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STUDIES ON THE INTERACTION OF SAFROLE WITH RAT HEPATIC MICROSOMES

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Abstract—(1) Similar to previous results with methylenedioxyphenyl compounds microsomes from safrole pretreated rats showed, on reduction with NADH, NADPH or $Na_2S_2O_4$, characteristic absorption maxima at 427 and 455 nm. The same spectrum can be obtained after incubation *in vitro* of control microsomes with safrole, NADPH and oxygen. (2) Subsequent addition of carbon monoxide to microsomes of safrole pretreated rats causes an absorption maximum at 448 nm, characteristic of the 3-methylcholanthrene type of induction of microsomal hydroxylase protein. (3) The suspected cytochrome P-450–safrole metabolite complex, which can be visualized only in the reduced state of cytochrome P-450, is very stable as witnessed by its preservation through the preparation procedure for microsomes or after dialysis or detergent treatment. However, when safrole or ethylbenzene is added, both absorption maxima decrease in a time dependent manner. This can be measured for each time point after complete reduction of the microsomal preparation by adding $Na_2S_2O_4$. (4) From this it is concluded that the carcinogen safrole leads to the biosynthesis of a 3-methylcholanthrene type cytochrome P-450 and formation of a safrole metabolite–cytochrome P-450 complex which in turn can be cleaved *in vitro* by safrole or other lipophilic compounds.

Safrole (4-allyl-1,2-methylenedioxybenzene) occurs in many essential oils and flavours. It is a hepatocarcinogen [1] and an inducer of hepatic microsomal drug metabolizing enzymes, including biphenyl hydroxylase, benzpyrene hydroxylase [2, 3], glucuronyl transferase [2], and acetamidofluorene 3- and 5-hydroxylase [4]. This pattern of induction is similar to that obtained with 3-methylcholanthrene [5].

On the other hand, methylenedioxyphenyl compounds are also potent inhibitors of mixed function oxidation, both *in vivo* and *in vitro*, for they inhibit the *in vitro* metabolism of parathion [6], carbamate [7], ethylmorphine, *p*-nitroanisole and aniline [8], and *in vivo* they prolong hexobarbital narcosis and zoxazolamine paralysis (Anders [8] and Fujii *et al.* [9]).

The mechanism of their inhibition of microsomal xenobiotic metabolism has been variously reported to be competitive [8–11], partially competitive, curvilinear, and noncompetitive [3, 11–13]. These apparent anomalies have been explained by Franklin [13] and Philpot and Hodgson [14] as being due to a dual inhibitor effect involving both competitive inhibition due to competition between the substrate and the methylenedioxyphenyl inhibitor for metabolism, and noncompetitive inhibition due to the "essentially irreversible" binding of a methylene dioxyphenyl metabolite to reduced cytochrome P-450. The characteristics of this metabolite binding have been well documented for piperonyl butoxide [14–16]. It is manifested optically in its interaction with reduced cytochrome P-450 by absorption maxima at 427 and 455 nm. A similar interaction of safrole and isosafrole has been demonstrated after administration *in vivo* to rats [15, 17].

These observations demonstrate that methylenedioxyphenyl compounds can have a high reactivity with hepatic microsomes. The present paper indicates a complex effect of safrole on hepatic microsomal drug metabolism, involving both the induction *in vivo* of a "P-448" type of cytochrome, similar to that induced by 3-methylcholanthrene, and also a strong interaction of a safrole metabolite with cytochrome P-450. Furthermoe, this work indicates that the safrole metabolite–cytochrome P-450 complex, under certain conditions, is not as stable as previously supposed [15, 16], because it can be dissociated by type I substrates such as ethylbenzene or safrole itself.

MATERIALS AND METHODS

Chemicals. Glucose-6-phosphate, glucose-6-phosphate dehydrogenase, NADP, NADPH, NADH and glutamate dehydrogenase were obtained from Boehringer–Mannheim (Mannheim, Germany). Safrole was purchased from Hopkins and Williams Ltd. (Essex, U.K.), and was shown to be 99·8% pure by gas–liquid chromatography. All other reagents were from either Merck (Darmstadt, Germany) or BDH (Poole, Dorset, U.K.).

Animal pretreatments. Male Sprague–Dawley rats of approximately 200 g were used. Phenobarbital stimulated microsomes were obtained after i.p. injections of sodium phenobarbital (80 mg/kg) in 0·9% saline daily for 3 days prior to sacrifice. Safrole stimulated microsomes were obtained from rats fed safrole (2% w/w) in a powdered standard diet (Altromin®, Altromin, Lage, Germany) for 2 weeks before they

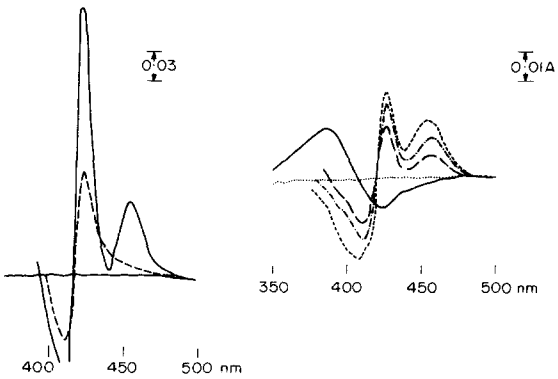

Fig. 1. The 427/455 nm absorptions after formation *in vivo* and *in vitro*. (a) Reduced difference spectra of safrole- and control-microsomes. Safrole- (——) or control- (– – – –) microsomes (2 mg/ml) in 66 mM Tris–HCl buffer (pH 7·4) and dithionite added to sample cuvettes. (b) Formation of 427/455 absorbing species *in vitro* from PB-microsomes. PB-microsomes (1 mg/ml) in 66 mM Tris–HCl buffer (pH 7·4) containing 3·3 mM G-6-P, 2 mM nicotinamide and 0·5 units G6PDH were divided between two cuvettes. 417 μM safrole was added to sample cuvette and the difference spectrum recorded (——). 60 μM NADPH was added to each cuvette and spectrum recorded at 1 (——); 3 (— · — · —) and 5 (– – –) min.

were killed. All animals were allowed water and food *ad lib.*

Microsomal preparations. Animals were killed by cervical dislocation, livers were excised into ice-cold 1·15% KCl and microsomes were prepared as previously described by Netter [18]. Microsomes were finally suspended in 0·25 M sucrose, 5·4 mM EDTA, 20 mM Tris–HCl buffer (pH 7·4) and stored at −20°. Microsomal protein was determined by the method of Lowry *et al.* [19]. All microsomes were used within 2 weeks of preparation.

Spectrophotometry. All spectra were recorded using a Perkin–Elmer 356 dual-beam dual-wavelength spectrophotometer. A temperature-controlled cell holder was used, enabling all measurements to be made at 37°. In all experiments microsomes were suspended in 66 mM Tris–HCl (pH 7·4) buffer to a protein concentration of 1 or 2 mg/ml.

Binding spectra caused by the interaction with oxidized cytochrome P-450 were measured by the method of Schenkman *et al.* [20]. Safrole and potential displacers were dissolved in dimethylformamide (Merck, Spectrosil grade) or water as applicable. Cuvette-volumes were always 3·0 ml.

In order to follow the displacement of the 427/455 nm species by type I agents the time dependent increase of type I binding spectra ($\lambda_{max} \approx 390$; $\lambda_{min} \approx 430$) with safrole-microsomes was measured after the addition of 0·1 mM final concentration of the potential displacer. This was done either in the dual-beam dual-wavelength mode (λ_1 = peak; λ_2 = trough) by recording the increase in absorbance vs time, or in the split-beam mode ($\lambda_1 = \lambda_2$) by repetitive scanning between 500 and 350 nm every 2 min. For measurement of the decrease of the 455 nm absorption during the displacement reaction, the reaction was stopped after various time periods by the addition of a few grains of dithionite to the sample cuvette. Then a spectrum was recorded and the size of the 455 peak determined.

On the other hand, the formation of the 427/455 nm absorbing species from safrole *in vitro* was tested by incubating metabolically active phenobarbital stimulated microsomes with safrole. Phenobarbital microsomes (1 mg/ml) in 66 mM Tris–HCl buffer containing 3·3 mM glucose-6-phosphate, 2 mM nicotinamide and 0·5 units of glucose-6-phosphate dehydrogenase were divided between two cuvettes. Various concentrations of safrole (0–833 μM) were added to the sample cuvette and the difference spectrum recorded. NADPH (60 μM) was then added to each cuvette and the spectrum recorded at various time intervals (1–10 min).

Cytochrome P-450 was measured by the method of Omura and Sato [21].

Changes in NADPH concentrations were measured in the dual beam mode ($\lambda_1 = 390$ nm; $\lambda_2 = 340$ nm) with respect to time. When required, NADPH was removed by the addition of 2·5 mM Na-α-ketoglutarate, 5·0 M NH_4Cl and 1·0 mg of glutamate dehydrogenase [22].

Nomenclature. The term "control-microsomes" refers to microsomes obtained from rats with no pretreatment, "safrole-microsomes" to those from rats pretreated with safrole, and "phenobarbital (PB)-microsomes" obtained from rats pretreated with phenobarbital. "Potential displacer" refers to a compound which was examined for its ability to decrease the magnitude of the 427/455 spectrum.

RESULTS

Solvent effects. Since safrole is almost insoluble in water, it was routinely dissolved in DMF. Control experiments have shown that DMF under the experimental conditions used (less than 10 μl DMF/cuvette) has no displacing effect of its own nor does it modify binding spectra or oxygen consumption.

Reduced difference spectrum of safrole-microsomes. Hepatic microsomes obtained from rats pretreated with safrole (safrole-microsomes) show unusual spectral characteristics when reduced. On recording the difference spectrum when the sample cuvette is reduced by $Na_2S_2O_4$, NADH gr NADPH an absorption maximum at 455 nm, together with a very marked absorption at 427 can be observed (Fig. 1a). Both absorption bands can be reproduced *in vitro* by the incubation of safrole with control- or PB-microsomes, NADPH and O_2 (Fig. 1b). When NADPH is removed, the 427 and 455 nm absorptions disappear (Fig. 2) while on addition of $Na_2S_2O_4$ or NADH, or readdition of NADPH they reappear (Fig. 2). However, safrole will not elicit the 427/455 absorption bands with control-microsomes on addition of either NADH or $Na_2S_2O_4$. These observations indicate that a metabolite or intermediate is involved in the production of the abnormal absorption bands and that the phenomenon is not due to safrole itself interacting with reduced cytochrome P-450. After this metabolite has interacted with cytochrome P-450 the 427/455 bands can be visualized under both reduced anaerobic (i.e. in the presence of $Na_2S_2O_4$) or reduced aerobic conditions (using NADH or NADPH). Similar effects *in vitro* have been reported by Franklin

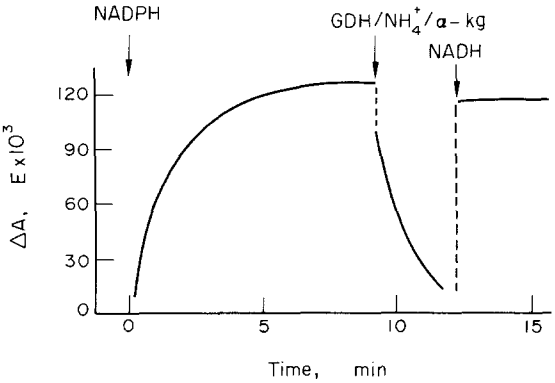

Fig. 2. The ability of NADH to completely reduce the proposed metabolite–cytochrome P-450 complex. PB-microsomes (1 mg/ml) in 66 mM Tris–HCl buffer (pH 7·4) were incubated with 417 μM safrole and 250 μM NADPH at 37°. The formation of the 455 nm absorption was followed in the dual-beam dual-wavelength mode ($\lambda_1 = 490$, $\lambda_2 = 455$). At the maximum of the 455 nm absorption, glutamate dehydrogenase (GDH), ammonium chloride (NH_4^+) and α-ketoglutarate (α-KG) were added as described in Methods. After loss of the 455 nm absorption 2–3 mg of NADH was added.

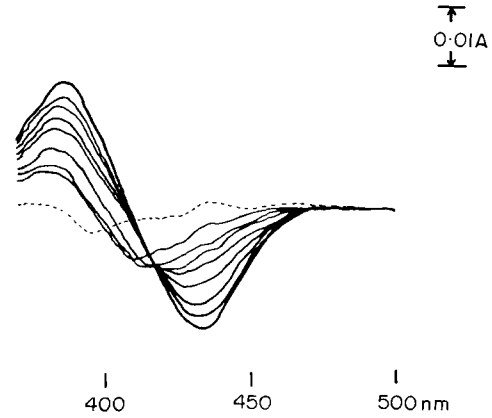

Fig. 4. Time-dependent increase of the safrole (displacer) type I binding spectrum with safrole-microsomes. Safrole-microsomes (1 mg/ml) in 66 mM Tris–HCl buffer (pH 7·4) were divided between two cuvettes and 41·7 μM safrole was added to the sample cuvette. Repetitive scans were obtained between 500 and 350 nm every 2 min for 30 min (only every 2nd scan is shown).

using piperonyl butoxide as substrate. However the ratio of the 427/455 peak height for safrole is far greater than that observed for piperonyl butoxide which may indicate subtle mechanistic differences between these two phenomena.

After generation *in vitro* of the 427/455 species and subsequent removal of the NADP reducing conditions a new peak at 438 nm can be observed in the oxidized spectrum. This is believed to be the spectrum of the oxidized cytochrome P-450–metabolite complex.

Reduced CO-difference spectra. The reduced CO-difference spectrum of control- or PB-microsomes shows the usual absorption maximum at 450 nm, whereas using safrole-microsomes the absorption maximum was observed at 448 nm (Fig. 3a). This blue shift could have been due to the interaction of cytochrome P-450 with the safrole metabolite. However, after generation of the 427/455 nm absorption bands *in vitro* from PB- or control-microsomes the reduced CO-difference spectrum remained at 450 nm. Furthermore, after "displacement" (see below) of the 455 nm absorbing species of safrole-microsomes, the reduced CO-difference spectrum still showed a maximum absorption at 448 nm (Fig. 3b).

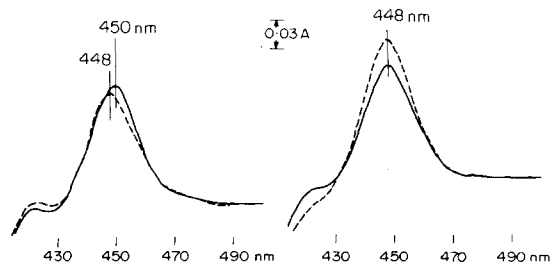

Fig. 3. Reduced CO-difference spectra of safrole- and PB-microsomes. (a) Safrole- (– – –) or PB- (——) microsomes (1 mg/ml) in 66 mM Tris–HCl buffer (pH 7·4) divided between two cuvettes, dithionite added to each, followed by CO to sample cuvette. (b) Conditions as in Fig. 3a. (——) before displacement; (– – –) after displacement by the addition of 833 μM safrole to sample cell and allowed to stand for 30 min before addition of $Na_2S_2O_4$ and CO.

Hence it is apparent that safrole has multiple effects upon rat hepatic microsomes *in vivo*, causing both the formation of a safrole metabolite–cytochrome P-450 adduct and also the induction of the P-448 type cytochrome.

Stability of the safrole metabolite-cytochrome P-450 adduct. The adduct is remarkably stable as judged by its presence in isolated safrole-microsomes. Dialysis of safrole-microsomes against 0·1 M phosphate buffer (pH 7·4) at 4° for 6 h, resulted in only a 5–10 per cent loss in the level of the 455 nm absorption.

Displacement of the safrole metabolite from safrole-microsomes. Various substrates of the mixed function oxidase system when added to oxidized but not to reduced safrole-microsomes show only a very weak difference spectrum. This can be taken as evidence that their binding site might possibly be occupied. As time progresses the difference spectra become increasingly pronounced (Fig. 4). A corresponding decrease in the 455 nm absorption band can be observed by reducing the contents of the sample cuvette at suitable time intervals. An inverse relationship between the decrease at 455 nm and the increase of the substrate binding spectrum with time is observed (Fig. 5). Compounds eliciting this phenomenon have been termed "displacers" [24]. Safrole, biphenyl and ethylbenzene have been particularly studied in this respect.

The degree of gradual spectral enhancement of the type I binding spectrum of the displacer (safrole etc.) was found to be directly correlated to the initial absorption at 455 nm (Fig. 6). This emphasizes the strict proportionality between the original concentration of the 455 nm absorbing species and the increment of the type I spectrum.

Kinetic analysis indicated that the rate and magnitude of spectral enhancement is directly related to the displacer concentration (Fig. 7). Double reciprocal plots of the initial rate of enhancement against displacer concentration yield straight lines (Fig. 8). However, with safrole as displacer at low concentrations

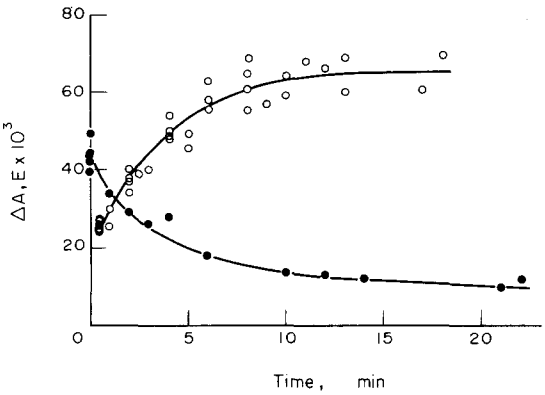

Fig. 5. The correlation between increase of safrole (displacer) type I binding spectrum and decrease of 455 nm absorption. Safrole-microsomes (1 mg/ml) in 66 mM Tris–HCl buffer (pH 7·4) were divided between two cuvettes. Safrole (167 μM) was added to sample cuvette at zero time. The spectrum between 500 and 350 nm was recorded every 2 min and the sample cuvette reduced with $Na_2S_2O_4$ at suitable times. Number of experiments = 15. O——O, Development of type I safrole (displacer) binding spectrum with time (see Fig. 4); ΔA = 386–434 nm. ●——●, Magnitude of 455 nm absorption measured after addition of $Na_2S_2O_4$ to sample cuvette at the times given in the diagram; ΔA = 455–500 nm.

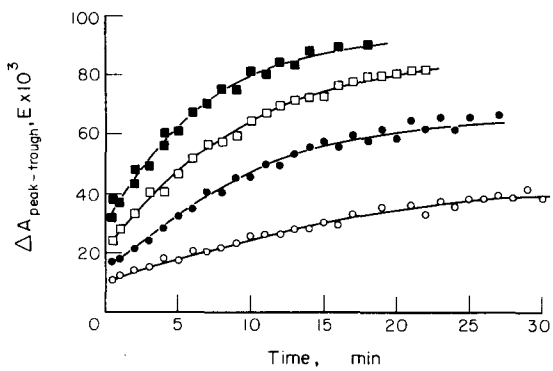

Fig. 7. The effect of safrole (displacer) concentration on the rate of enhancement of the safrole type I binding spectrum. Conditions as in Fig. 4. Safrole (displacer) concentrations: ■, 417 μM; □, 167 μM; ●, 83·3 μM; O, 41·7 μM. ΔA = 386–434 nm.

(<80 μM) non-linearity is observed, possibly indicating that safrole under these conditions is acting as a positive allosteric modifier. By analogy with the Lineweaver–Burk plot [25] the reciprocal of the negative intercept on the x axis can be taken to represent the apparent K_m for the displacement reaction (about 10^{-4}M). This is independent of the original magnitude of the absorption at 455 nm.

By utilization of a double reciprocal plot of ΔA peak-trough for the displacer binding spectrum versus time, accurate A_{max} values for displacer binding can be obtained for each displacer concentration. Also a direct relationship between the time taken for 50 per cent of the maximum displacement ($t_{1/2}$) and displacer concentration can be observed (Fig. 9).

The affinity of safrole (as a displacer) for safrole-microsomes before and after displacement can be calculated utilizing double reciprocal plots of ΔA peak-trough vs displacer concentration (Fig. 10). The ΔA final peak-trough values are derived from Fig. 9, and the ΔA initial peak-trough values are obtained from extrapolation of the curves, in Fig. 7, to zero time. Figure 10 shows a distinct difference between the initial and final K_s and A_{max} for safrole as a displacer. The K_s for safrole with PB-microsomes is $0{\cdot}2 \times 10^{-4}$M. Hence the final K_s of safrole with safrole-microsomes ($0{\cdot}46 \times 10^{-4}$M) is intermediate between the K_s with PB-microsomes and the initial K_s of safrole with safrole microsomes ($1{\cdot}3 \times 10^{-4}$M).

Many compounds have been screened as displacers, but no clear correlations between displacing activity and other parameters (e.g. partition coefficient or K_s value) are apparent, although lipophilicity and the absence of sterically bulky groups appear to be important. Furthermore, all displacers so far examined are type I compounds, no type II compounds (e.g. aniline, nicotinamide, 4,4′-dipyridyl) have been found to be effective ([24] and unpublished observations).

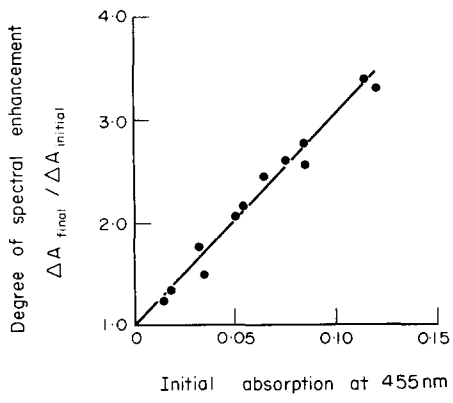

Fig. 6. The relationship between the degree of spectral enhancement of type I displacer binding and initial absorption at 455 nm. Each point represents a different safrole-microsomal preparation at a concentration of 2 mg/ml. Complete displacement was effected by the addition of 833 μM safrole, the initial and final type I displacer spectra were recorded (ΔA = 386–434 nm). Parallel samples were reduced with $Na_2S_2O_4$ to determine the initial 455 absorption.

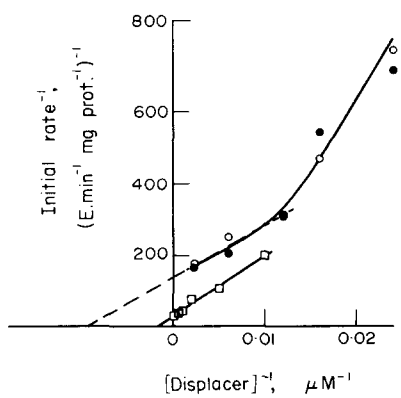

Fig. 8. Double reciprocal plot of the rate of enhancement of displacer type I binding with safrole-microsomes. Conditions as Fig. 4. ●, O two different safrole-microsomal preparations with safrole as displacer; □, ethylbenzene as displacer.

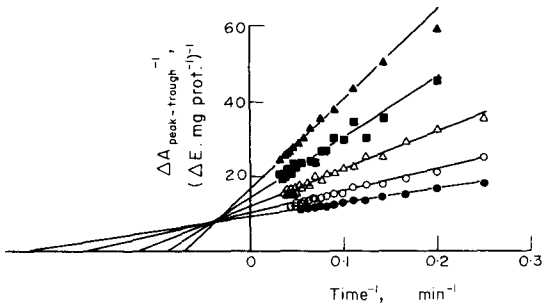

Fig. 9. Double reciprocal plot for time-dependent increase of safrole type I binding with safrole microsomes. Conditions as Fig. 4. Safrole (displacer) concentrations: ▲, 41·7 μM; ■, 62·5 μM; △, 83·3 μM; ○, 167 μM; ●, 417 μM. ΔA = 383–434 nm.

Displacement of the in vitro *generated* 427/455 *species from phenobarbital-microsomes.* Displacement has also been shown using phenobarbital-microsomes exhibiting the 427/455 nm absorption bands generated from safrole *in vitro*. Formation of the complex of cytochrome P-450 with the postulated metabolite in the presence of safrole, NADPH and O_2 is described in Fig. 11. In order to eliminate the NADPH-induced spectrum of reduced microsomes this cofactor was added to both cuvettes. At the maximum of the 427/455 formation, $Na_2S_2O_4$ was added to each cuvette and the spectrum re-recorded between 500 and 350 nm (Fig. 11a). The resultant spectrum was stable for 1 hr. In both cases maxima at 427 and 455 nm are visible.

An identical experiment was performed but, at the maximum of 427/455 generation, glutamate dehydrogenase, NH_4Cl and Na-α-ketoglutarate were added to oxidize any remaining NADPH [22]. This was shown to oxidize the total NADPH within 90 sec. The microsomal suspensions were allowed to stand for 20 min in the oxidized state, $Na_2S_2O_4$ was added to both cuvettes, and the difference spectrum recorded. A loss of the 427 and 455 nm absorptions was observed which was complete after 20 min (Fig. 11b). The 455 nm absorption is replaced by a maximum at 450 nm, which can always be found in drug oxidizing microsomes after reduction. It is due to the endogenous generation of carbon monoxide during haem breakdown [29]. Hence it is probable that the excess

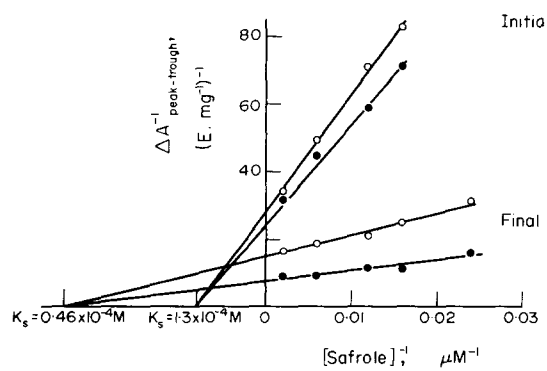

Fig. 10. K_s (initial and final) plots for safrole (displacer) type I binding with safrole-microsomes. Conditions as Fig. 4. Curves are shown for two representative safrole-microsomal preparations (● and ○).

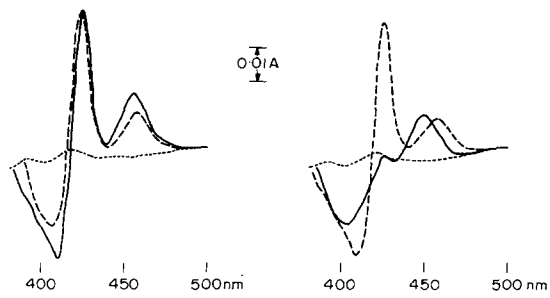

Fig. 11. Demonstration of the displacement phenomenon after formation *in vitro* of 427/455 nm absorbing species from PB-microsomes. (a) PB-microsomes (1 mg/ml) suspended in 66 mM Tris–HCl (pH 7·4) divided between two cuvettes and baseline recorded (·····). Safrole (833 μM) was added to the sample cuvette and 250 μM NADPH added to both cuvettes and the spectrum again recorded (– – –). After 5 min. $Na_2S_2O_4$ was added to both cuvettes and spectrum recorded again (——). (b) A similar experiment was carried out and the NADPH reduced spectrum recorded (– – –), then GDH/αKG/NH_4^+ (as described in Methods) were added and the suspensions allowed to stand 20 min before the addition of $Na_2S_2O_4$ to both cuvettes (——).

safrole in the microsomal suspensions displaced the safrole metabolite from the cytochrome P-450, when reducing conditions were lost. This time period corresponds well with that required for complete displacement from safrole-microsomes (Fig. 5).

It must be emphasized that no displacement by alternative substrates, or by safrole itself, can be obtained when the microsomes are in the reduced state (i.e. in the presence of NADPH, NADH or $Na_2S_2O_4$) but the removal of reducing conditions will immediately result in the gradual dissociation of the 427/455 nm absorbing species, by displacers.

Effect of displacement upon reduced CO-difference spectrum of safrole-microsomes. As shown in Fig. 3, no qualitative changes occurred in the Soret absorption band at 448 nm after displacement of the safrole-metabolite from safrole-microsomes. However, after removal of the 427/455 nm absorbing species by displacement the magnitude of the 448 nm CO-Soret band increased by between 20 and 50 per cent, depending on the individual safrole-microsomal preparations (Fig. 3b). This inhibition of CO binding by the proposed safrole metabolite is in agreement with the data obtained *in vivo* and *in vitro* using piperonyl butoxide [14, 26]. It demonstrates the release of previously complexed cytochrome, thereby making possible further CO-binding, and is probably an indication of the interaction of the safrole-metabolite with haem.

DISCUSSION

The present work clearly indicates that safrole pretreatment of rats has both qualitative and quantitative effects upon the cytochrome P-450 of hepatic microsomes. The nature of the reduced CO-spectrum before and after removal of the 455 nm-absorbing species indicates two things. First, that the magnitude of the reduced CO–cytochrome absorption is increased after displacement, thus demonstrating that the 455 nm-absorbing species probably involves a

haem interaction thereby inhibiting CO binding; and second that the Soret absorption maximum of the reduced CO–cytochrome complex, before and after displacement, is at 448 nm. This indicates that safrole preferentially induces the "P-448" type of microsomal cytochrome, as does 3-methylcholanthrene and other carcinogens. In support of this view Lake *et al.* [5] showed that the induction-pattern (with respect to substrates) of *iso*-safrole was more similar to that of 3-methylcholanthrene than to that of phenobarbital. Furthermore, Lotlikar and Wassermann [4] have demonstrated that safrole, in common with 3-methylcholanthrene and *iso*-safrole, induces the 3- and 5-hydroxylation of acetamidofluorene to a greater extent than the *N*-hydroxylation reaction. This effect was prevented by the prior treatment of the animals with ethionine to inhibit *de novo* protein synthesis.

Evidence that a metabolite of safrole is concerned, rather than safrole itself, comes from the prerequisite *in vitro* of oxygen and NADPH for 427/455 nm formation. This suggestion is also supported by the results of Franklin [13, 15, 16] using piperonyl butoxide.

Finally, the observation that certain compounds which are substrates for cytochrome P-450 are able to dissociate the 427–455 nm-absorbing complex is in support of the proposed metabolite–cytochrome P-450 interaction. Both the present and previous work [14–16] have shown the relatively high stability of the species absorbing at 427/455 nm under reduced conditions. Our work has now demonstrated that under oxidized conditions certain other substrates of the mixed function oxidase system (such as ethylbenzene, biphenyl or safrole) will dissociate the safrole-metabolite–cytochrome P-450 complex. The displacement reactions described obey typical enzymic kinetics. For example, the degree of type I spectral enhancement is proportional to the original magnitude of the 455 nm absorption; and the rate of displacement is proportional to the concentration of displacing substrate. The observed behaviour is entirely consistent with the binding of a metabolite of safrole as being the reason for the unusual spectral pattern.

It is striking that the A_{max} and K_s values of the displacer (e.g. safrole) change (Fig. 10) during the displacement reaction. The increase of the A_{max} could be explained by the release of cytochrome P-450 from its metabolite complex thereby making more cytochrome available for the type I substrate binding. However the decrease of the K_s value ($1{\cdot}3 \times 10^{-4}$ to $0{\cdot}46 \times 10^{-4}$ M) for safrole is not so easily explained. On the basis of classical kinetic analysis it could be suggested as being due to the removal of a competitive inhibitor. However, after metabolism (i.e. as would happen during safrole pretreatment) the methylenedioxyphenyl compounds exhibit non-competitive inhibition of the mixed function oxidases [3, 13]. Alternatively, it is possible that the initial K_s refers to the "free" cytochrome (i.e. not complexed with safrole metabolite) and that the final K_s represents a combination of the "free" and "released" cytochromes. Possibly the "free" cytochrome is the newly induced cytochrome P-448 (plus perhaps some P-450) and that after displacement a mixture of "old" cytochrome P-450 and new cytochrome P-448 is available to interact with the displacer. This possibly is supported by evidence of Franklin [13] and Anders [8] showing that the K_i for piperonyl butoxide inhibition of drug metabolism was higher in 3-methylcholanthrene induced animals than in control or PB-induced animals, indicating that cytochrome P-448 is less sensitive to the metabolite–cytochrome interactions than is cytochrome P-450. This could represent an interesting example of a biological protection mechanism directed against inhibition of drug metabolism.

Displacement has been qualitatively shown after formation *in vitro* of the 427/455 nm absorbing species from safrole, indicating that the displacement of the metabolite from the metabolite–cytochrome complex does not require the induction process as a prerequisite.

The nature of the safrole metabolite responsible for the 427/455 nm absorbing species is the subject of much speculation. The metabolism of safrole is not well documented and reactive metabolites could potentially arise in several ways. Hansch [27] suggested a free radical produced by homolytic cleavage of a proton from the methylene moiety of the 1:3 dioxalane ring, while Hennessy [28] proposed the loss of a hydride ion from the methylene bridge leading to formation of the electrophilic benzodioxolium ion. However, both of these possibilities would probably result in covalent binding to the microsomes, in which case the displacement reactions described above would be unlikely to occur. Ullrich and Schnabel [23] have suggested a third possibility, namely the abstraction of a proton from the methylene group by the strongly nucleophilic active oxygen formed during metabolism. They have presented evidence in favour of this hypothesis for fluorene which gives a 446 nm absorption in the reduced difference spectrum after oxidative metabolism. Using this hypothesis for the safrole metabolite–cytochrome P-450 interaction, displacement could occur due to the acceleration of the reprotonation of the carbanion. This would explain the relatively long time periods required for the displacement process, since hydrophobic environments are not conducive to rapid protonation reactions. A conformational change elicited by the displacer could result in an increase in the hydrophilicity of the cytochrome P-450 environment thereby aiding the reprotonation reaction.

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