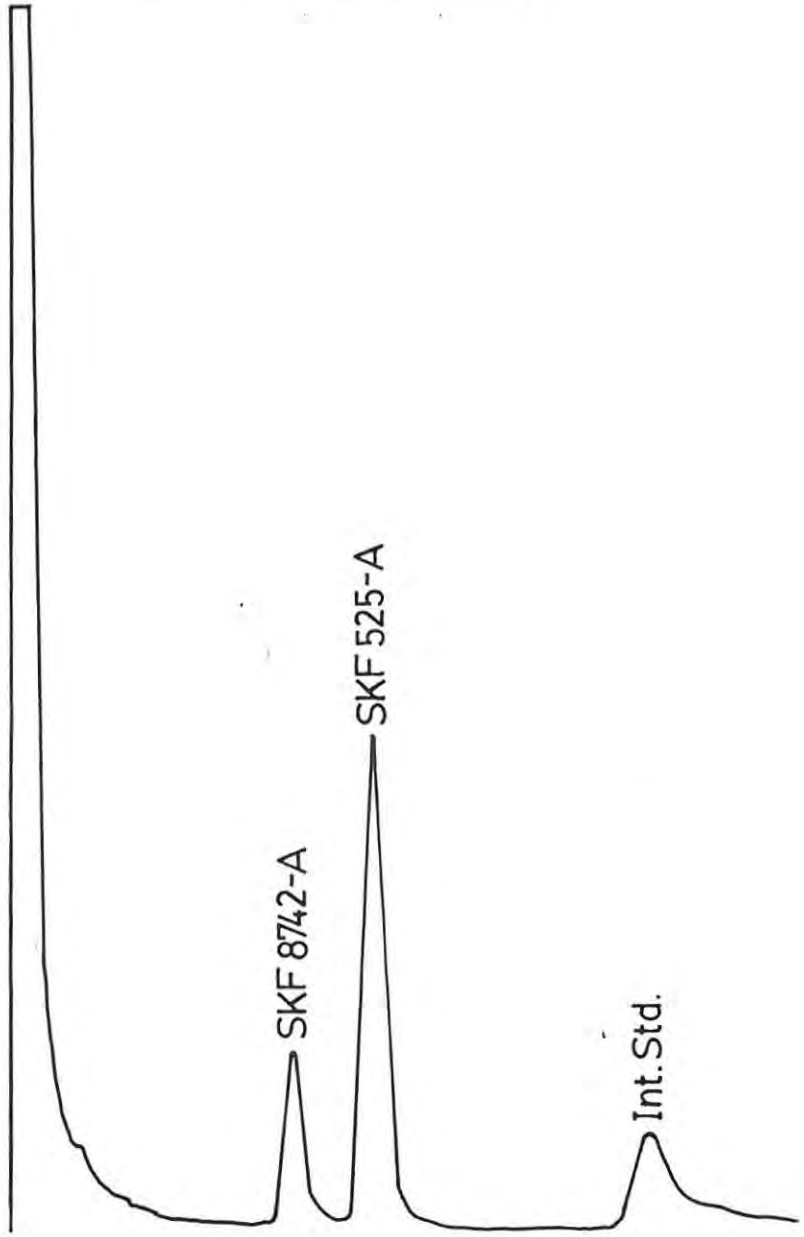
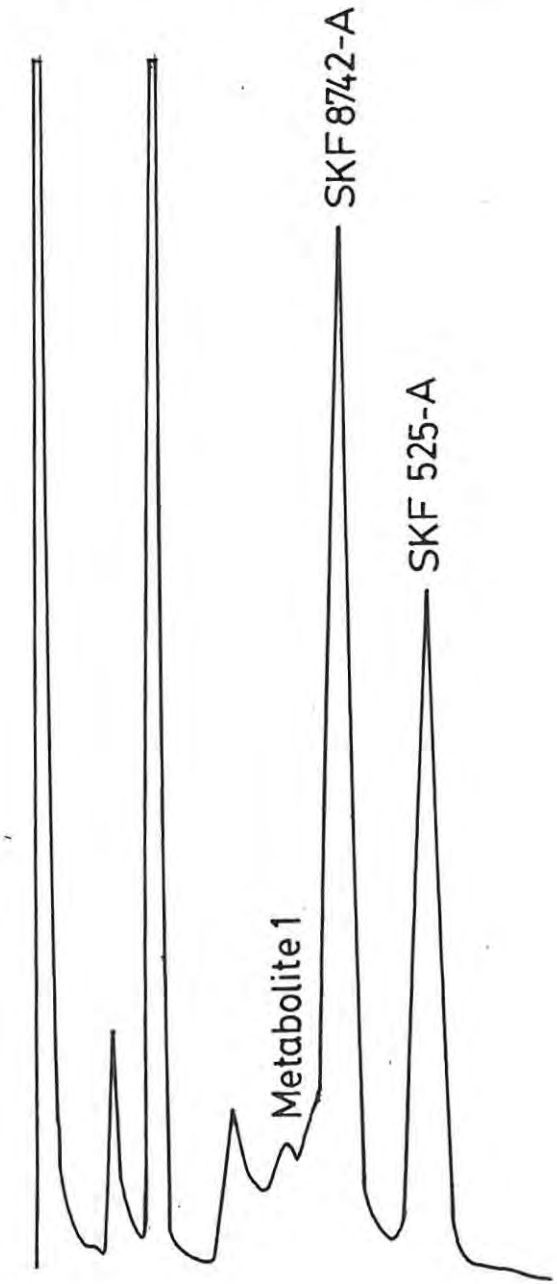


Fig 8b Chromatogram of the Products of Oxidation of SKF 525-A



Materials and Methods

One g of SKF 525 A was dissolved in a minimum quantity of distilled water and the pH was adjusted to 7,8 with dilute NaOH. The resulting suspension was extracted with diethyl ether and the extract was evaporated to leave the oily base. This was dissolved in 8ml analytical grade methanol and the solution incubated with 2,4ml 30% H_2O_2 at room temperature for three days.

The oxidation mixture was stirred and cooled while 0,02g platinum oxide was added. The mixture was stirred for 3h, then filtered and the filtrate concentrated under vacuum to c. 30% of its original volume. This concentrated solution was saturated with NaCl, giving rise to an oily layer, which was removed, dried and analysed by g.l.c.

Results

See chromatogram Fig. 8b. Oxidation of SKF 525 A gave rise to a number of different products, and the chromatogram indicated that both SKF 8742 A and the minor metabolite had been produced. Without authentic N-oxide samples, it was difficult to identify peaks and attempts to purify the components of this mixture by column chromatography were unsuccessful.

Due to the many contaminants, this approach, which apparently succeeded in the production of both metabolites of SKF 525 A, was not pursued.

CHAPTER 4

STUDIES ON THE MECHANISM OF HEPATIC MICROSOMAL SKF 525 A METABOLISM

Objectives

The metabolism of nitrogen containing compounds, and particularly tertiary amines, had received increasing attention in recent years and attempts to isolate and characterise the enzyme system(s) responsible for the N-oxidation of tertiary amines have been made. Zeigler and co-workers[112,113] succeeded in purifying and characterising a mixed function amine oxidase which was devoid of cytochrome P-450 and NADPH-cytochrome c reductase and which is responsible for the N-oxidation of some tertiary and secondary amines.

Use of n-octylamine to stimulate the microsomal amine oxidase system is well documented[216,241,255,257]. Jefcoate et al.[86] have shown that this agent inhibits cytochrome P-450 mediated reactions and Prough and Zeigler[255] showed that the N-demethylation of four tertiary amines was inhibited from 74 to 93% but that the N-demethylation of secondary amines was only partially inhibited. Poulsen et al.[257] found that n-octylamine causes 0-10% activation of demethylaniline N-oxidase in rat hepatic microsomes whereas it stimulates the same system by 146% in pork liver microsomes. Thus, it was decided to use both these species in these studies.

Evidence indicating participation of cytochrome P-450 in the N-oxidation of tertiary amines has been presented[132] and recent studies[254,255] confirm this participation. Hlavica and Kehl[254] have shown that cytochrome P-450 accounts for 50-60% of the total N,N-dimethylaniline N-oxide formed in the rabbit liver microsomal fraction, the remainder arising from the action of the mixed function amine oxidase. Similarly, Prough and Zeigler[255]

have shown considerable participation by amine oxidase in the N-demethylation of benzphetamine and p-chloro-N-methylaniline, reactions previously considered to be cytochrome P-450 dependent.

Thus, it appears that there is considerable overlap in the activity of these enzymes, and their influence on the metabolism of SKF 525 A, a widely used experimental tool, requires investigation.

Materials and Methods

Microsomes and incubation mixtures were prepared as previously described (see chapter II). Microsomal preparations were obtained from a) male, 300-350g rats b) male swine and c) induced microsomes from female, 200-250g rats injected i/p with 80mg/kg phenobarbital for three days before sacrifice.

In all experiments, the microsomal protein concentration was adjusted to c. 5mg/ml.

Solutions of dithiothreitol (10^{-3}M and 10^{-2}M) were added to the test incubation mixtures before the addition of microsomes or isocitrate dehydrogenase.

Microsomes were gassed with carbon monoxide for 1 min and were protected from light (which may cause dissociation of the cytochrome P-450 CO complex). The CO gassed microsomes were added to the test incubation flasks (which were wrapped in aluminium foil to prevent penetration of light) before the addition of isocitrate dehydrogenase. The flasks were finally perfused with CO instead of oxygen and stoppered.

Three mM n-octylamine (1 µl) was added to each test incubation flask after the addition of the distilled water and Mg^{++}/Mn^{++} solution. The flasks were agitated to disperse the immiscible liquid.

Studies with induced microsomes were performed like those using normal rat microsomes with the exception of the pretreatment of the rats with phenobarbital.

Results

Quantitative and qualitative differences in the metabolism of SKF 525 A and SKF 8742 A by induced rat hepatic microsomes are shown in the g.l.c. traces. See Figs. 9 and 10.

SKF 525 A:- Induction causes a c. 100% increase in the production of SKF 8742 A but the formation of metabolite I appears to be little affected. Induction does appear to cause the formation of another metabolite (termed metabolite IV) whose formation is inhibited by carbon monoxide.

SKF 8742 A:- Induction greatly increases the formation of metabolite I and causes the formation of another metabolite (termed metabolite III) whose formation is inhibited by carbon monoxide. It is not possible to comment on the presence of metabolite IV under these conditions as the size of the metabolite I peak obscures its detection.

The results (see Figs 11, 12, 13, 14 and 15) of duplicate experiments with rat hepatic microsomes are expressed as a percentage of the control (100%) value ± standard deviation. Each experiment comprised four test and four control incubations. Single experiments were performed with

Chromatograms Showing Metabolites Produced with Induced Microsomes

Fig 9

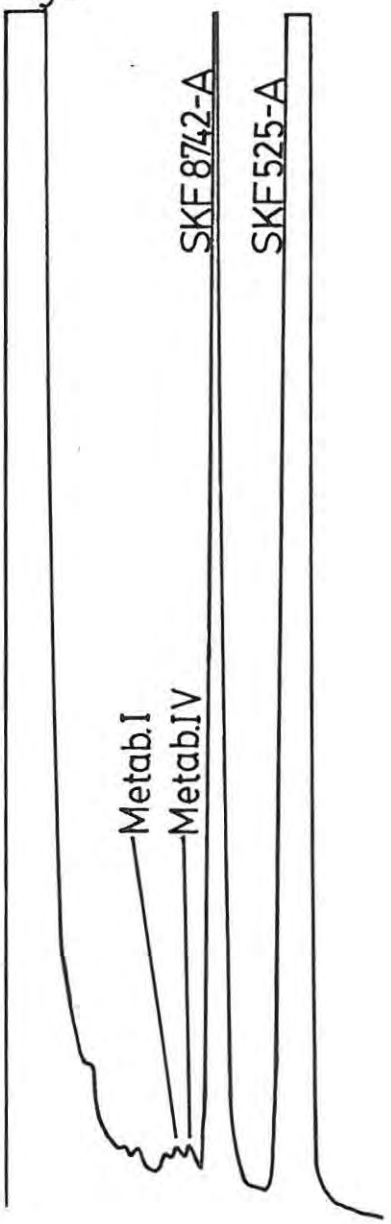


Fig 10

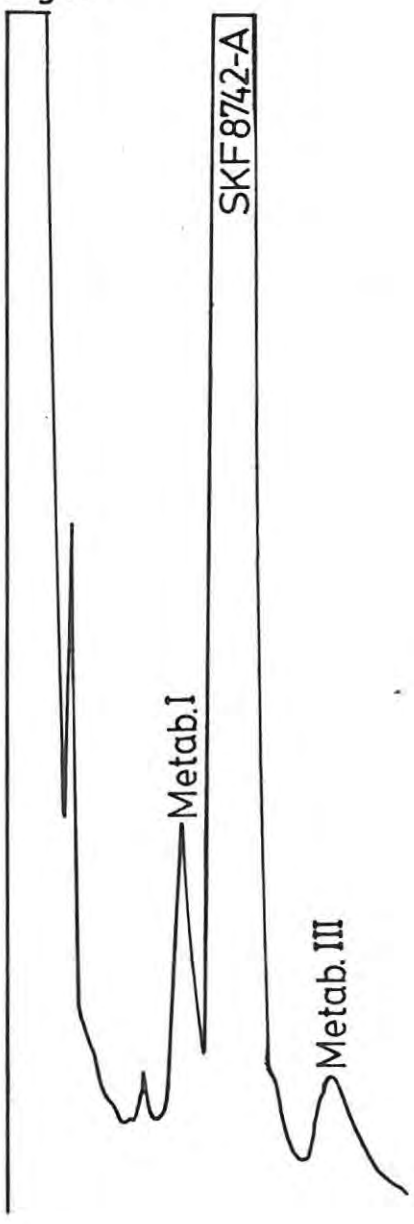


Fig 11 Metabolism of SKF 525-A Using Rat Hepatic Microsomes

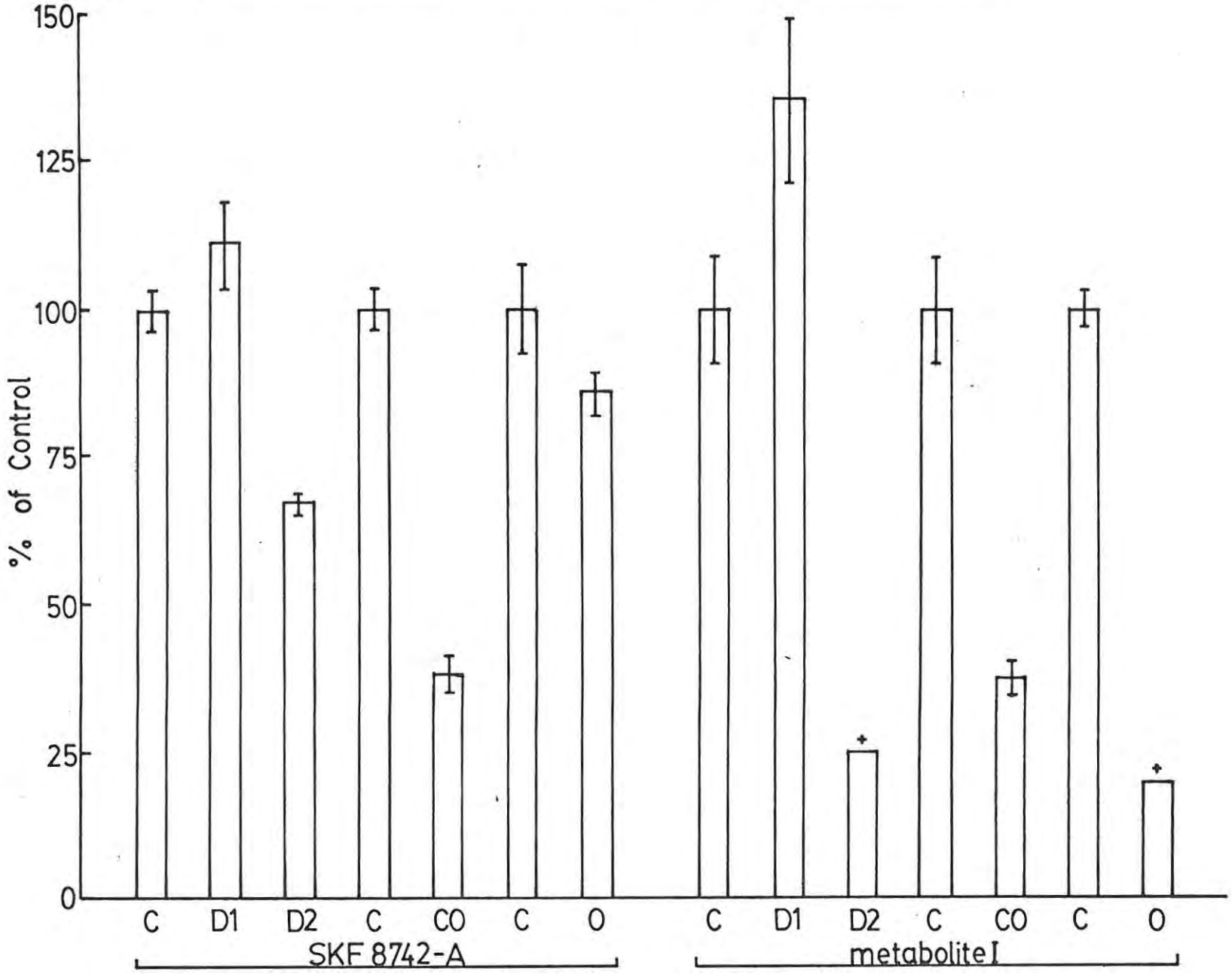


Fig12 Metabolism of SKF 8742-A Using Rat Hepatic Microsomes

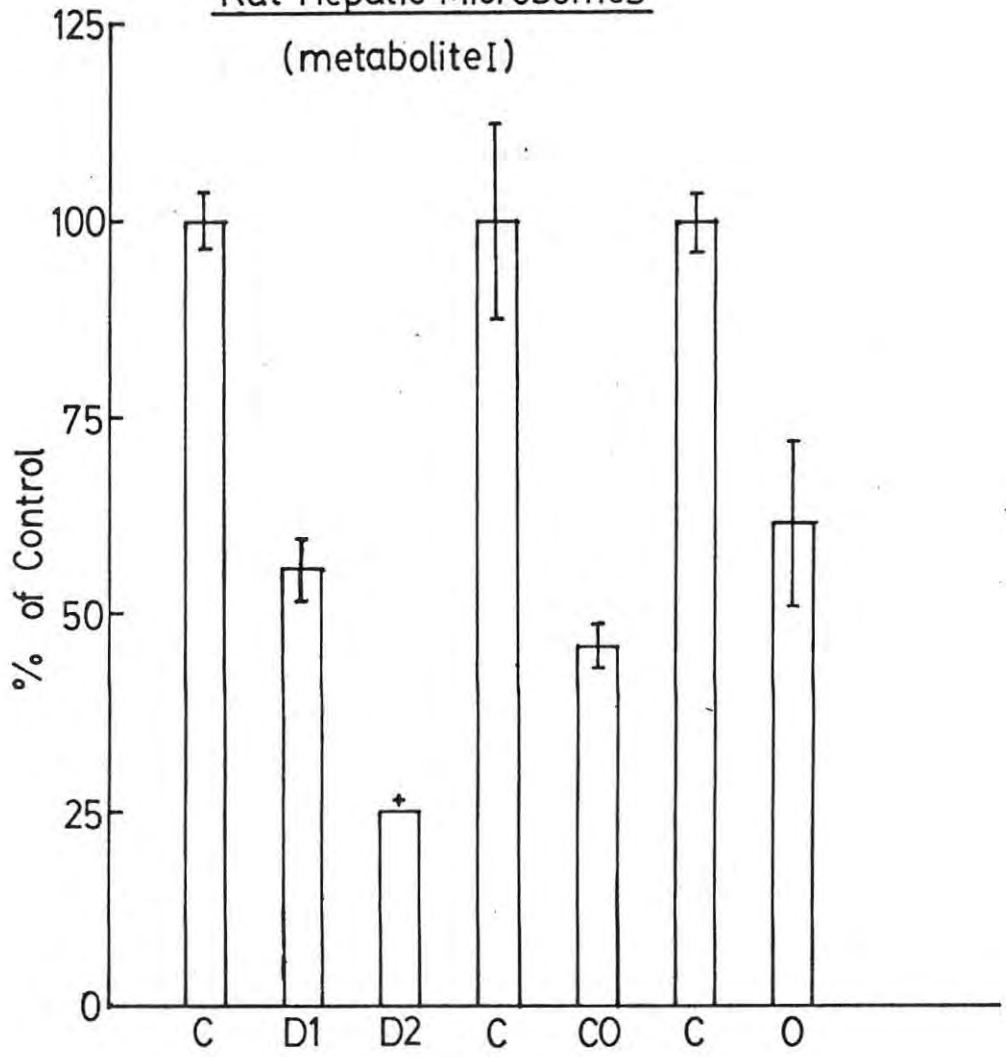
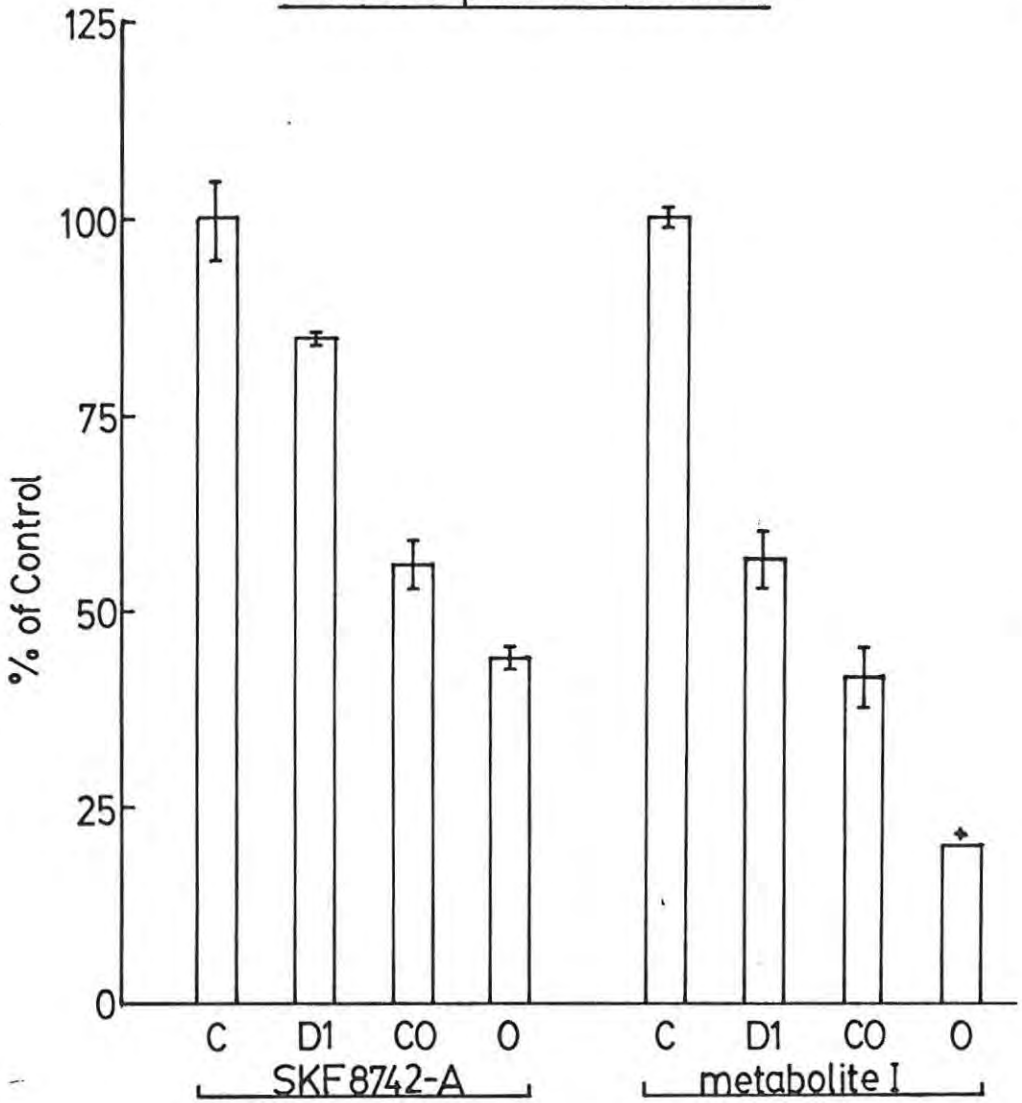


Fig13 Metabolism of SKF 525-A Using Swine Hepatic Microsomes



Metabolism of SKF 8742-A Using Swine Hepatic Microsomes

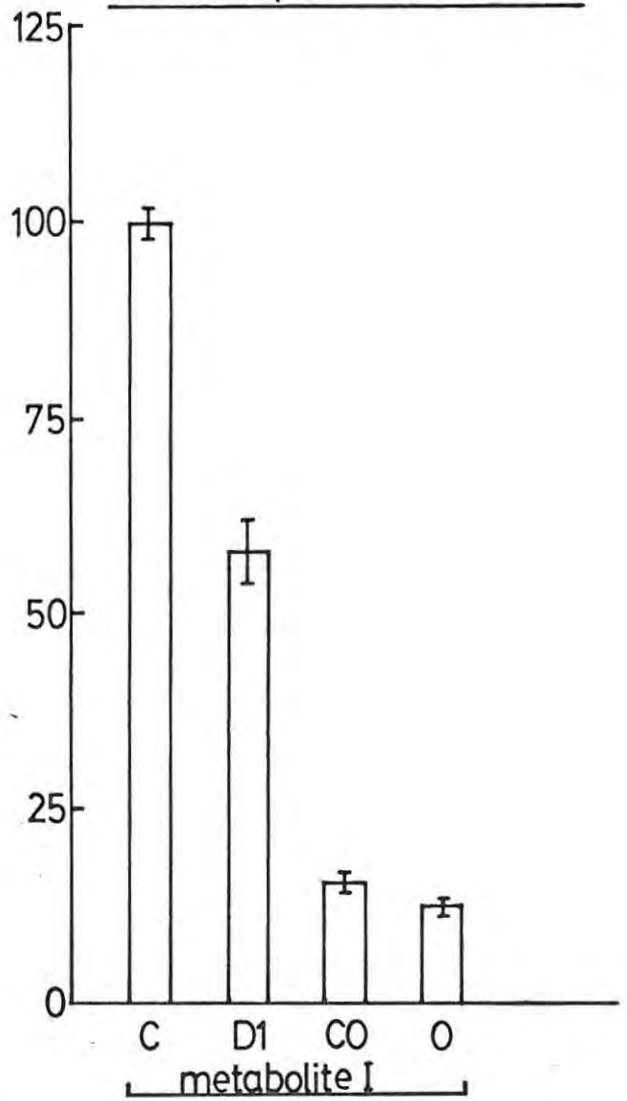


Fig14 Metabolism of SKF 525-A Using Induced Rat Hepatic Microsomes

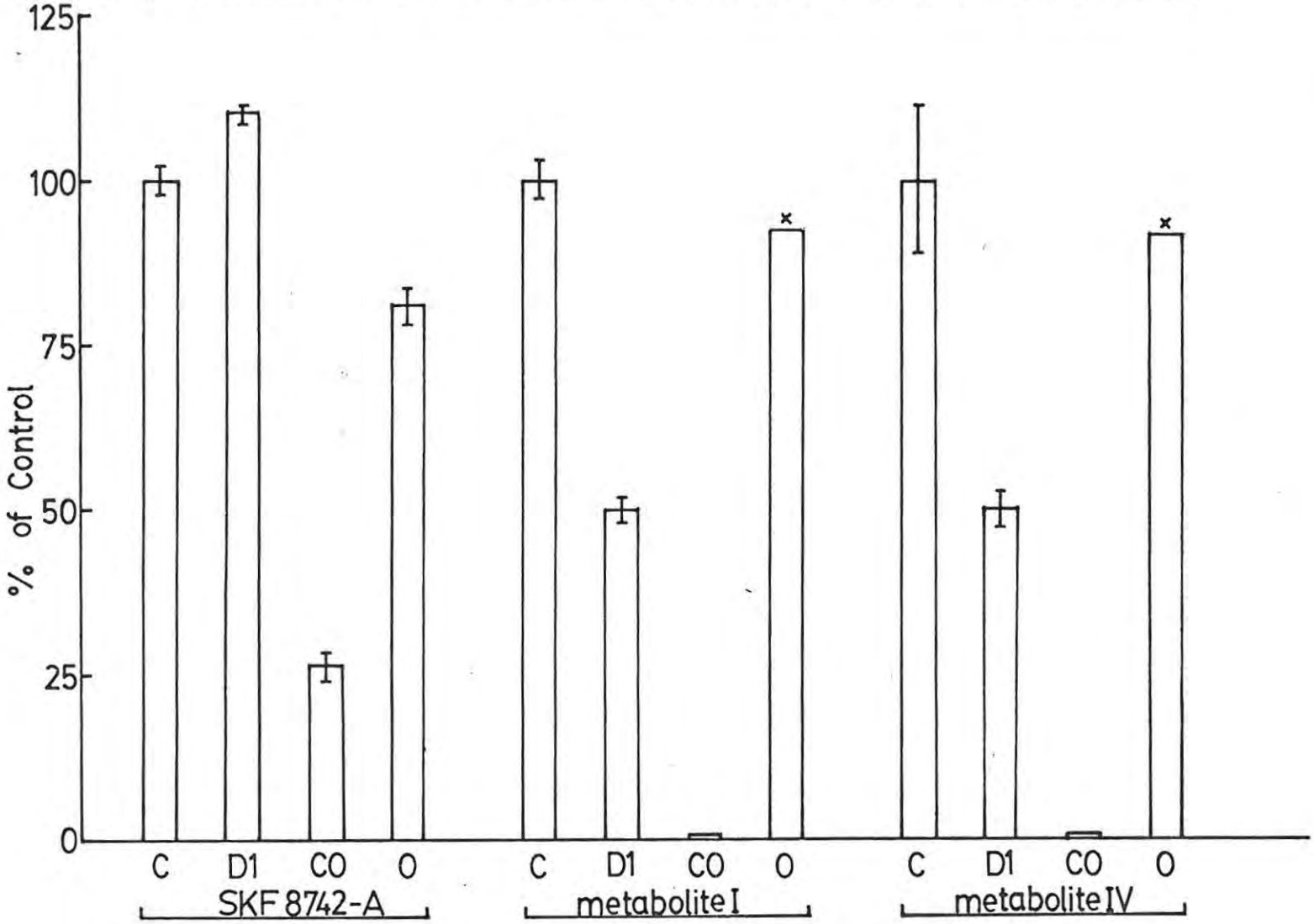
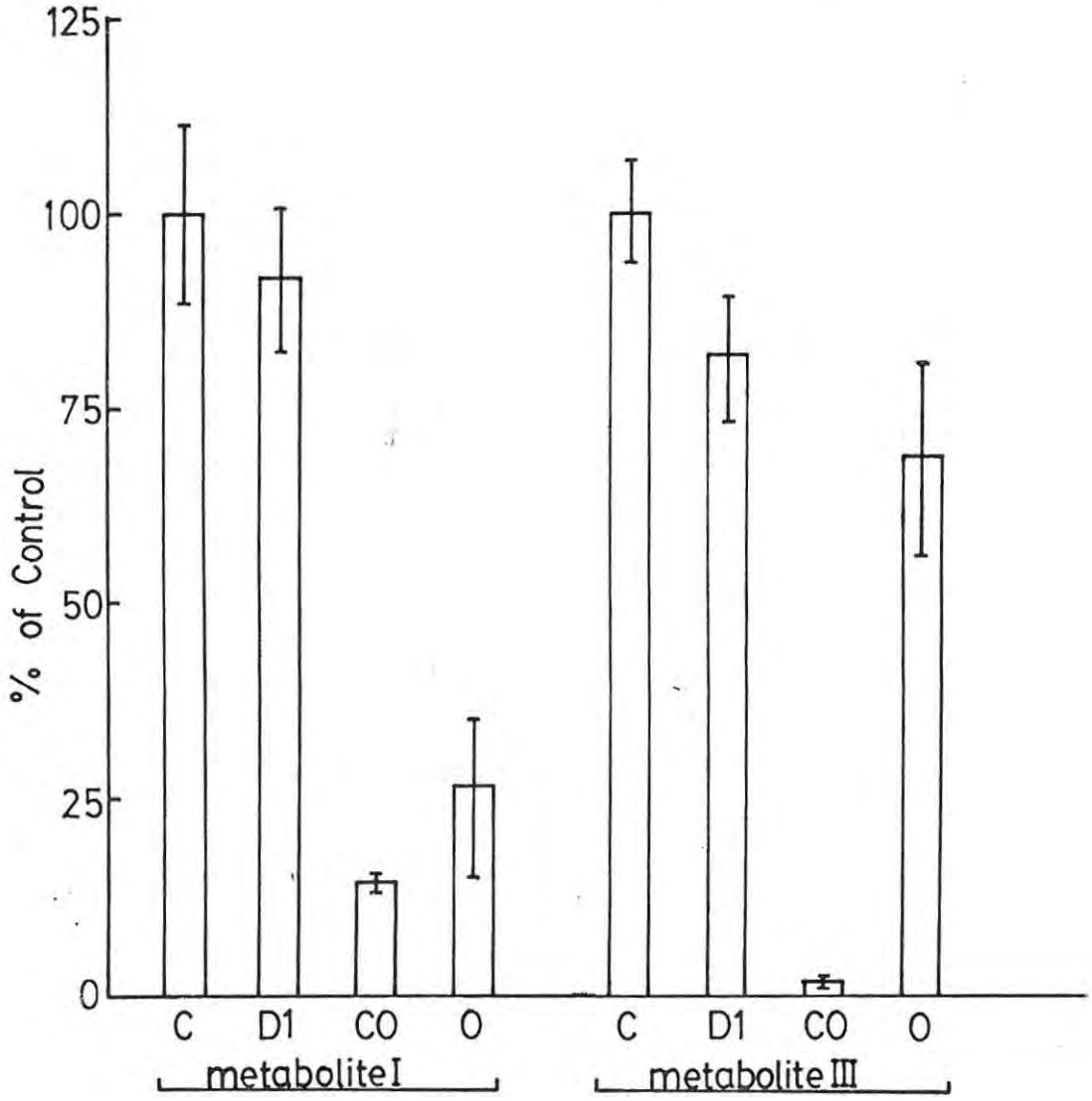


Fig 15 Metabolism of SKF 8742-A Using Induced Rat Hepatic Microsomes



swine hepatic microsomes and induced rat hepatic microsomes and the results were expressed as above.

Key C - control
D1 - 1mM dithiothreitol
D2 - 10mM dithiothreitol
CO - carbon monoxide
O - 3mM n-octylamine.

+ These values were too small to be measured with g.l.c. and the listed percentage control values are estimates.

x The metabolites I and IV peaks merged and were thus expressed as one value.

Discussion

As induction of rats with phenobarbital will stimulate cytochrome P-450 but not amine oxidase mediated reactions, microsomes prepared from induced rats serve as a useful tool to evaluate the enzyme systems involved in metabolism.

The results of these studies performed with phenobarbital induced microsomes indicate that production of SKF 8742 A from SKF 525 A is mediated by cytochrome P-450. As metabolite IV is only present when induced microsomes are used, its formation probably is also catalysed by cytochrome P-450. Similarly, all metabolites of SKF 8742 A appear to be produced in cytochrome P-450 mediated reactions.

A problem arises however when consideration is given to the fact that metabolite I, derived from SKF 525 A, and metabolite I, derived from SKF 8742 A, are not produced in similar quantitative ratio when induced microsomes are used. Furthermore, it is apparent that metabolite III arises from conversion of SKF 8742 A by induced microsomes and, thus, the presence of this metabolite would be expected in metabolic extracts derived from SKF 525 A (due to formation of SKF 8742 A). This is not the case.

The inhibition studies strongly suggest that SKF 8742 A is produced from SKF 525 A by normal rat liver microsomes in a cytochrome P-450 mediated reaction. Dithiothreitol (10^{-3}M) failed to inhibit SKF 8742 A formation significantly, n-octylamine failed to stimulate it and CO significantly inhibited the formation. Similarly, metabolite I also appeared to be formed in a cytochrome P-450 catalysed reaction.

The production of metabolite I from SKF 8742 A also appears to be mediated by cytochrome P-450 and, while 10^{-3}M dithiothreitol inhibits metabolite I formation by c.40%, only values as high as c.90% inhibition would be indicative of amine oxidase participation[251].

Use of swine liver microsomes failed to show amine oxidase involvement as n-octylamine did not stimulate metabolite production.

Inhibition studies involving phenobarbital induced microsomes did not indicate amine oxidase participation in the metabolism of SKF 525 A and SKF 8742 A.

CHAPTER 5

PREPARATIVE TECHNIQUES

Objectives

Anders et al.[26] succeeded in separating the metabolites of SKF 525 A using thin layer chromatography, but the minor metabolite was never identified. It is therefore the aim of this work to separate the metabolites by non-destructive processes and to identify them.

1. Thin Layer Chromatography (t.l.c.)

Materials and Methods

The following equipment was used. Gelman silicic acid and silica gel instant thin layer chromatography (I.T.L.C.) plates, Gelman developing tank[238], Hewlett Packard 5830A g.l.c. All solvents used were of analytical grade.

Spray Reagents

i. Potassium iodoplatinate reagent for amines.

Spray: 3ml of 10% platinum chloride and 97ml water + 100ml 6% potassium iodide.

ii. Sodium nitroprusside reagent for secondary aliphatic and alicyclic amines.

Spray: 5g sodium nitroprusside in 100ml of a 10% aqueous solution of acetaldehyde. One volume of this is mixed before use with 1 volume of 1% sodium carbonate. Primary amines react to produce a red colour and secondary amines produce a blue colour.

iii. Folin Ciocalteau reagent for phenols.

iv. Diazotized p-nitro-aniline solution for phenols.
Stock solution: dissolve 0,7g p-nitro-aniline in 9ml concentrated HCl and make up to 100ml with water.
Spray: 4ml of stock solution added dropwise to 5ml of an ice-cooled 1% sodium carbonate solution and made up to 100ml with iced water.
N.B. prepare freshly before use.

A dual wavelength ultra-violet light source was also used to detect the presence of SKF 525 A and SKF 8742A.

Pilot studies were run in microscope slide staining tanks using small strips of the I.T.L.C. plate. Solvent systems which produced promising results were re-tested in full scale in the Gelman tank after the atmosphere above the solvent had equilibrated for 10 minutes. On development, the strip was visualized under uv light and the spots marked. However, if the concentration of metabolite was too small to show up under uv then the strip was cut in half longitudinally and one half was sprayed for amine to visualize separation of the major components. Unlike the results of Anders et al.[26], the minor metabolite did not show up as a blue spot on treatment with Folin reagent and this necessitated a g.l.c. check for separation. The unsprayed half of the plate was cut up transversely in regular sections, which were numbered. These were alkalinised with ammonia gas, extracted with diethylether and evaporated to dryness in a sample tube. The extracts were redissolved in methanol and analysed by g.l.c.

Results

A total of 89 solvent systems were used in an attempt to separate the metabolites of SKF 525 A. See Table 1.

TABLE 1

I.T.L.C. OF SKF 525 A AND METABOLITES

No.	S.A.	S.G.	Solvent System	Results
1		*	EtOH:HAc:H_2O (60:30:10)	No separation, tailing.
2	*		$CHCl_3$:MeOH (95:5)	Slight separation,tailing.
3	*		" (90:10)	Slight separation,less tailing.
4	*		Benzene: Acetone (50:50)	No separation, tailing.
5*	*		$CHCl_3$:EtOH:NH_3 (95,5:4,5:0,5)	Good separation.
6		*	$CHCl_3$ (100%)	No separation, tailing.
7		*	$CHCl_3$:EtOH (98:2)	No separation, tailing.
8		*	" (96:4)	No separation.
9		*	" (95:5)	No separation.
10		*	$CHCl_3$:Diethylamine (99:1)	No separation.
11*	*		" (98:2)	Some separation.
12	*		" +EtOH (98:1:1)	No separation, tailing.
13	*		$CHCl_3$:Diethylamine (99:1)	No separation,less tailing.
14	*		" +MeOH (97:2:1)	No separation,tailing.
15	*		$CHCl_3$:Diethylamine:acetone (94:1:5)	Better separation, tailing.
16	*		" (97:1:2)	Some separation.
17		*	Benzene:Diethylamine (98:2)	No separation.
18	*		" (98:2)	Some separation.
19	*		" (96:4)	Better separation.
20	*		" (94:6)	Separation, Rf too high.
21	*		" (94:6 + 1 drop NH_3)	Ammonia immiscible.
22*	*		" (95:5)	Good separation.
23	*		$CHCl_3$:Diethylamine (99,9:0,1)	No separation.
24		*	Benzene:$CHCl_3$ (50:50)	Tailing.
25	*		" (50:50)	Tailing.

No.	S.A.	S.G.	Solvent System		Results
26	*		$CHCl_3$:EtOH:NH_3	(93:4:3)	Tailing.
27	*		"	(90:6:4)	Tailing.
28*	*		$CHCl_3$:EtOH:Diethylamine	(96:1:3)	Good separation.
29	*		"	(94:1:5)	Slight separation.
30	*		Benzene:EtOH:Diethylamine	(95:2:3)	Slight separation.
31	*		"	(94:3:3)	Better separation.
32	*		"	(92:5:3)	Less separation.
33	*		"	(90:5:5)	Some separation.
34	*		"	(87:5:8)	Some separation.
35	*		"	(89:5:6)	Some separation.
36	*		"	(89:6:5)	No separation.
37	*		"	(88:7:5)	No separation.
38	*		Pet.Ether:Diethylamine	(97:3)	Separation, tailing.
39	*		" + EtOH	(96:3:1)	Separation, tailing.
40	*		Pet.Ether (100%)		No separation.
41	*		" + EtOH	(98:2)	No separation.
42	*		Pet.Ether:Diethylamine	(94:6)	Separation.
43*	*		"	(95:5)	Good separation.
44	*		Pet.Ether:HAc	(98:2)	No separation.
45		*	"	(98:2)	No separation.
46	*		" + EtOH	(96:2:2)	No separation.
47		*	"	(96:2:2)	No separation.
48	*		$CHCl_3$:HAc	(98:2)	Tailing.
49		*	"	(98:2)	Tailing.
50		*	" + EtOH	(96:2:2)	No separation.
51		*	"	(98,5:1:0,5)	Tailing.
52	*		"	(98,5:1:0,5)	Tailing.
53	*		$CHCl_3$:HAc:acetone	(98:1:1)	Tailing.

No.	S.A.	S.G.	Solvent System		Results
54		*	$CHCl_3$:HAc:acetone	(98:1:1)	Tailing.
55	*		Benzene:EtOH + 1d HCl dil.	(98:2)	HCl immiscible.
56		*	"	(98:2)	HCl immiscible.
57	*		Benzene:HAc:EtOH	(97:1:2)	No separation.
58		*	"	(97:1:2)	No separation.
59	*		"	(96:1:3)	No separation.
60		*	"	(96:1:3)	No separation.
61	*		Benzene: HAc:Dioxane	(97:1:2)	No separation.
62		*	"	(97:1:2)	Tailing.
63	*		"	(95:1:4)	No separation.
64		*	"	(95:1:4)	Tailing.
65	*		"	(93:1:6)	No separation.
66	*		"	(91:3:6)	No separation.
67		*	"	(91:3:6)	Tailing.
68	*		Toluene:HAc:EtOH	(97:1:2)	No separation.
69		*	"	(97:1:2)	Tailing.
70	*		Pet.Ether:HAc:EtOH	(94:2:4)	No separation.
71		*	"	(94:2:4)	Tailing.
72		*	"	(92:2:6)	Tailing.
73		*	Pet.Ether:$CHCl_3$:HAc:EtOH	(70:25:2:3)	Tailing.
74	*		Et Ac:Benzene:HAc	(70:28:2)	No separation.
75		*	"	(70:28:2)	Tailing.
76[X]	*		Pet.Ether (100%)		No separation.
77[X]		*	" (100%)		Tailing.
78[X]	*		Pet.Ether:Diethylamine	(95:5)	Some separation.
79[X]		*	"	(95:5)	No separation.
80[X]	*		"	(99:1)	No separation.
81[X]		*	"	(99.1)	No separation.

No.	S.A.	S.G.	Solvent System		Results
82*X	*		Pet-Ether:EtOH	(98:2)	Separation.
83X		*	"	(98:2)	No separation.
84X	*		Hexane:EtOH	(98:2)	No separation.
85X		*	"	(98:2)	No separation.
86X	*		Toluene:EtOH	(98:2)	Separation.
87X		*	"	(98:2)	No separation.
88X		*	Benzene:acetone:EtOH	(89:9:2)	Some separation.
89X	*		"	(89:9:2)	No separation.

S.A. - Silicic acid plate

S.G. - Silica gel plate

5* - Full size plate run, cut into strips and analysed by g.l.c.

76X - Plates saturated with 0,95% NaOH spray before spotting

EtOH - 95% Ethanol

HAc - Glacial acetic acid

Pet.Ether - petroleum ether

$CHCl_3$ - chloroform

MeOH - methanol

Et Ac - ethyl acetate.

1d - one drop

5* In spite of the good separation apparently obtained using the I.T.L.C. method with this solvent, the g.l.c. strip analysis of the plate showed considerable contamination of the SKF 525 A and SKF 8742 A spots with one another.

11* This solvent system produced a chromatogram in which the minor metabolite was still contaminated with SKF 8742 A but it trailed behind the secondary amine to a large degree.

22* This solvent system was used to separate a mixture of pure SKF 525 A and SKF 8742 A. These two spots had an Rf value of 0,87 and 0,79 respectively. When metabolic extracts were run on the plate and analysed by g.l.c. there was considerable contamination of each component and the minor metabolite did not separate at all from SKF 8742 A.

28* This solvent system gave much the same result as solvent 11* where the minor metabolite lagged behind SKF 8742 A. Thus, it appeared that metabolite I was less polar than SKF 8742A. The polarity of the solvent system was varied with ethanol in solvents 30-37 but this had no separative effect.

43* This solvent system produced excellent separation of the tertiary and secondary amine but did not separate the minor metabolite from SKF 8742 A. As neutral or basic solvents had been used up to this stage, it was decided to test the effects of acidic systems. These were not at all successful.

82* Plates sprayed with sodium hydroxide as described were spotted and run in this solvent system. SKF 525 A and SKF 8742 A were apparently well separated with Rf values of 0,98 and 0,60 respectively. When the thin layer chromatogram was examined by g.l.c. strip analysis, contamination was evident.

In view of the excellent separation obtained on the I.T.L.C. plates but the lack of purity of the amine spots it was felt that other techniques should be investigated in an attempt to separate the metabolites as a sample with a high degree of purity was required.

2. Paper Chromatography

Materials and Methods

Medium flow rate Whatman chromatography paper was used in preference to fast flow rate paper as the former was likely to afford better separation.

Paper chromatography was conducted in the same manner as the I.T.L.C. and analytical grade solvents were used.

Results

TABLE II

	Solvent Systems		Result
1	$CHCl_3$:MeOH	(90:10)	Tailed.
2	MeOH (100%)		Tailed.
3	Pet.Ether (100%)		No separation.
4	MeOH:NH_3	(80:20)	Tailed.
5	MeOH:HAc	(80:20)	Tailed.
6	Pet.Ether:NH_3	(97:3)	No separation.
7	Pet.Ether:Diethylamine	(97:3)	No separation.
8	Dioxane:H_2O:NH_3	(90:8:2)	Tailed.
9	Acetone:NH_3	(90:10)	Tailed.
10	EtOH:NH_3	(98:2)	Tailed.

This technique did not look at all promising and was thus abandoned.

3. Column Chromatography

Materials and Methods

The following equipment was used in these studies. Glass chromatography

columns,an L.K.B. 7000 Ultrarac fraction collector and the Hewlett Packard 5830 A g.l.c.

A glass column, 80cm long and 1,5cm in diameter, was constructed with a No. 1 sinter fused into one end. Proximal to this, leaving the minimum of dead space, a teflon tap was affixed. At the other end of the column, a Quick-fit joint was fitted. The purpose of the sinter was to prevent gel leakage from the column. Previously glass wool had been used for this purpose but was unsuitable. The dead space after the sinter was kept as small as possible to prevent mixing and tailing of the separated components. The inlet to the column was constructed with a Quick-fit glass stopper through which a thin glass tube passed. The outer end of the tube was drawn fine and teflon tubing (1mm I.D.) was attached to this. Similarly teflon tubing was attached to the bottom end of the column leading to the fraction collector.

Sephadex L.H. 20 was allowed to swell for 90 minutes in a mixture of chloroform:methanol (1:1). At the end of this period, 2 volumes of hexane were added and the gel was degassed under vacuum for 3 minutes. After the column had been washed, the slurry was gently passed into the column to avoid the incorporation of air bubbles. Solvent was run through the column for 24h to allow it to settle and stabilize then a solution of Sudan III was passed through to check for aeration. A circular disc of filter paper was cut to the correct size and placed on top of the gel bed to prevent foreign particles from entering the gel.

Sample application was done in one of two ways. 1. The sample was dissolved in chloroform and, with the use of a pasteur pipette, was allowed to form a denser layer above the gel. 2. The solvent layer above the gel was removed and as the filter paper was exposed, so

the sample, dissolved in a minimal volume of solvent, was applied. As this ran dry it was "washed" into the gel by successive applications of small volumes of solvent. The latter method was preferred as the former involved the use of a different solvent to that used for elution and further, while the dense chloroform layer apparently remained intact diffusion of the sample could have occurred to cause tailing.

Once the sample had been successfully applied, the elution solvent was siphoned into the top of the column and by removing air bubbles a continuous flow system was established. Flow rate was set by altering the height of the solvent reservoir. Fractions were collected using an L.K.B. 7000 Ultrarac fraction collector. Each fraction was evaporated to dryness, diluted to a specific volume with methanol and analysed using g.l.c.

All solvents used were analytical reagent grade.

Results

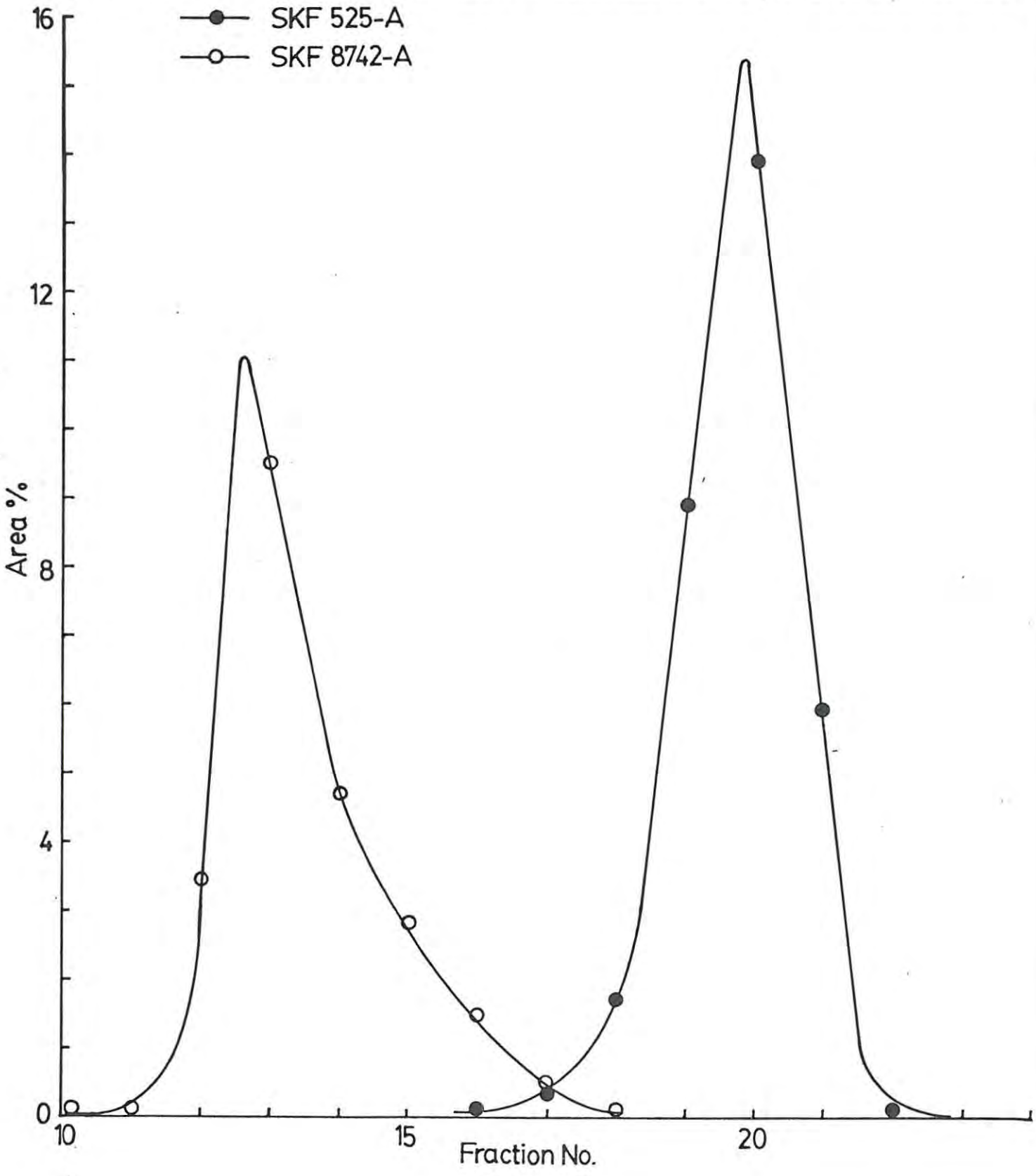
1. When chloroform:methanol:hexane (1:1:2) was used, the separation achieved between the two components in a mixture of SKF 525 A and SKF 8742 A is shown on the following graph (see Fig. 16).

Flow rate = 4 drops/min (c. 46 drops = 1ml).

2. Metabolites were prepared and a sample was applied to the column. The same conditions as in 1 were used. The minor metabolite was not separated from SKF 8742 A.

3. A new solvent system comprising methanol:hexane:chloroform (4:2:1) was used after the column had been conditioned for 24h.

Fig16 Seperation Between SKF 525-A & SKF 8742-A Using Sephadex LH 20



Area % is a measure of the g.l.c. detector response.

Flow rate 4 drops/min. The minor metabolite was not separated from SKF 8742 A.

4. The solvent system comprising chloroform:hexane:methanol (4:2:1) was used after the column had been conditioned for 24h.

Flow rate 4 drops/min. The minor metabolite was not separated from SKF 8742 A.

5. Solvent system 1 appeared to afford the best separation and so this was reused with a slower flow rate.

Flow rate 2 drops/min. This did not noticeably improve separation.

It was decided to increase column length to 2 m. A 2m, coiled, 6mm/O.D., glass, g.l.c. column was converted for this purpose by embedding a sinter at one end and introducing a Y tube at the other. On one arm of the Y tube was fitted a Quick-fit joint and stopper while the other was drawn out to accommodate the teflon tube. Dead space below the sinter was kept to a minimum and a tap was not fitted. This column was difficult to pack due to its length and small diameter and negative and positive pressure had to be used appropriately. The column was fitted to just below the level of the Y tube and the sample was introduced via the Quick-fit joint. The sample was encouraged to enter the gel by applying positive pressure. The column was conditioned for 24h and the following solvent systems were used.

1. Chloroform:methanol:hexane (1:1:2)

   Flow rate 1 drop every 90s.

   No separation.

2. Methanol:hexane:chloroform (4:2:1)

   Flow rate 1 drop every 90s.

   No separation.

3. Chloroform:hexane:methanol (4:2:1)

   Flow rate 1 drop every 90s.

   No separation.

4. Pyridine:methanol:hexane (3:1:1)

   Flow rate 1 drop every 90s.

   No separation.

This technique was abandoned as it was time consuming and did not look promising.

G.l.c./mass spectrometry will have to be relied on to elucidate the structure of the minor metabolite.

CONCLUSION

The pH optima for CO and for drug binding to cytochrome P-450, not unexpectedly, differ from one another. Different binding sites on the enzyme are involved and it is quite possible that, at different pHs the conformation of the protein changes, thus hindering or facilitating access of the agent to its binding site.

The observed difference in pH optima for the formation of SKF 8742 A and metabolite 1 is more difficult to explain. Gorrod[132] has pointed out that pH dependent metabolic studies may yield inconclusive results since the stability of the products will affect the quantity of metabolites formed. Metabolite I may be more stable at lower pH values and this increased stability would explain its higher incidence at pH 7,5. The results obtained in this study point to the involvement of cytochrome P-450 since the pH optimum for the amine oxidase system is c. 8,6[215].

The stability tests performed on the metabolites indicate that they are stable to further oxidation at room temperature. These tests were performed using g.l.c. analysis and consideration must be given to the high temperatures involved in this process as it is unlikely that a room temperature dependent change will be effectively monitored with g.l.c.

Possibly, the ideal method for studying these metabolites would be high pressure liquid chromatography. In fact, pilot studies were performed with a Varian 8500 but teething troubles after its recent installation and the expense of purchasing the ideal column precluded pursuit in this area.

The physical properties of the metabolites of SKF 525 A appear to be similar in that a) both the secondary amine and metabolite I were efficiently extracted by the process used and b) extensive investigation of techniques for separation were all unsuccessful. These similarities in behaviour make unlikely the possibility that metabolite I may be a polar N-oxide.

The search for a suitable internal standard for g.l.c. may have an added value as many of the compounds tested were well resolved by the columns used and, with little further effort, could quite possibly be analysed by the methods outlined for other studies.

Contrary to the findings of Anders et al.[26], this study has shown SKF 8742 A to be metabolised to a compound with an identical g.l.c. retention time to that of metabolite I derived from SKF 525 A. In fact, this metabolite of the secondary amine has been referred to in this work as metabolite I. However, these two compounds cannot be fully accepted as being identical until further studies have been performed on them. Unfortunately, information from samples submitted to another institution for g.l.c./mass spectrographic analysis has not been forthcoming.
A further query as to the identical nature of metabolites I is raised when biotransformation of these compounds is undertaken with phenobarbital induced microsomes. Metabolism of SKF 8742 A is then enhanced, whereas production of metabolite I from SKF 525 A remains unchanged.

As metabolite I is the main metabolite of SKF 8742 A, it may be expected to increase when induced microsomes are used, more than the expected production of a secondary metabolite which would be the case if SKF 525 A was converted to SKF 8742 A, from which metabolite I was formed.

The pattern of metabolites from the two compounds differ quantitatively and qualitatively on induction and this may be indicative of some change taking place in the mixed function oxidase system.

Zeigler and Pettit[107] have suggested that two somewhat different enzyme systems may be active in the metabolism of some drugs and this situation may apply to SKF 525 A.

The results of the inhibitor studies favour the involvement of cytochrome P-450 alone, but the failure to increase production of metabolite I from SKF 525 A with induced microsomes raises some doubt about this conclusion.

Viewing the problem of enzyme involvement from a theoretical standpoint, one expects that the size of the molecule[220], its basicity[132] and the fact that it is a tertiary amine[103,105] would favour the involvement of the amine oxidase system. However, it has been shown that diethyl substitution on the nitrogen of at least some compounds gives rise to derivatives which cannot be converted to an N-oxide. Furthermore, it is well established that SKF 525 A is N-dealkylated to produce SKF 8742 A in what may be the first stage in the biotransformation. It is not possible to say whether metabolite I is produced directly from SKF 525 A or SKF 8742 A or whether it is a stable intermediate involved in the conversion of the former to the latter molecule. Carefully conducted kinetic studies on the metabolism of these compounds should cast more light on this matter.

Activities of the mixed function oxidase and of the amine oxidase systems have been shown to overlap, but uncertainty over which enzyme may be involved in a particular metabolic step does not prevent speculation as

to the structure of the metabolites. In fact, the same products may arise *via* different pathways[254]. The following possibilities may occur as the result of metabolism of SKF 525 A. See Fig. 17.

i) Conversion of SKF 525 A to an α-carbinolamine intermediate, which, if stable, may comprise metabolite I. Dealkylation would follow to form the secondary amine.

ii) If an N-oxide is implicated, then the following pathway is likely: SKF 525 A would be converted to the N-oxide, which would form the α-carbinolamine and this would dealkylate to form SKF 8742 A.

iii) SKF 8742 A may be converted to the hydroxylamine derivative.

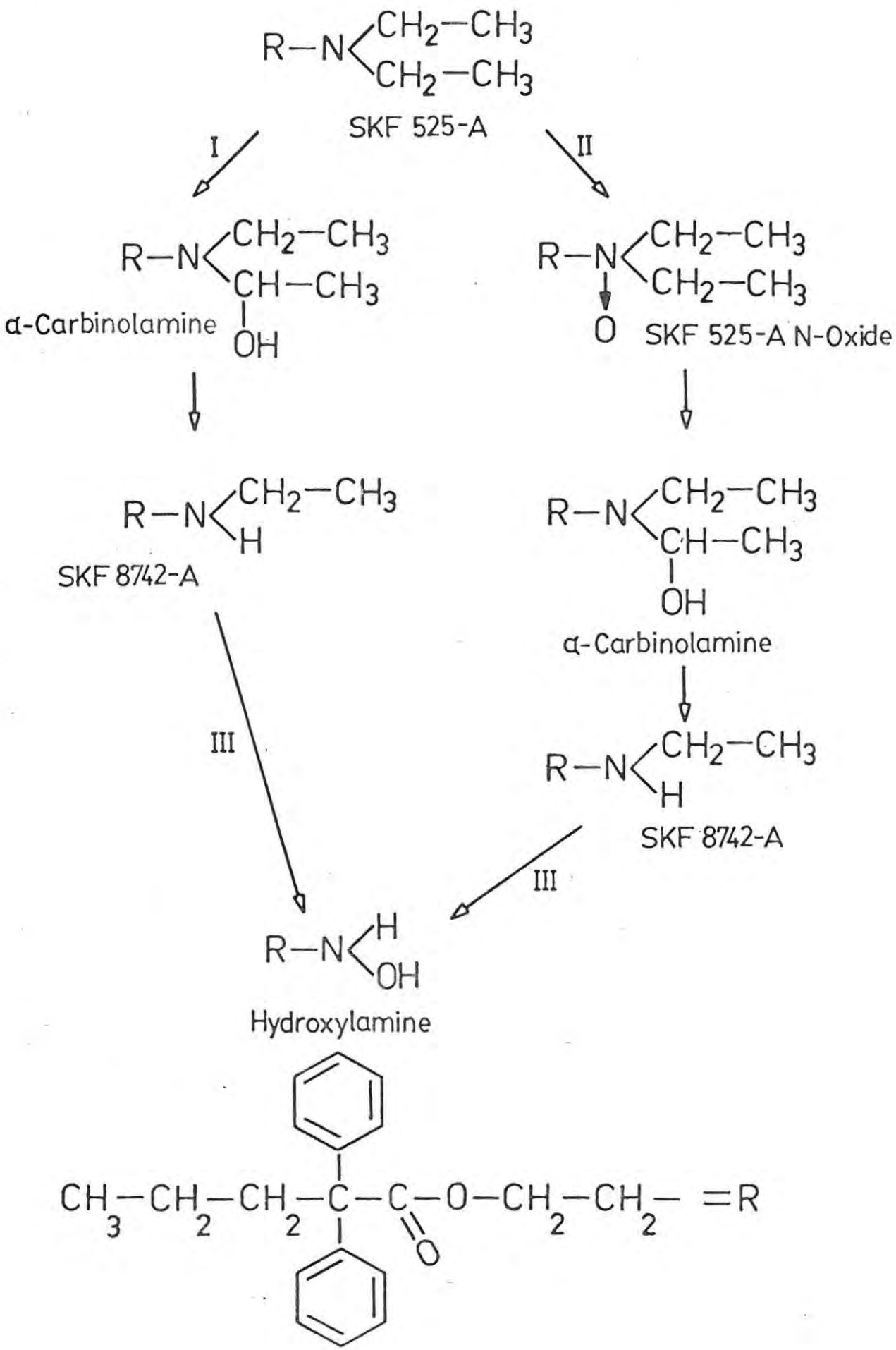
These possibilities lead to the suggestion that the unidentified minor metabolite of SKF 525 A may be either a stable α-carbinolamine intermediate or the hydroxylamine derivative. It is felt that the latter product is a more likely candidate as SKF 8742 A has been shown to be susceptible to further metabolic conversion.

This study is incomplete, and apart from g.l.c./mass spectrographic analysis of metabolites, a number of avenues are open for further investigation.

Methimazole may possibly be the most specific amine oxidase inhibitor used to date[255]. It may prove to be more satisfactory than dithiothreitol in implicating this oxidase for, as Mull *et al.*[204] have found, cysteamine (a compound with a similar function to dithiothreitol) has been shown to inhibit cytochrome P-450 mediated reactions as well. This fact may

Possible Metabolic Pathways For SKF 525-A

Fig 17



explain some of the inhibitory effects of dithiothreitol in this study, effects which were greater than might otherwise have been expected for a cytochrome P-450 dependent reaction.

Pretreatment of animals with $CoCl_2$[285] has been shown to reduce levels of cytochrome P-450, and a study with this compound may prove useful in further evaluating enzyme pathways involved.

Treatment of microsomes with detergents may also be a useful tool as this procedure decreases cytochrome P-450 activity but has little effect on the amine oxidase system[107].

No induction study is really complete until the effects of both phenobarbital and the polycyclic hydrocarbons have been evaluated since induction with benzpyrene is known to alter metabolite patterns in many cases.

BIBLIOGRAPHY

1. Cook, L., Toner, J.J., Fellows, E.J. J.Pharmac.exp.Ther. 111, 131, 1954.
2. Cook, L., Macko, E., Fellows, E.J. J.Pharmac.exp.Ther. 112, 382, 1954.
3. Cook, L., Navis, G., Fellows, E.J. J.Pharmac.exp.Ther. 112, 473, 1954.
4. Brodie, B.B. J.Pharm.Pharmac. 8, 1, 1956.
5. Axelrod, J., Reichenthal, J., Brodie, B.B. J.Pharmac.exp.Ther. 112, 49, 1954.
6. Cooper, J.R., Axelrod, J., Brodie, B.B. J.Pharmac.exp.Ther. 112, 55, 1954.
7. Fouts, J.R., Brodie, B.B. J.Pharmac.exp.Ther. 115, 68, 1955.
8. Gaudette, L.E., Brodie, B.B. Biochem.Pharmac. 2, 89, 1959.
9. Fouts, J.R., Brodie, B.B. J.Pharmac.exp.Ther. 116, 480, 1956.
10. Takemori, A.E., Mannering, G.J. J.Pharmac exp.Ther. 123, 171, 1958.
11. Axelrod, J. Biochem.J. 63, 634, 1956.
12. Mitoma, C., Posner, H.S., Reid, H.C. Udenfriend, S. Arch.Biochem. Biophys. 61, 431, 1956.
13. Gillette, J.R., Kamm, J.J. J.Pharmac.exp.Ther. 130, 262, 1960.
14. Fouts, J.R., Brodie, B.B. J.Pharmac.exp.Ther. 119, 197, 1957.
15. Kamm, J.J., Gillette, J.R. Progr.Drug Res. 6, 11, 1963.
16. Burns, J.J. Adv.Pharmac. 4, 219, 1966.
17. Sladek, N.E., Mannering, G.J. Biochem.Biophys.Res.Commun. 24,668, 1966.
18. Alvares, A.P., Mannering, G.J. Unpublished observations. Ref. cited by Mannering, G.J. Ann.N.Y.Acad.Sci. 123, 108, 1965.
19. Brodie, B.B. Ciba Foundation Symposium on Enzymes and Drug Action, Little Brown Boston, 1962.
20. Gilette, J.R. Progr.Drug Res. 6, 17, 1963.
21. Netter, K.J. Proc.Ist Int.Pharmac.Meeting 6, 213, 1962.
22. Gillette, J.R., Sasame, H.A. Fedn.Proc. 23, 537, 1964.
23. Schenkman, J.B., Wilson, B.J., Cinti, D.L. Biochem.Pharmac. 21, 2372, 1972.
24. Jenner, P., Netter, K. Biochem.Pharmac. 21, 1921, 1972.
25. Anders, M.W., Alvares, A.P., Mannering, G.J. Mol.Pharmac. 2, 328, 1966.
26. Anders, M.W., Alvares, A.P., Mannering, G.J. Mol.Pharmac. 2, 335, 1966.
27. Ikeda, M. Tanaka, S., Katayama, T. Mol.Pharmac. 4, 38, 1968.
28. Franklin, M.R. Xenobiotica 2, 517, 1972.

29. Philpot, R.M., Hodgson, E. Chem-Biol.Interactions 4, 185, 1971/1972.
30. Ullrich, V., Schnabel, K.H. Drug Metab.Dispos. 1, 176, 1973.
31. Franklin, M.R. Biochem.Pharmac. 21, 3287, 1972.
32. Elcombe, C.R., Bridges, J., Nimmo-Smith, R.H., Werringloer, J. Biochem.Soc.Transac. 3, 967, 1975.
33. Ullrich, V., Schnabel, K.H. Arch.Biochem.Biophys. 159, 240, 1973.
34. Omura, T., Sato, R. J.Biol.Chem. 237, PC 1375, 1962.
35. Omura, T., Sato, R. J.Biol.Chem. 239, 2370, 1964.
36. Omura, T., Sato, R. Biochim.Biophys.Acta. 71, 224, 1963.
37. Omura, T., Sato, R. J.Biol.Chem. 239, 2379, 1964.
38. Nishibayashi, H., Omura, T., Sato, R. Biochim.Biophys.Acta. 118, 651, 1966.
39. Imai, Y., Sato, R. J.Biochem. 62, 464, 1967.
40. Imai, Y., Sato, R. J.Biochem. 64, 147, 1968.
41. Imai, Y., Sato, R. Biochem.Biophys.Res.Commun. 25, 80, 1966.
42. Franklin, M.R. Pharmacologist 15, 169, 1973.
43. Franklin, M.R. Xenobiotica 4, 143, 1974.
44. Franklin, M.R. Drug.Metab.Dispos. 2, 321, 1974.,
45. James, R.C., Franklin, M.R. Biochem.Pharmac. 24, 835, 1975.
46. Buening, M.K., Franklin, M.R. Drug Metab.Dispos. 2, 386, 1974.
47. Franklin, M.R. Chem.-Biol.Interactions 14, 337, 1976.
48. Mansuy, D., Beaune, P., Chattard, J.C., Bartoli, J.F., Gans, P. Biochem.Pharmac. 25, 609, 1976.
49. Franklin, M.R. Mol.Pharmac. 10, 975, 1974.
50. Jonsson, J., Lindeke, B. Acta Pharm. Suecica 13, 313, 1976.
51. Chance, B., Williams, G.R. J.Biol.Chem. 209, 945, 1954.
52. Klingenberg, M. Arch Biochem. 75, 379, 1958.
53. Garfinkel, D. Arch Biochem. 77, 493, 1958.
54. Mason, H.S., North, J.C. Vanneste, M. Fedn.Proc. 24, 1172, 1965.
55. Schoeman, D.W., White, J.G., Mannering, G.J. Science 165, 1371, 1969.
56. Mason, H.S. Advances in Enzymology 21, Ed. F.F. Nord. Chider Science Publ., Inc. New York, 1957.
57. Cooper, D.Y., Estabrook, R.W., Rosenthal, O. J.Biol.Chem. 238, 1320, 1962.
58. Estabrook, R.W., Cooper, D.T., Rosenthal, O. Biochem.Z. 338, 741, 1963.
59. Cooper, D.T., Levine, S., Narasimhulu, S., Rosenthal, O., Estabrook, R.W. Science 147, 400, 1965.
60. Narasimhulu, S., Cooper, D.T., Rosenthal, O. Life Sci. 4, 2101, 1965.

61. Hildebrandt, A., Remmer, H., Estabrook, R.W. Biochem.Biophys.Res. Commun. 30, 607, 1968.
62. Strittmatter, P., Velick, S.F. J.Biol.Chem. 221, 253, 1956.
63. Remmer, H., Schenkman, J.B. Estabrook, R.W., Sasame, H., Gillette, J.R., Narasimhulu, S., Cooper, D.Y., Rosenthal, O. Mol.Pharmac. 2, 187, 1966.
64. Schenkman, J.B., Remmer, H., Estabrook, R.W. Mol.Pharmac. 3, 113, 1967.
65. Schenkman, J.B. Biochem. 9, 2081, 1970.
66. Schenkman, J.B., Cinti, D.L., Orrenius, S., Moldeus, P., Kraschnitz, R. Biochem.11, 4243, 1972.
67. Schenkman, J.B., Sato, R. Mol.Pharmac. 4, 613, 1968.
68. Schoeman, D.W., Chaplin, M.D., Mannering, G.J. Mol.Pharmac. 5, 412, 1969.
69. Conney, A.H., Davison, C., Gastel, R., Burns, J.J. J.Pharmac.exp.Ther. 130, 1, 1960.
70. van der Hoeven, T.A., Coon, M.J. J.Biol.Chem. 249, 6302, 1974.
71. Haughen, D.A., Van der Hoeven, T.A., Coon, M.J. J.Biol.Chem. 250, 3567, 1975.
72. Levin, W., Ryan, D., West, S., Lu, A. Y.H. J.Biol.Chem. 249, 1747, 1974.
73. Kwalek, J.C., Levin, W., Ryan, D., Thomas, P.E., Lu, A.Y.H. Mol. Pharmac. 11, 874, 1975.
74. Kwalek, J.C., Levin, W., Ryan, D., Lu, A.Y.H. Drug Metab.Dispos. 4, 190, 1976.
75. Estabrook, R.W., Cohen, B. Microsomes and Drug Oxiation. Ed. J. Gillette et al. Academic Press, New York, 1969, p.95.
76. Waterman, M., Mason, H.S. Biochem.Biophys.Res.Commun. 39, 450, 1970.
77. Orrenius, S., Ernster, L. Biochem.Biophys.Res.Commun. 16, 60, 1964.
78. Hildebrandt, A., Estabrook, R.W. Fedn Proc. 29, 738, 1970.
79. Cooper, D.Y., Narasimhulu, S., Slade, A., Raich, W. Foroff, O., Rosenthal, O. Life Sci. 4, 2109, 1965.
80. Oldham, S.B., Wilson, L.D., Landgraf, W.L., Harding, B.W. Arch.Biochem. Biophys. 123, 484, 1965.
81. Hildebrandt, A.G., Estabrook, R.W. Microsomes and Drug Oxidation. Ed. J. Gillette et al. Academic Press, New York, 1969, p.331.
82. Conney, A.H., Levin, W., Jacobson, M. Kuntzman, R. Microsomes and Drug Oxidation. Ed. J. Gillette et al. Academic Press, New York, 1969, p.279.
83. Mannering, G.J., Sladek, N.E., Parli, C.J., Schoeman, D.W. Microsomes and Drug Oxidation. Ed. J. Gillette et al. Academic Press, New York, 1969, p.303.

84. Kuntzman, R., Levin, W., Schilling, G., Alvares, A.P. Microsomes and Drug Oxidation. Ed. J. Gillette et al. Academic Press, New York, 1969, p.349.
85. Leibman, K.C., Hildebrandt, A.G., Estabrook, R.W. Biochem.Biophys. Res.Commun. 36, 789, 1969.
86. Jefcoate, C.R.E., Gaylor, J.C., Calabrese, R.L. Biochem. 8, 3455, 1969.
87. Gorrod, J.W., Disley, L.G., Temple, D.J. Xenobiotica 1, 521, 1971.
88. Gorrod, J.W., Temple, D.J. Chem.-Biol.Interactions 6, 203, 1973.
89. Estabrook, R.W., Baron, J., Peterson, J., Ishimura, Y. Biochim.J. 125, 3P, 1971.
90. Orrenius, S., Wilson, B.J., Von Bahr, C., Schenkman, J.R. Biochem.J. 126, 80, 1971.
91. Schenkman, J.B., Griem, H., Zange, M., Remmer H. Biochim.Biophys. Acta. 171, 23, 1969.
92. Wilson, B.J., Orrenius, S. Biochim.Biophys.Acta. 261, 94, 1972.
93. Hashimoto, Y., Yamano, T., Mason, H.S. J.Biol.Chem. 237, P.C. 3843, 1962.
94. Cammer, W., Schenkman, J.B., Estabrook, R.W. Biochem.Biophys.Res. Commun. 23, 264, 1966.
95. Whysner, J.A., Ramseyer, J., Kazmi, G.M., Harding, B.W. Biochem. Biophys.Res.Commun. 36, 795, 1969.
96. Misselwitz, R., Janig, G-R., Rein, H., Buder, E., Zirwer, D., Ruckpaul, K. Acta Biol.Med.Germ. 35, K19, 1976.
97. Jefcoate, C.R.E., Gaylor, J.L. Biochem. 8, 3464, 1969.
98. Peisach, J., Blumberg, W.E. Proc.National Academy of Sciences 67, 172, 1970.
99. Jefcoate, C.R.E., Gaylor, J.L. Biochem. 9, 3816, 1970.
100. Reichman, L.M., Annaev, B., Rozantzev, E.G. Biochem.Biophys.Acta. 263, 41, 1972.
101. Reichman, L.M., Annaev, B., Rozantzev, E.G., Shapiro, A.B. Chem.-Biol. Interactions 5, 243, 1972.
102. Machinist, J.M., Orme-Johnson, W.H., Zeigler, D.M. Biochem. 5, 2939, 1966.
103. Baker, J.R., Chaykin, S. J.Biol.Chem. 237, 1309, 1962.
104. Zeigler, D.M., Pettit, F.H. Biochem. 5, 2932, 1966.
105. Norris, E.R., Benoit, G.J. J.Biol.Chem. 158, 443, 1945.
106. McMahon, R.E., Sullivan, H.R. Life Sci. 3, 1167, 1964.
107. Zeigler, D.M., Pettit, F.H. Biochem.Biophys.Res.Commun. 15, 188, 1964.
108. Dehner, E.W., Machinist, J.M., Zeigler, D.M. Life Sci. 7, 1135, 1968.

109. Mueller, G.C., Miller, J.A. J.Biol.Chem. 202, 579, 1953.
110. Battersby, A.R. Proc.Chem.Soc. 72, 189, 1963.
111. Hodgson, E., Casida, J.E. Biochem.Pharmac. 9, 179, 1961.
112. Zeigler, D.M., Mitchell, C.H., Jollow, D. Microsomes and Drug Oxidation. Ed. J. Gillette et al. Academic Press, New York, 1969, p.173.
113. Zeigler, D.M. Microsomes and Drug Oxidation. Ed. J. Gillette et al. Academic Press New York, p.188.
114. Bickel, M.H., Weder, H.J., Aebi, H. Biochem.Biophys.Res.Commun. 33, 1012, 1968.
115. Bickel, M.H. Arch.Biochem.Biophys. 148, 54, 1971.
116. Gigon, P.L., Bickel, M.H. Biochem.Pharmac. 20, 1921, 1971.
117. Uehleke, H. Xenobiotica 1, 327, 1971.
118. Uehleke, H. Arch.Exp.Path.Pharmak. 259, 66, 1967.
119. Masters, B.S.S., Nelson, E.B., Zeigler, D.M., Baron, J., Raj, P.P., Isaacson, E.G. Chem.-Biol.Interactions 3, 296, 1971.
120. Reiner, O., Uehleke, H. Xenobiotica 1, 329, 1971.
121. Beckett, A.H., Van Dyck, J.M. Chissick, H.H., Gorrod, J.W. J.Pharm. Pharmac. 23, 809, 1971.
122. Beckett, A.H., Van Dyck, J.M., Chissick, H.H., Gorrod, J.W. J.Pharm. Pharmac. 23, 560, 1971.
123. Beckett, A.H., Al-Sarraj, S. J.Pharm.Pharac. 24, 176, 1972.
124. Bickel, M.H. Pharmacol.Rev. 21, 325, 1969.
125. Bickel, M.H. Unpublished observations cited in Pharmacol.Rev. 21, 325, 1969.
126. Beckett, A.H., Hewick, D.S. J.Pharm.Pharmac. 19, 134, 1967.
127. Beckett, A.H., Eissen, E.E. J.Pharm.Pharmac. 25, 188, 1973.
128. Beckett, A.H., Al-Sarraj, S. J.Pharm.Pharmac. 24, 916, 1972.
129. Beckett, A.H., Salami, M.A. J.Pharm.Pharmac. 24, 900, 1972.
130. Murray, K.N., Chaykin, S. J.Biol.Chem. 241, 2029, 1966.
131. Murray, K.N., Chaykin, S. J.Biol.Chem. 241, 3468, 1966.
132. Gorrod, J.W. Chem.-Biol.Interactions 7, 289, 1973.
133. Gorrod, J.W., Temple, D.J., Beckett, A.H. Xenobiotica 5, 465, 1975.
134. Beckett, A.H., Belanger, P.M. J.Pharm.Pharmac. 26, 558, 1974.
135. Beckett, A.H., Belanger, P.M. J.Pharm.Pharmac. 27, 547, 1975.
136. Beckett, A.H., Belanger, P.M. J.Pharm.Pharmac. 28, 2, 1976.
137. Gorrod, J.W., Temple, D.J. Xenobiotica, 6, 265, 1976.
138. Jori, A., Die Salle, E., Santini, V. Biochem.Pharmac. 20, 2965, 1971.

139 Brown, R.R., Miller, J.A., Miller, E.C. J.Biol.Chem. 209, 211, 1954.
140. Cramer, J.W., Miller, J.A., Miller, E.C. J.Biol.Chem. 235, 250, 1960.
141. Conney, A.H., Miller, E.C. Miller, J.A. J.Biol.Chem. 228, 753, 1957.
142. Arcos, J.C., Conney, A.H., Buu-Hoi, N.P. J.Biol.Chem. 236, 1291, 1961.
143. Conney, A.H., Gillette, J.R., Inscoe, J.K., Trams, E.R., Posner, H.S. Science 130, 1478, 1959.
144. Griem, H., Schenkman, J.B., Klotzbucker, M., Remmer, H. Biochim. Biophys.Acta. 201, 20, 1970.
145. Levin, W., Kuntzman, R. J.Biol.Chem. 244, 3671, 1969.
146. Fouts, J.R., Rogers, L.A. J.Pharmac.exp.Ther. 147, 112, 1965.
147. Neale, M.G., Parke, D.V. Biochem.J. 113, 12p. 1969.
148. Welton, A.F., O'Neal, F.O., Chaney, L.C., Aust, S.A. J.Biol.Chem. 250, 5631, 1975.
149. Parli, C.J., Mannering, G.J. Mol.Pharmac. 6, 178, 1970.
150. Bidleman, K., Mannering, G.J. Mol.Pharmac. 6, 697, 1970.
151. Gnosspelius, Y., Thor, H., Orrenius, S. Chem.-Biol.Interactions 1, 125, 125, 1969.
152. Alvares, A.P., Mannering, G.J. Mol.Pharmac. 6, 206, 1970.
153. Nebert, D.W., Gelboin, H.V. J.Biol.Chem. 245, 160, 1970.
154. Nebert, D.W. J.Biol.Chem. 245, 519, 1970.
155. Nebert, D.W., Brausserman, L.L. Mol.Pharmac. 6, 293, 1970.
156. Nebert, D.W., Brausserman, L.L. Mol.Pharmac. 6, 304, 1970.
157. Gielen, J.E., Nebert, D.W. J.Biol.Chem. 246, 5189, 1971.
158. Nebert, D.W., Gielen, J.E. J.Biol.Chem. 246, 5199, 1971.
159. Nebert, D.W., Kon, H. J.Biol.Chem. 248, 169, 1973.
160. Gielen, J.E., Goujon, F.M., Nebert, D.W. J.Biol.Chem. 247, 1125, 1972.
161. Nebert, D.W., Gielen, J.E., Goujon, F.E. Mol.Pharmac. 8, 651, 1972.
162. Nebert, D.W., Robinson, J.R. J.Biol.Chem. 248, 7633, 1973.
163. Gielen, J.E., Nebert, D.W. Science, 172, 167, 1971.
164. Conney, A.H., Gilman, A.G. J.Biol.Chem. 238, 3682, 1963.
165. Jick, H., Schuster, L. J.Biol.Chem. 241, 5366, 1966.
166. Pelkonen, O., Karki, N.T. Chem.-Biol.Interactions 7, 93, 1973.
167. Fujita, T., Schoeman, D.W., Mannering, G.J. J.Biol.Chem. 248, 2192, 1973.
168. Imai, Y., Mason, H.S. J.Biol.Chem. 246, 5970, 1971.
169. Lu, A.Y.H., Kuntzman, R., West, S., Jacobson, M., Conney, A.H. J.Biol.Chem. 247, 1727, 1972.
170. Philpot, R.M., Hodgson, E. Mol.Pharmac. 8, 204, 1972.

171. Schoeman, D.W. Mol.Pharmac. 9, 372, 1973.
172. Comai, K., Gaylor, J.L. J.Biol.Chem. 248, 4947, 1973.
173. Guenther, T.M., Mannering, G.J. Biochem.Pharmac. 26, 567, 1977.
174. Guenther, T.M., Mannering, G.J. Biochem.Pharmac. 26, 577, 1977.
175. Glauman, H. Chem.-Biol.Interactions 2, 369, 1970.
176. Hook, G.R.E., Orton, T.C., Moore, J.A., Lucier, G.W. Biochem. Pharmac. 24, 335, 1975.
177. Stonard, M.D., Nenon, P.Z. Biochem.Pharmac. 23, 2175, 1974.
178. Stonard, M.D., Biochem.Pharmac. 24, 1959, 1975.
179. Conney, A.H., Levin, W., Ikeda, M., Kuntzman, R., Cooper, D.Y., Rosenthal, O. J.Biol.Chem. 243, 3912, 1968.
180. Conney, A.H., Levin, W., Jacobson, M., Kuntzman, R. Microsomes and Drug Oxidation. Ed. J. Gillette et al. Academic Press, New York, 1969, p.279.
181. Ryan, D., Lu, A.Y.H., West, S., Levin, W. J.Biol.Chem. 250, 2157, 1975.
182. Kwalek, J.C., Lu, A.Y.H. Mol.Pharmac. 11, 201, 1975.
183. Thomas, P.E., Lu, A.Y.H., Ryan, D., West, S.B., Kwalek, J., Levin, W. J.Biol.Chem. 251, 1385, 1976.
184. Haughen, D.A., Coon, M.J., Nebert, D.W. J.Biol.Chem. 251, 1817, 1976.
185. Huang, M.T., West, S.B., Lu, A.Y.H. J.Biol.Chem. 251, 4659, 1976.
186. Mailman, R.B., Tate, L.G., Muse, K.E., Coons, L.B., Hodgson, E. Chem.-Biol.Interactions 10, 215, 1975.
187. Anders, M.W., Mannering, G.J. Mol.Pharmac. 2, 341, 1966.
188. Stanton, R.H., Khan, M.A.Q. Biochem.Biophys.Res.Commun. 72, 1278, 1976.
189. Jernstrom, B., Capdevila, J., Jakobsson, S., Orrenius, S. Biochem. Biophys.Res.Commun. 64, 814, 1975.
190. Arinc, E., Philpot, R.M. J.Biol.Chem. 251, 3213, 1976.
191. Nebert, D.W., Heidema, J.K., Strobel, H.W., Coon, M.J. J.Biol.Chem. 248, 7631, 1973.
192. Capdevila, J., Ahmed, N., Agosin, M. J.Biol.Chem. 250, 1048, 1975.
193. Wagstaff, D.J., Short, C.R. Toxic.App.Pharmac. 19, 54, 1971.
194. Yu, S.J., Terriere, L.C. Pest.Biochem.Phys. 4, 160, 1974.
195. Ryan, D., Lu, A.Y.H., Kwalek, J., West, S., Levin, W. Biochem. Biophys.Res.Commun. 64, 1134, 1975.
196. Imai, Y., Sato, R. Biochem.Biophys.Res.Commun. 60, 8, 1974.
197. Johnson, E.F., Muller-Eberhard, U. Fedn.Proc. 36, 833, 1977.
198. Peterson, J.A., White, R.E., Yasukochi, Y., Coon, M.J.J. Biol.Chem. 252, 4431, 1977.

199. Buening, M.K., Franklin, M.R. Drug Metab.Dispos. 4, 244, 1976.
200. Philpot, R.M., Arinc, E. Mol.Pharmac. 12, 483, 1976.
201. Ingleman-Sundberg, M., Gustaffson, J.A. Febs Letters. 74, 103, 1977.
202. Jenner, P. Xenobiotica 1, 399, 1971.
203. Hewick, D.S., Beckett, A.H. Biochem.J. 122, 59, 1971.
204. Mull, R., Hinkelbein, W., Gertz, J., Flemming, K. Biochim.Biophys. Acta. 481, 407, 1977.
205. Nery, B. Biochem.J. 106, 1, 1968.
206. Booth, J., Boyland, E. Biochem.J. 91, 362, 1964.
207. Hlavica, P., Kiese, M. Biochem.Pharmac. 18, 1501, 1969.
208. Weisburger, J.H., Weisburgher, E.K. Concepts in Biochemical Pharmacology. Eds. B.B. Brodie and J.R. Gillette. Vol. XXVIII, Part 2 pp.312-333. Springer, New York, 1971.
209. Matsushima, T., Grantham, P.H., Weisburger, E.K., Weisburger, J.H. Biochem.Pharmac. 21, 2043, 1972.
210. Thorgeirsson, S.S., Jollow, D.J., Sasame, H.A., Green, I., Mitchell, J.R. Mol.Pharmac. 9, 398, 1973.
211. Lotlikar, P.D., Wasserman, M.B. Biochem.J. 129, 937, 1972.
212. Matsushima, T., Weisburger, J.H. Xenobiotica 2, 423, 1972.
213. Lotlikar, P.D. Biochem.J. 118, 513, 1970.
214. Zeigler, D.M., Mitchell, C.H. Arch.Biochem.Biophys. 150, 116, 1972.
215. Zeigler, D.M., McKee, E.M., Poulsen, L.L. Drug Metab.Dispos. 1, 315, 1973.
216. Zeigler, D.M., Poulsen, L.L., McKee, E.M. Xenobiotica, 1, 523, 1971.
217. Beckett, A.H., Coutts, R.T., Ogunbona, F.A. J.Pharm.Pharmacol. 25, 190, 1973.
218. Beckett, A.H., Coutts, R.T., Ogunbona, F.A. J.Pharm.Pharmacol. 26, 312, 1974.
219. Beckett, A.H., Haya, K. J.Pharm.Pharmacol. 29, 89, 1977.
220. Testa, B., Jenner, P. Drug Metabolism. Ed. J. Swarbrick. Marcel Dekker Inc. New York, p.67, 1976.
221. Khan, F. Ph.D. Thesis, University of London 1972. Ref. cited by B. Testa and P. Jenner in Drug Metabolism Ed. J. Swarbrick. Marcel Dekker Inc., New York, 1976.
222. McMahon, R.E. J.Pharm.Sci. 55, 457, 1966.
223. Testa, B. Acta Pharm.Suecica 10, 441, 1973.
224. Hucker, H.B., Michniewicz, B.M., Rhodes, R.E. Biochem.Pharmac. 20, 2123, 1971.
225. Hucker, H.B. Drug Metab.Dispos. 1, 332, 1973.

226 Parli, C.J., McMahon, R.E. Drug Metab.Dispos. 1, 337, 1973.

227. Parli, C.J., Wong Lee, N., McMahon, R.E. Drug Metab.Dispos. 1, 628, 1973.

228. Hes, J., Sternson, L.A. Drug Metab.Dispos. 2, 345, 1974.

229. Walle, T., Ishizaki, T., Gaffney, T.E. J.Pharm.exp.Ther. 183, 508, 1972.

230. Becket, A.H., Huizing, G. J.Pharm.Pharmac. 27, 42p, 1975.

231. Gillette, J.R., Brodie, B.B., La Du, B.N. J.Pharm.exp.Ther. 119, 532, 1957.

232. Lee, I.P., Yamamura, H.I., Dixon, R.L. Biochem.Pharmac. 17, 1671, 1968.

233. Testa, B., Jenner, P. Drug Metabolism. Ed. J. Swarbrick, Marcel Dekker Inc., New York, 1976, p.357.

234. Fischer, P.W.F., Spencer, T. Chem.-Biol.Interactions 8, 253, 1974.

235. Mueller, G.C., Miller, J.A. J.Biol.Chem. 176, 535, 1948.

236. La Du, B.N., Gaudette, L., Trousof, N., Brodie, B.B. J.Biol.Chem. 214, 741, 1955.

237. Schenkman, J.B., Ball, J.A., Estabrook, R.W. Biochem.Pharmac. 16, 1071, 1967.

238. Gelman chromatography system. Tehcnical Bull. 17R Gelman Instrument Co. Ann Arbor, Michigan.

239. Burke, M.D., Mayer, R.T. Drug Metab.Dispos. 2, 583, 1974.

240. Poulsen, L.L., Kadlubar, F.F., Zeigler, D.M. Arch.Biochem.Biophys. 164, 774, 1974.

241. Poulsen, L.L., McKee, E.M., Zeigler, D.M. Fedn.Proc. 32, 666, 1973.

242. Hewlett Packard 5830A Operating Supplement (18800-90100).

243. Hammarstrand, K. Varian Instrumental Applications 10, 10, 1976.

244. Cinti, D.L., Moldeus, P., Schenkman, J.B. Biochem.Pharmac. 21, 3249, 1972.

245. Goa, J. Scand.J.Clin.Lab.Invest. 5, 218, 1953.

246. Good, N.E., Winget, G., Winter, W., Connolly, T.N., Izawa, S., Singh, R.M.M. Biochem. 5, 467, 1966.

247. Fundamentals of Drug Metabolism and Disposition. Ed. B.N. La Du, H.G. Mandel, E.L. Way. Williams & Wilkins, Baltimore, 1971, p.538.

248. Documenta Geigy Scientific Tables. Ed. K. Diem, C. Lenther. 7th Edition. Ciba Geigy Switzerland. p.280.

249. Siebert, G., Carsiotis, M., Plant, G.W.E. J.Biol.Chem. 226, 977, 1957.

250. Fundamentals of Drug Metabolism and Disposition. Ed. B.N. La Du, H.G. Mandel, E.L. Way. Williams & Wilkins, Baltimore, 1971, p.539.

251. Beckett, A.H., Mitchard, K., Shihab, A.A. J.Pharm.Pharmac. 23, 941, 1971.
252. Gorrod, J.W., Jenner, P., Keysell, G., Beckett, A.H. Chem.-Biol. Interactions, 3, 269, 1971.
253. Schenkman, J.B., Remmer, H., Estabrook, R.W. Mol.Pharmac. 3, 516, 1967.
254. Hlavica, P., Kehl, M. Biochem.J. 164, 487, 1977.
255. Prough, R.A., Zeigler, D.M. Arch.Biochem.Biophys. 180, 363, 1977.
256. Beckett, A.H. Xenobiotica 1, 365, 1971.
257. Poulsen, L.L., Hyslop, R.M., Zeigler, D.M. Biochem.Pharmac. 23, 3431, 1974.
258. Holmes, W.L., Bentz, J.D. J.Biol.Chem. 11, 3118, 1960.
259. Dick, E.C., Greenburg, S.M., Herndon, J.F., Jones, M., Van Loon, E.J. Proc.Soc.Exptl.Bio & Medicine, 104, 523, 1960.
260. Lena, E.T. Boll.Chim.Farm. 98, 201, 1959.
261. Goudie, J.A., Kelley, M., Taylor, M., Wheeler, T.J. Psychopharmacologia (Berl.) 41, 291, 1975.
262. Magus, R.D., Fouts, J.R. Biochem.Pharmac. 16, 1323, 1967.
263. Magus, R.D., Rickert, D.E., Fouts, J.R. Biochem.Pharmac. 17, 2071, 1968.
264. Lu, A.T.H., West, S.B., Vore, M., Ryan, D., Levin, W. J.Biol.Chem. 249, 6701, 1974.
265. Spatz, L., Strittmatter, P. J.Biol.Chem. 248, 793, 1973.
266. Pederson, T.C., Buege, J.A., Aust, S.D. J.Biol.Chem. 248, 7134, 1973.
267. Anders, M.W., Mannering, G.J. Fedn.Proc. 23, 537, 1964.
268. Takanaka, A., Ho, I.K. Pharmacologist 18, 235, 1976.
269. Rogers, L.A., Fouts, J.R. Fedn.Proc. 23, 537, 1964.
270. Fundamentals of Drug Metabolism and Disposition. Ed. B.N. La Du, H.G. Mandel, E.C. Way. Williams & Wilkins Co. Baltimore, 1971, p.187.
271. Parke, D.V. Biochem.J. 77, 493, 1960.
272. Creaven, P.J., Parke, D.V., Williams, R.T. Biochem.J. 96, 879, 1965.
273. Jori, A., Bernardi, D., Pugliatti, C., Garrattini, S. Biochem. Pharmac. 19, 1315, 1970.
274. Jori, A., Pescador, R. Chem.-Biol.Interactions 8, 297, 1974.
275. Conney, A.H. Pharmacol.Rev. 19, 317, 1967.
276. Knutt, J.C.A., Wills, E.D. Biochem.Pharmac. 23, 793, 1974.
277. Gram, T.E., Guarino, A.M., Schroeder, D.H., Gillette, J.R. Biochem.J. 113, 681, 1969.

278. Basu, F.K., Dickerson, J.W.T., Parke, D.V. Biochem.J. 124, 19, 1971.
279. Neale, M.G., Parke, D.V. Biochem.Pharmac. 22, 1451, 1973.
280. McCarthy, J.S., Furner, R.C. van Dyke, K., Stitzel, R.E. Biochem. Pharmac. 19, 1341, 1970.
281. Lotter, A.P., Goosens, A.P. S.A.Pharm.J. 44, 282, 1977.
282. Anders, M.W., Mannering, G.J. Analyt.Chem. 34, 730, 1962.
283. Albro, P.W. Analyt.Biochem. 64, 485, 1975.
284. Sutton, F. United States Patent Office Patent No. 3174994, 1965.
285. De Matteis, F., Gibbs, A.H. Biochem.J. 162, 213, 1977.