Fd Cosmet Toxicol Vol 19 pp 657 to 666. 1981
Printed in Great Britain

0015-6264/81/050657-10$02.00/0

SAFROLE: ITS METABOLISM, CARCINOGENICITY AND INTERACTIONS WITH CYTOCHROME *P*-450

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(Received 13 April 1981)

Summary—A review of studies on safrole metabolism shows that the compound gives rise to a large number of metabolites by two major pathways, oxidation of the allyl side chain and oxidation of the methylenedioxy group with subsequent cleavage to form a catechol. The mechanism by which safrole exerts the weak hepatocarcinogenicity that has been demonstrated in rats and mice is considered on the basis of published work and recent studies by the authors. Metabolic conversion of the allyl group gives rise to intermediates capable of covalent binding with DNA and protein, and recent findings are compatible with conversion of the methylenedioxy group to a carbene, which forms ligand complexes with the haem moiety of cytochromes *P*-450 and *P*-448. It is suggested that while the allyl group is responsible for the mutagenic potential of safrole, the methylenedioxy moiety may be associated with epigenetic aspects of carcinogenicity.

Introduction

Safrole (4-allyl-1,2-methylenedioxybenzene) is a natural plant constituent, found in oil of sassafras and certain other essential oils (Arctander, 1960; Fishbein & Falk, 1969; Friedman & Shibko, 1969; Guenther, 1948–1952). It is a member of the methylenedioxybenzene group of compounds, many of which (e.g. piperonyl butoxide) are extensively used as insecticide synergists.

A major source of human exposure to safrole is through consumption of spices, such as nutmeg, cinnamon and black pepper, in which safrole is a constituent (*Fenaroli's Handbook of Flavor Ingredients*, 1971; Friedman & Shibko, 1969; Synerholm & Hartzell, 1945; Weil, 1965). Safrole is also present in root beer, and has been used as an additive in chewing gum, toothpaste, soaps and certain pharmaceutical preparations (Fishbein & Falk, 1969).

Safrole is a weak hepatocarcinogen (Homburger, Kelley, Friedler & Russfield, 1961; Long, Nelson, Fitzhugh & Hansen, 1963) and it is a matter of considerable interest whether the allyl moiety or the methylenedioxy group, or both, are involved in the mechanism of its carcinogenesis.

Metabolism

The metabolism of safrole both *in vivo* and *in vitro*, using hepatic homogenates and cell cultures, has been the subject of many studies (Borchert, Wislocki, Miller & Miller, 1973b; Janiaud, Delaforge, Levi, Maume & Padieu, 1977; Stillwell, Carman, Bell & Horning, 1974), and a single study dealing with its metabolism in humans has also been reported (Benedetti, Malnoë & Broillet, 1977). Safrole is extensively metabolized, giving rise to a large number of metabolites. Metabolism involves essentially two major routes, oxidation of the allyl side chain, and oxidation of the methylenedioxy group with subsequent cleavage to form the catechol (Fig. 1).

Oxidation of the allyl chain

Safrole undergoes oxidation of the allylic group to yield the 2',3'-epoxide (safrole epoxide) in both rat and guinea-pig (Janiaud *et al.* 1977; Stillwell *et al.* 1974). This epoxide is only a minor metabolite, possibly because of its slow rate of formation, or because of its further metabolism by epoxide hydratase to the corresponding dihydrodiol (Delaforge, Janiaud, Chessebeuf, Padieu & Maume, 1976; Delaforge, Janiaud, Levi & Morizot, 1980c). The dihydrodiol is one of the metabolites of safrole in rats and guinea-pigs, and presumably arises from the hydration of the 2',3'-epoxide, as administration of the epoxide to these species resulted in excretion of the dihydrodiol in the urine (Borchert *et al.* 1973b; Delaforge *et al.* 1980c; Stillwell *et al.* 1974); the unchanged epoxide was also excreted in the urine, demonstrating its relative stability. Furthermore, the epoxide was detected in the liver microsomes of rats pretreated with safrole and in rat hepatocytes incubated with safrole (Delaforge, Janiaud, Maume & Padieu, 1978).

It is believed that the carcinogenicity of safrole is at least partly mediated through its metabolite, 1'-hydroxysafrole. This metabolite has been detected in the liver, urine and bile of animals, and is also found in the urine conjugated with glucuronic acid (Borchert, Miller, Miller & Shires, 1973a; Borchert *et al.* 1973b; Janiaud *et al.* 1977; Stillwell *et al.* 1974). The 1'-hydroxysafrole forms an ester, when incubated with cytosolic fractions from mouse and rat liver in the presence of 3'-phosphoadenosine-5'-phosphosulphate (Wislocki, Borchert, Miller & Miller, 1976). These workers also reported that alkaline digestion of hepatic protein, isolated from animals treated with 1'-hydroxysafrole, released a metabolite which appeared to be 3'-methylmercaptoisosafrole, indicating the further reaction of the hydroxylated metabolite with tissue *S*-proteins or with glutathione. 1'-Hydroxysafrole, like its parent compound, safrole, undergoes oxidation of the allyl group to yield 1'-hydroxy-2',3'-epoxide (Wis-

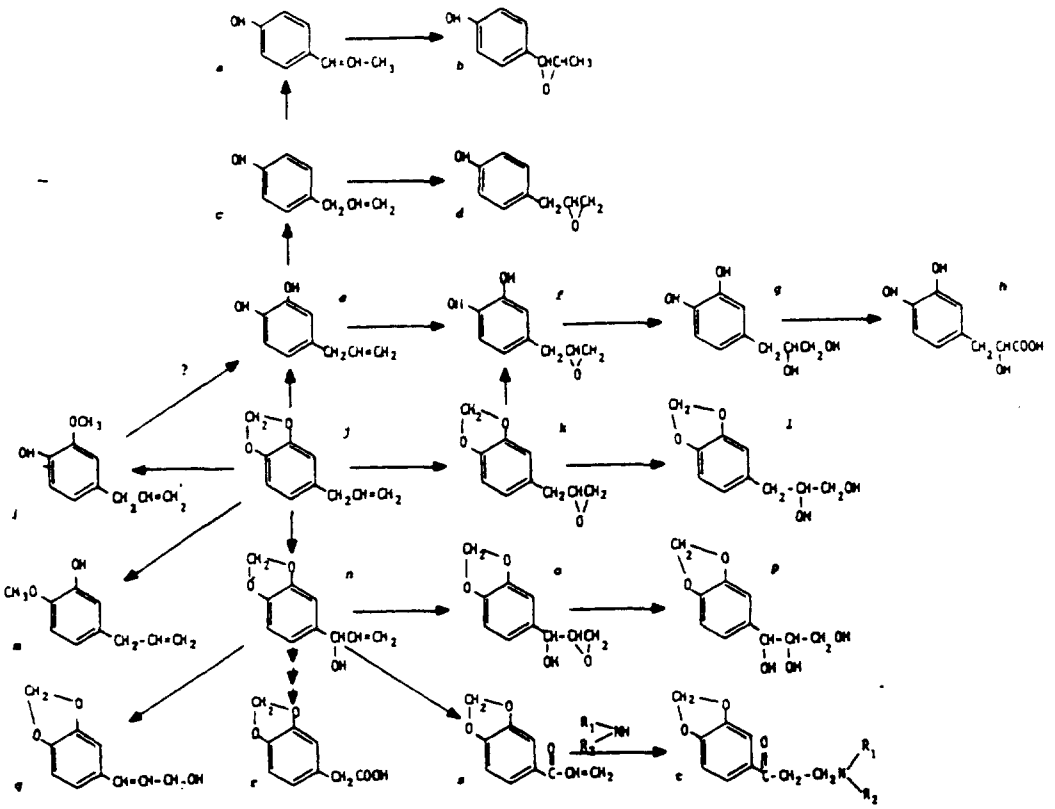

Fig. 1. Major routes of safrole metabolism: (a) propen-1'-ylphenol; (b) 1',2'-epoxypropylphenol; (c) allylphenol; (d) 2',3'-epoxypropylphenol; (e) allylcatechol; (f) 2',3'-epoxypropylcatechol; (g) 2',3'-dihydroxypropylcatechol; (h) 2'-hydroxy-3'-(3,4-dihydroxyphenyl)propanoic acid; (i) eugenol—(4-allyl-2-methoxyphenol); (j) safrole—(4-allyl-1,2-methylenedioxybenzene); (k) safrole epoxide; (l) dihydroxysafrole; (m) 1-methoxy-2-hydroxy-4-allylbenzene; (n) 1'-hydroxysafrole; (o) 1'-hydroxy-2',3'-epoxysafrole; (p) trihydroxysafrole; (q) 3'-hydroxysafrole; (r) 1,2-methylenedioxybenzene-4'-acetic acid; (s) 1'-oxosafrole; (t) 3'-dialkylamino-2',3'-dihydro-1'-oxosafrole.

lock) *et al.* 1976), and incubation of 1'-hydroxysafrole with mouse- and rat-liver microsomal preparations and NADPH generated the hydroxy-epoxide. However, free 1'-hydroxyepoxysafrole has not been detected *in vitro*, although the glucuronic acid conjugate has been detected by gas-liquid chromatography–mass spectrometry of its trimethylsilyl derivative in the urine of animals dosed with safrole (Levi, Janiaud, Delaforge, Morizot, Maume & Padieu, 1977). This hydroxy-epoxide also serves as a substrate of epoxide hydratase, being converted to the trihydroxysafrole (Delaforge *et al.* 1976; Stillwell *et al.* 1974).

Administration of safrole or 1'-hydroxysafrole to animals or man gave rise to the excretion of its isomer, 3'-hydroxysafrole (Benedetti *et al.* 1977; Janiaud *et al.* 1977; Peele & Oswald, 1978), which is believed to result from rearrangement of the 1'-hydroxysafrole during enzymic hydrolysis with β-glucuronidase (Borchert, Miller & Miller, 1971). Other metabolites of 1'-hydroxysafrole include 3,4-methylenedioxyphenyl vinyl ketone, also known as 1'-oxosafrole (Peele & Oswald, 1978), and the formation of this metabolite was also indicated by the excretion of small amounts of adducts of oxosafrole with secondary amines (McKinney, Oswald, Fishbein & Walker, 1972; Oswald, Fishbein & Corbett, 1969; Oswald, Fishbein, Corbett & Walker, 1971).

Oxidation of the methylenedioxy group

The principal route of metabolism of safrole is through cleavage of the methylenedioxy group, the major metabolites being allylcatechol and its isomer, propenylcatechol. Eugenol and its isomer 1-methoxy-2-hydroxy-4-allylbenzene have been detected as minor metabolites in the rat, mouse and man (Benedetti *et al.* 1977; Janiaud *et al.* 1977; Stillwell *et al.* 1974). The intact allyl side chain of allylcatechol may be oxidized to yield 2',3'-epoxypropylcatechol, which serves as a substrate for epoxide hydratase and is hydrated to 2',3'-dihydroxypropylcatechol; this in turn is oxidized to the corresponding propanoic acid (Delaforge *et al.* 1976; Stillwell *et al.* 1974). The epoxide of allylcatechol may also be generated from the cleavage of the methylenedioxy group of the safrole epoxide. Using sensitive techniques, such as high-resolution capillary columns, 1',2'-epoxypropylphenol and its isomer 2',3'-epoxypropylphenol, as well as 2',3'-epoxypropylcatechol, have been detected as minor metabolites in rat urine (Delaforge *et al.* 1976). These epoxides are

poor substrates for epoxide hydratase. Finally the acids, methylene-3,4-dioxyphenylacetic acid and 3,4-dihydroxyphenylacetic acid, have been detected in the urine and bile of animals treated with safrole.

The cleavage of the methylenedioxy ring and the metabolism of the allyl group involve the hepatic microsomal mixed-function oxidases (Casida, 1970; Hodgson & Philpot, 1974). Administration to rats of the typical inducers of the mixed-function oxidases, phenobarbital and 3-methylcholanthrene, resulted in increased urinary excretion of epoxypropylphenol, epoxysafrole and 1'-hydroxysafrole (Borchert *et al.* 1973b; Janiaud *et al.* 1977), and phenobarbital stimulated the production of other epoxy and hydroxy derivatives and the conjugation of metabolites with glucuronic acid (Janiaud *et al.* 1977).

Carcinogenicity of safrole

The carcinogenic properties of safrole have been the subject of several studies and the compound is described as a weak hepatocarcinogen in both mice and rats (Borchert *et al.* 1973a; Epstein, Fujii, Andrea & Mantel, 1970; Hagan, Jenner, Jones, Fitzhugh, Long, Brouwer & Webb, 1965; Homburger, Kelley, Baker & Russfield, 1962; Homburger *et al.* 1961; Long, Hansen & Nelson, 1961; Parke & Gray, 1978). When fed to rats for 2 yr, a diet containing less than 1000 ppm safrole caused minimal hepatic damage, malignant changes being evident only at higher doses (Hagan *et al.* 1965; Long *et al.* 1963). The isomer, isosafrole, and dihydrosafrole exhibited even weaker hepatocarcinogenicity (Hagan *et al.* 1965); however, the latter compound fed for long periods to rats at a dose of 5000 ppm in the diet produced benign and malignant oesophageal tumours. Short-term administration of high doses of all three compounds resulted in extensive gross pathological damage (Taylor, Jenner & Jones, 1964). Liver is not the only tissue damaged by safrole; lymphomas and adenomas of the lung have also been described (Taylor *et al.* 1964). Neither allylbenzene nor methylenedioxybenzene show any significant toxicity when compared with safrole (Hagan *et al.* 1965), indicating that both the methylenedioxybenzene group and the allyl side chain are concerned in the manifestation of safrole toxicity.

The monohydroxylated derivative of safrole, 1'-hydroxysafrole, is more hepatotoxic and hepatocarcinogenic to animals than is the parent compound when fed at the same dietary level, indicating that it may act as a proximate carcinogen of safrole (Borchert *et al.* 1973a,b; Wislocki *et al.* 1976; Wislocki, Miller, Miller, McCoy & Rosenkranz, 1977). When administered to rats, 1'-hydroxysafrole labelled with tritium gives rise to tritium-labelled DNA, RNA and protein, indicating the covalent binding of the compound or of a further metabolite. Furthermore, application of the electrophilic 1'-hydroxysafrole epoxide, derived from 1'-hydroxysafrole, to mouse skin followed by repeated applications of the tumour promoter, croton oil, resulted in the formation of skin papillomas (Wislocki *et al.* 1977). The reactivity of 1'-acetoxysafrole with nucleosides and with methionine led to speculation that this might also act as an ultimate carcinogen (Borchert *et al.* 1973b; Wislocki *et al.* 1976), and when injected into newborn mice this compound gave rise to liver tumours, similar to those induced by 1'-hydroxysafrole (Borchert *et al.* 1973a). However, this metabolite could not be detected following incubation of 1'-hydroxysafrole with microsomes or cytosol from rat or mouse liver in the presence of acetyl-CoA (Wislocki *et al.* 1976). Another possible ultimate carcinogen, 1'-oxosafrole, did not result in carcinogenicity when administered orally to rats (Wislocki *et al.* 1977).

The bacterial system devised by Ames (Ames, McCann & Yamasaki, 1975) has been used extensively in studies to establish the identity of the ultimate carcinogen(s) of safrole. With the standard test system, safrole does not give a positive mutagenic response (McCann, Choi, Yamasaki & Ames, 1975; Swanson, Chambliss, Blomquist, Miller & Miller, 1979; Wislocki *et al.* 1977). However, when safrole was pre-incubated with liver microsomes from 3-methylcholanthrene-treated animals, a positive mutagenic response was obtained (Dorange, Janiaud, Delaforge, Levi & Padieu, 1978), although these findings could not be reproduced by other workers (Swanson *et al.* 1979). Green & Savage (1978) obtained a positive mutagenic response for safrole in the Ames test using other bacterial strains and a mouse-liver microsomal preparation, and also in a host-mediated system *in vivo*.

1'-Hydroxysafrole is more mutagenic than safrole but is still a relatively weak mutagen. Its mutagenicity was increased in the presence of an activating system, indicating that it is metabolized, at least partly, to more potent mutagen(s) (Swanson *et al.* 1979). However, other workers reported no positive mutagenic response either in the presence or absence of an activation system (Dorange, Delaforge, Janiaud & Padieu, 1977; McCann *et al.* 1975; Wislocki *et al.* 1977). The low mutagenicities of safrole and its 1'-hydroxy derivative probably reflect the low rates of metabolism observed *in vitro* with hepatic microsomes (Wislocki *et al.* 1976). In contrast, all of the epoxides of safrole that were investigated were found to be mutagenic in the Ames test, in the absence of an activation system (Dorange *et al.* 1977; Swanson *et al.* 1979; Wislocki *et al.* 1977). 1'-Acetoxysafrole, a postulated ultimate carcinogen, was also directly mutagenic in the Ames test (McCann *et al.* 1975; Wislocki *et al.* 1977) but no significant mutagenic response was obtained from dihydrosafrole, 3'-hydroxyisosafrole, 3'-acetoxyisosafrole and 1'-oxosafrole, with or without activation (Wislocki *et al.* 1977).

Interactions of safrole with cytochrome *P*-450

Safrole, as a substrate of the hepatic microsomal mixed-function oxidases, interacts with cytochrome *P*-450, giving rise to a type I spectral change (Franklin, 1971). In the presence of either NADPH and oxygen, or cumene hydroperoxide, it is transformed into a reactive intermediate which interacts with the sixth ligand of ferri- and ferrocytochrome *P*-450 to yield characteristic complexes (Elcombe, Bridges, Gray, Nimmo-Smith & Netter, 1975; Franklin, 1971 & 1976; Kulkarni & Hodgson, 1978; Parke & Rahman, 1971). The complex formed with reduced cytochrome

P-450 exhibits absorption maxima at 427 and 455 nm, while only one absorption maximum at 437 nm is observed with the oxidized cytochrome (Elcombe *et al.* 1975; Gray & Parke, 1973; Hodgson & Philpot, 1974; Lake & Parke, 1972). Safrole does not form the complex when incubated with microsomes plus dithionite or NADH, indicating that the ligand is formed by a reactive intermediate of safrole and not by safrole itself (Elcombe *et al.* 1975). The formation of the complex *in vivo* has also been shown to occur in rats and mice following administration of safrole (Delaforge, Ioannides & Parke, 1980a; Fennell, Sweatman & Bridges, 1980; Parke & Rahman, 1971).

The nature of the reactive safrole intermediates remains unclear, but it is generally believed that the complex is formed between the cytochrome and a carbene generated from the hydroxylation of the methylenedioxy group with subsequent loss of water (Mansuy, Battioni, Chottard & Ullrich, 1979; Nastainczyk, Ullrich & Sies, 1978; Ullrich, Nastainczyk & Ruff, 1975). Carbenes are known to have a high affinity for reduced cytochrome *P*-450 (Mansuy, Nastainczyk & Ullrich, 1974). Other possible reactive intermediates for safrole have been suggested, such as carbanions formed by removal of a proton from the methylene group (Ullrich & Schnabel, 1973), radicals generated following the homolytic scission of the methylenedioxy group (Hansch, 1968), and benzodioxolium ions produced following the loss of a hydride ion from the methylenedioxy bridge (Hennessy, 1965).

Experimental evidence for the involvement of hydroxyl radicals in the formation of the ligand complex of safrole and cytochrome P-450

The formation of the reactive intermediate that gives rise to the formation of the safrole–cytochrome *P*-450 ligand complex may involve hydroxylation by cytochrome *P*-450 or interaction with hydroxyl or other radicals.

Recent work has demonstrated that NADPH-cytochrome *P*-450 reductase can generate hydroxyl radicals (Lai, Grover & Piette, 1979). To investigate the role of hydroxyl radicals in the formation of the safrole ligand complex, we incubated hepatic microsomes with NADPH and safrole in the presence of scavengers of the active forms of oxygen. In addition to the study of the effects on safrole, the microsomal oxidation of methional to ethylene, a reaction effected by hydroxyl radicals, was also investigated.

Male Wistar albino rats (150–200 g) received a daily intraperitoneal administration of phenobarbital (80 mg/kg) for 3 days and were killed 24 hr after the last administration. Hepatic microsomal preparations were prepared as previously described (Ioannides & Parke, 1975). To study the generation of the ligand complex *in vitro*, microsomal fractions (1 mg protein/ml) were incubated with safrole (0·04–0·5 mM) and various oxygen scavengers at concentrations shown in Table 1 (with the data obtained with 0·3 mM-safrole). Following addition of NADPH (0·5 mM), the formation of the ligand complex was monitored for 2 min by measuring the optical difference between 455 and 490 nm using a double-beam dual-wavelength recording spectrophotometer.

The antioxidants butylated hydroxytoluene (BHT) and ascorbic acid inhibited the formation of the safrole complex with cytochrome *P*-450, suggesting the requirement for oxidation in the formation of the complex. The hydroxyl-radical scavengers mannitol, dimethylsulphoxide and methional inhibited the formation of the ligand complex by 30% (Table 1). Lineweaver-Burk presentations, such as that for methional in Fig. 2, showed that with these four compounds inhibition was likely to be of the competitive type. In contrast, the inhibition by catalase and superoxide dismutase was uncompetitive, indicating that these do not compete directly with safrole for hydrogen peroxide. It is possible that catalase, by removing H_2O_2, prevents its further conversion to hydroxyl radicals in the presence of ferrous ions (Walling, Partch & Weil, 1975).

The involvement of the OH· radical in the formation of the ligand complex was further indicated by the decreased evolution of ethylene from methional in the presence of safrole. Aliquots of a liver-microsomal preparation from phenobarbital-treated rats (3 mg protein/ml 0·1 M-phosphate buffer, pH 7·4) were incubated with an NADPH-generating system (2 μmol NADP, 20 μmol glucose 6-phosphate and 2 units glucose 6-phosphate dehydrogenase) in rubber-sealed tubes and the reaction was started by addition of methional (0–0·4 mM). Ethylene evolution was determined by removing 1-ml aliquots and injecting them into a Packard 409 gas chromatograph equipped with

Table 1. *Effect of oxygen scavengers on the formation of the safrole complex with cytochrome* P-450 *in rats pretreated with phenobarbital*

Oxygen scavenger	Concentration	Complex formation (% of control)
Butylated hydroxytoluene	60 μm	55
Ascorbic acid	600 μm	68
Dimethylsulphoxide	10 mM	70
Mannitol	6 mM	71
Methional	600 μm	66
Catalase	3000 IU	72
Superoxide dismutase	500 IU	80

Incubations were carried out at a safrole concentration of 0·3 mM. Reactions were started by addition of NADPH (0·5 mM). Control incubations contained 0·3 mM safrole and no oxygen scavenger.

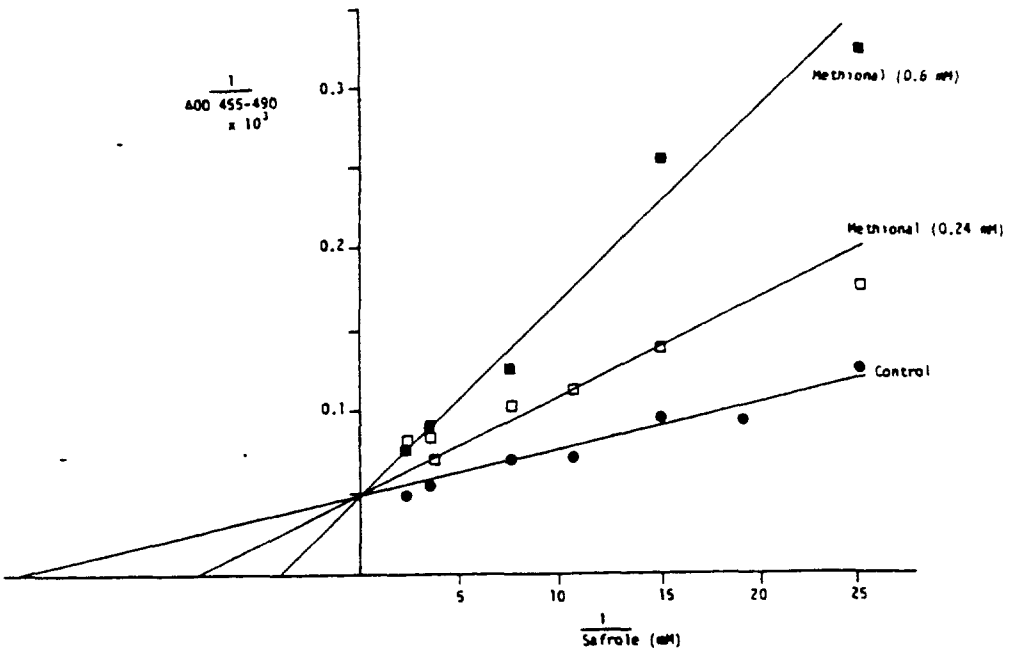

Fig. 2. Liver microsomes from phenobarbital-treated rats (1 mg protein/ml) were incubated with safrole (0·04–0·5 mM). The reaction was commenced by addition of NADPH (0·5 mM) and the initial velocity of the ligand complex formation was monitored for 2 min.

a 2 m × 2 mm glass column packed with Carbosieve B (60-80 mesh). Analysis was carried out isothermally at 170°C using a flame ionization detector and helium or oxygen-free nitrogen as carrier gas (25 ml/min); air and hydrogen flows were 300 and 30 ml/min respectively, and the chromatograph was operated with an injection temperature of 200°C and detection temperature of 250°C; under these conditions ethylene had a retention time of 2·2 min, and the response was linear from 0·5 pmol to at least 200 pmol/ml. Safrole at concentrations of 2 and 5 mM inhibited, possibly competitively, the release of ethylene from methional, confirming the involvement of the OH· radical in the metabolism of safrole (Fig. 3). This involvement of the OH· radical in the formation of a ligand complex between safrole and cytochrome *P*-450 is compatible with the formation of a carbene (Fig. 4).

Inhibition of mixed-function oxidase activity by safrole

The formation of the safrole carbene complex is accompanied by a decrease in the concentration of free cytochrome *P*-450 and by a concomitant loss of mixed-function oxidase activity (Anders, 1968; Franklin, 1972; Parke & Rahman, 1971). The characteristic ligand complex of reduced cytochrome *P*-450 and carbon monoxide, with the absorption maximum at 450 nm, is decreased, showing that carbon monoxide cannot displace the carbene from the sixth ligand of the haem iron. An inverse linear relationship ($r = 0{\cdot}96$) exists between the ratio of free and total cytochrome *P*-450 concentrations, and the ratio of carbene-bound and total cytochrome *P*-450 concentrations *in vivo* when safrole was administered to the rat (Delaforge *et al.* 1980a). A similar relationship was reported by the same workers for the formation of the complex *in vitro*.

In the presence of some type I and reverse type I substrates, but not of type II substrates (which ligand to the haem moiety), the ferricytochrome carbene complex dissociates, resulting in removal of the 455-nm peak and restoration of the catalytic activity of the cytochrome (Delaforge, Ioannides & Parke, 1980b; Dickins, Elcombe, Moloney, Netter & Bridges, 1979; Elcombe *et al.* 1975; Elcombe, Bridges & Nimmo-Smith, 1976; Gray & Parke, 1973; Ullrich, 1977), presumably because of removal of the haem-bound safrole metabolite. The extent of the increase in mixed-function oxidase following displacement is dependent on the amount of complex formed and the displacing conditions (Elcombe, Dickins, Sweatman & Bridges, 1977). Furthermore, when the substrates that act as displacers are incubated with microsomal preparations isolated from animals pretreated with safrole or isosafrole, only very weak binding spectra with oxidized cytochrome *P*-450 are exhibited, but these undergo a time-dependent intensification resulting from the displacement of the safrole complex (Gray & Parke, 1973). Displacement occurs anaerobically and does not involve competition for the type I binding site, since the carbene ligands to the haem iron and is not bound to the apoprotein. However, as no type II substrates act as displacers, the dissociation of the complex may be initiated by the breaking of some additional linkage of the carbene with the apoprotein, possibly involving the allyl side chain. Alternatively, the interaction of the displacing sub-

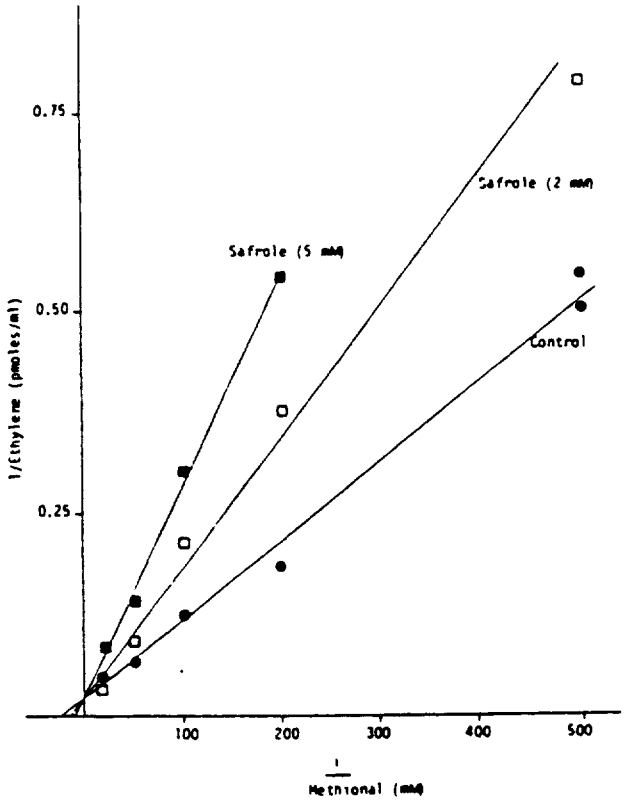

Fig. 3. Incubation mixtures comprised 10 ml liver microsomes from phenobarbital-treated rats (3 mg/ml), and methional (0·002–0·4 mM). The reaction was commenced by addition of an NADPH-generating system (2 μmol NADP, 20 μmol glucose 6-phosphate and 2 units glucose-6-phosphate dehydrogenase). Evolution of ethylene was determined by GLC as described.

strate with the type I site may lead to a conformational change of the cytochrome, resulting in the loss of the 'safrole' ligand and conversion of the cytochrome to the high-spin state.

In our studies (unpublished data, 1981), we have found that the carcinogen benzo[*a*]pyrene can also act as a displacer to remove the carbene and restore mixed-function oxidase activity. The ability of benzo[*a*]pyrene to displace the carbene, and then of the free cytochrome to convert the benzo[*a*]pyrene to mutagenic intermediates was investigated in safrole-treated rats. Mutagenicity was determined by the Ames test (Ames *et al.* 1975) using *Salmonella typhimurium* strain TA98. The activation system was prepared with microsomes from safrole-pretreated rats (single daily intraperitoneal injections of 150 mg/kg for 3 days, the animals being killed 24 hr after the last administration). Displacement of the carbene was achieved by pre-incubation of the microsomes with benzo[*a*]pyrene (2 μg) for 15 min prior to addition of the NADPH-generating system to initiate metabolism. Following pre-incubation, the number of his-

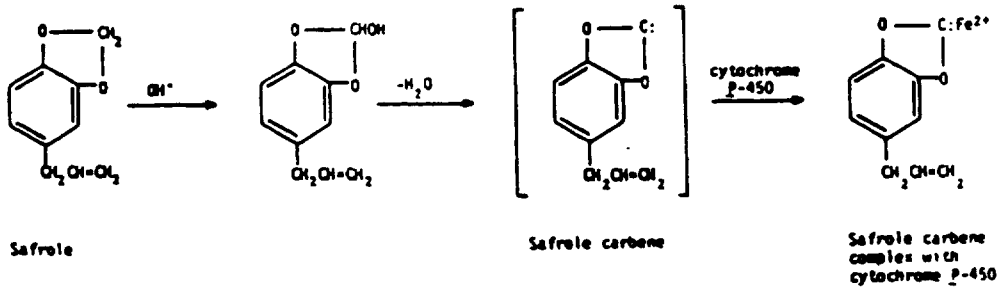

Fig. 4. Possible route of formation of a safrole carbene–cytochrome *P*-450 complex.

tidine revertants was more than doubled (from 128 to 288/plate, the number in the absence of any activation system being 47/plate), demonstrating that the function of the cytochrome was restored when the ligand complex was dissociated. When biphenyl, a non-mutagen, was used as the displacing agent and no benzo[*a*]pyrene was added, no mutagenic response was observed, demonstrating that the displaced carbene itself did not elicit the mutagenic response. Previous workers have failed to displace the safrole carbene with benzol[*a*]pyrene (Elcombe *et al.* 1977).

Dissociation of the safrole carbene ligand complex results spontaneously in the replacement of the 455 nm absorption maximum with a 450 nm peak, resembling the CO-difference spectrum with cytochrome *P*-450 (Delaforge & Coon, 1981; Hodgson & Philpot, 1974; Hodgson, Philpot, Baker & Mailman, 1973; Kulkarni & Hodgson, 1978). The presence of this CO was initially attributed to endogenous breakdown of haem (Schmid, 1973), but recent evidence suggests that it is generated from the methylenic carbon during the metabolism of methylenedioxyphenyl compounds by the cytochrome *P*-450 enzyme system (Yu, Wilkinson & Anders, 1980). Certain methylenedioxy compounds that generate CO do not elicit a 455 nm absorption maximum, showing that the carbene and CO are formed *via* different pathways, perhaps from a common unstable intermediate (Yu *et al.* 1980). The formation of CO subsequent to displacement of the safrole moiety from the ligand complex, indicates that the C atom of the methylenedioxy moiety is still intact, confirming the involvement of a carbene and indicating that the product of the displacement is likely to be an allylcatechol. The displaced product may then bind covalently to cytochrome *P*-450, inhibiting further formation of the ligand complex (Delaforge & Coon, 1981).

Ligand complex formation with cytochromes *P*-450 and *P*-448

Cytochrome *P*-450 exists in several forms, differing in their spectral, immunological and electrophoretic properties as well as in their structures and substrate specificity (Guengerich, 1979). One of these forms, namely, cytochrome *P*-448, predominates in foetal and neonatal rat liver and in malignant tissue, and is formed by the treatment of animals with hepatocarcinogens, including safrole and isosafrole (Parke, 1981). Cytochromes *P*-450 and *P*-448 form ligand complexes with safrole both *in vivo* and *in vitro*, showing that they can convert safrole to the carbene and then interact with it (Delaforge & Coon, 1981; Delaforge *et al.* 1980a). However, the safrole carbene complexes formed show different stability. The absorption maximum at 455 nm is formed less readily with cytochrome *P*-448, indicating that either the rate of generation of the carbene, or its interaction to form the complex, is slower with cytochrome *P*-448 than with cytochrome *P*-450. Furthermore, dissociation by biphenyl of the safrole complex formed *in vivo* in animals pretreated with the cytochrome *P*-450 inducer, phenobarbital, resulted in a large increase (150%) in the cytochrome *P*-450-mediated 4-hydroxylation of biphenyl but in only a small increase (20%) in the cytochrome *P*-448-mediated 2-hydroxylation of biphenyl (Delaforge *et al.* 1980b). In contrast, dissociation of the safrole complex formed in animals pretreated with the cytochrome *P*-448 inducer, 3-methylcholanthrene, resulted in a small increase (10%) in the 4-hydroxylation of biphenyl with no significant increase in the 2-hydroxylation, indicating that displacement of the ligand from the cytochrome *P*-448 form is less facile than displacement from the cytochrome *P*-450 complex. Inhibition of the mixed-function oxidases by piperonyl butoxide, another methylenedioxy compound, was more marked in animals pretreated with phenobarbital than in those treated with 3-methylcholanthrene, indicating that the piperonyl butoxide carbene also interacted more readily with cytochrome *P*-450 to form a complex (Anders, 1968; Franklin, 1972). Similar findings have been reported for SKF-525A and amphetamine ligand complexes (Buening & Franklin, 1974; Franklin, 1974).

The ligand complex formed with cytochrome *P*-450 is metastable, 50% being destroyed within 6 hr following safrole administration to rats (Delaforge *et al.* 1980a). In contrast, the complex with cytochrome *P*-448, although more slowly formed, is more stable, no degradation being evident in the first 6 hr. If indeed CO generation is directly related to the ligand complex formation (Yu *et al.* 1980) these findings may explain why rat-liver microsomes induced with phenobarbital, but not those induced with 3-methylcholanthrene, lead to CO production on dissociation of the safrole carbene complex.

A novel haemoprotein formed by safrole and isosafrole

Administration of safrole to animals leads initially to a marked inhibition of the mixed-function oxidases (Anders, 1968; Fujii, Jaffe, Bishop, Arnold, Mackintosh & Epstein, 1970; Hodgson & Casida, 1961; Nakatsugawa & Dahm, 1967). The inhibition is achieved through two different mechanisms, on the one hand competitive inhibition by safrole itself of the substrate for metabolism, and on the other formation of the safrole carbene ligand complex, preventing the ready hydroxylation of the substrate.

However, like many inhibitors, safrole and isosafrole also stimulate the synthesis of new enzymic protein and act as potent inducers of the mixed-function oxidase system (Lotlikar & Wasserman, 1972; Parke & Rahman, 1970). The pattern of induction has characteristics of both the barbiturate and the polycyclic aromatic hydrocarbon inducers (Fennell *et al.* 1980; Gray, Parke, Grasso & Crampton, 1972; Lake & Parke, 1972; Wagstaff & Short, 1971). Administration of a safrole-containing diet (0·25%) to rats resulted in induction of hepatic cytochrome *P*-450 and cytochrome *P*-448 activities within 7 days (Parke & Gray, 1978). Extrahepatic mixed-function oxidase activities, such as those of the small intestine and kidney, were also induced by isosafrole (Lake, Hopkins, Chakraborty, Bridges & Parke, 1973). The full extent of the inducibility of the mixed-function oxidases is only evident when the carbene ligand complex with cytochrome *P*-450 is dissociated to release the free cytochrome. In rats pretreated with safrole, there were

increases in both cytochrome *P*-450 and *P*-448 activities following dissociation of the ligand complexes with biphenyl (Delaforge *et al.* 1980b). These observations indicate that safrole induces a haemoprotein having overlapping activities of cytochrome *P*-450 and cytochrome *P*-448, or may induce a mixture of the two. Indeed, using SDS-disc gel electrophoresis, it has been demonstrated that isosafrole induces a novel haemoprotein, which is distinct from those induced by phenobarbital or by 3-methylcholanthrene but which may be similar to the minor protein band induced by 3-methylcholanthrene (Fennell, Dickins & Bridges, 1979). Further work has led to the isolation from isosafrole-pretreated rats, and the purification of a unique form of hepatic cytochrome *P*-450 existing in the form of an isosafrole metabolite complex (Ryan, Thomas & Levin, 1980). The cytochrome *P*-448 induced by 3-methylcholanthrene has also been shown to contain a bound moiety of the inducing agent (Schenkman, Greim, Zange & Remmer, 1969).

Conclusions

This ligand complexing and covalent binding of reactive intermediates with the haem and protein moieties, respectively, of cytochrome *P*-450, may be fundamental to the mechanism of carcinogenesis. The structural and functional properties of the cytochrome are substantially changed by this binding which, since ribosomes are attached to the endoplasmic reticulum at cytochrome *P*-450, may be associated with the loss of ribosomes known to occur following treatment with safrole or other carcinogens (Parke, 1981). Chemical carcinogenesis is known to be associated with increases in hepatic cytochrome *P*-448 and simultaneous loss of cytochrome *P*-450 activity (Parke, 1981) and could, through loss of ribosomes, result in impairment of glycoprotein synthesis and so contribute to the process of malignant transformation by epigenetic mechanisms (Parke, 1981). Hence, of the two functional groups of safrole, the allyl group has been shown to be associated with mutagenicity, and the methylenedioxy moiety to be associated with changes to cytochrome *P*-450 and possibly with epigenetic aspects of carcinogenicity.

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