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# FURTHER STUDIES ON THE DISSOCIATION OF THE ISOSAFROLE METABOLITE–CYTOCHROME P-450 COMPLEX

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**Abstract**—The dissociation of an *in vivo* isosafrole metabolite–cytochrome P-450 complex by a number of exogenous and endogenous compounds is described. The rate of dissociation is temperature-dependent, but the process does not require oxygen. Compounds which produce Type I (substrate) binding spectra with oxidized cytochrome P-450 facilitate displacement of the isosafrole metabolite from cytochrome P-450 whereas Type II (ligand-binding) compounds do not dissociate the complex. The factors necessary for dissociation of the complex are discussed and a model for the isosafrole metabolite–cytochrome P-450 complex is proposed.

Methylenedioxyphenyl (MDP) compounds interact with hepatic microsomes, in the presence of NADPH and molecular oxygen, to form metabolite or intermediate complexes with cytochrome P-450. These complexes have characteristic difference spectra. The ferrocytochrome P-450 ($Fe^{2+}$) complexes exhibit two Soret absorption maxima at 427 and 455 nm [1, 2], while the ferricytochrome ($Fe^{2+}$) complexes have a single absorption maximum at about 438 nm [3, 4]. The formation of these metabolite complexes is accompanied by the inhibition of monooxygenase reactions [5] and accounts for the insecticide synergist properties of MDP compounds (see ref. 6 for review).

The nature of the MDP metabolite–cytochrome P-450 complex is unclear, although evidence suggests that the metabolite is bound to the haem of cytochrome P-450 [1, 7, 8]. Several suggestions have been made as to the chemical nature of the particular metabolite or intermediate of the MDP compounds involved in the formation of the 427/455 nm absorbing complexes and the concomitant inhibition of monooxygenation. Hennessy [9] proposed the production of an electrophilic benzodioxolium ion *via* the loss of a hydride ion from the methylene bridge, while Hansch [10] suggested that a reactive metabolite could arise by the homolytic cleavage of the methylene bridge resulting in the formation of a free radical. More recently Ullrich, Nastainczyk and Ruf [11] have proposed that a carbene may be produced by hydroxylation of the methylene bridge followed by elimination of water. Since carbenes bind tightly to ferrocytochrome P-450 [12] a rather stable MDP metabolite complex would result from monooxygenation of the methylenedioxy ring system.

Certain MDP compounds also produce metabolite–cytochrome P-450 complexes *in vivo*. The $Na_2S_2O_4$-reduced difference spectrum of hepatic microsomes obtained from rats pretreated with safrole (4-allyl-1,2-methylenedioxybenzene) or isosafrole (4-propenyl-1,2-methylenedioxybenzene) exhibits an anomalous absorption maximum at 455 nm [13]. This spectrum is the optical manifestation of the safrole or isosafrole metabolite–cytochrome P-450 complex formed *in vivo* [14]. The high stabilities of these complexes are illustrated by their ability to survive the procedure involved in the isolation of the microsomes in addition to prolonged dialysis [14].

The oxidized binding spectra of certain substrates of the monooxygenase system undergo a time-dependent intensification on addition to a suspension of microsomes obtained from safrole or isosafrole pretreated rats (safrole- or isosafrole-microsomes) [15]. This process is accompanied by a loss of the spectrally observable isosafrole metabolite–cytochrome P-450 complex [14] and an expulsion of an isosafrole-related material from the microsomes into solution [16]. This phenomenon has been termed 'displacement' and compounds which elicit the dissociation of the complex are called 'displacers' [14, 15]. The displacement process, involving dissociation of the isosafrole metabolite–ferricytochrome P-450 complex, results in the increase of mono-oxygenation ability [17], presumably due to the removal of the haem-bound isosafrole metabolite and the consequential return of "oxygen activating" properties of the cytochrome.

The present study was embarked upon to investigate the mechanism by which a highly stable MDP metabolite–cytochrome P-450 complex can be dissociated by the mere addition of an alternative substrate of cytochrome P-450.

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## METHODS

*Chemicals.* Isosafrole and safrole were obtained from Eastman Kodak Co. (England) and Hopkins and Williams Ltd (England) respectively; dihydrosafrole was purchased from Merck GmbH (GFR); methylenedioxybenzene and piperonyl butoxide were gifts from the Pharmakologisches Institut, Mainz (GFR) and The Wellcome Research Laboratories (Berkhamsted, England) respectively; 2-methyl-, 2-propyl- and 2-nonylbenzimidazole were obtained from Aldrich Chemical Co. Ltd (England). The other 2-*n*-alkylbenzimidazoles used in this study were synthesized by Dr. S. R. Challand and Mr. G. Brown of The Wellcome Research Laboratories (Beckenham, England). The *n*-alkylcarbamates were generously donated by Dr. D. Upshall at the Chemical Defence Establishment (Porton Down, Salisbury, England). Carboxylic acids, cyclohexane, biphenyl, alkanes and *n*-alcohols were obtained from BDH (England) and Hopkins and Williams (England). 7-Ethoxycoumarin, 4-nitroanisole and the various steroids used were from the Department of Biochemistry, University of Surrey (Guildford, Surrey, England).

*Animals.* Male albino Wistar rats (180–200 g) were obtained from either Charles River Ltd (England) or the Zentralinstitut für Versuchstierzucht (GFR). The animals were allowed food and water *ad libitum*. An ambient temperature of 22–24° and a 12 hr (0700–1900) light cycle were maintained.

*Pretreatment of animals and preparation of microsomes.* Isosafrole-microsomes (i.e. microsomes obtained from animals pretreated with isosafrole) were prepared from the pooled livers of six rats after i.p. injection of isosafrole (150 mg/kg) in corn oil (2.5 ml/kg) once daily for three consecutive days. The rats were killed on the 4th day, livers excised into ice-cold 1.15% (w/v) KCl and scissor-minced. The minced livers were washed 3 times with ice-cold 1.15% (w/v) KCl containing 50 mM Tris–HCl (pH 7.4). Washed microsomes were then prepared as described previously [18]. The final microsomal pellet was resuspended in 20 mM Tris–HCl (pH 7.4) containing 250 mM sucrose and 5.4 mM EDTA to a concentration equivalent to about 1–1.5 g wet weight liver per ml (about 20–30 mg microsomal protein per ml). The microsomes were stored at −20° for a maximum of 2 weeks prior to use. Microsomal protein was estimated by the method of Lowry *et al.* [19] using bovine serum albumin standards.

*Spectrophotometry.* Binding spectra [20] were obtained at 37° (unless otherwise stated) using either an Aminco DW-2 spectrophotometer or a Perkin Elmer 356 spectrophotometer. Substrates were utilized as 250 mM or 500 mM stock solutions in *N*,*N*-dimethylformamide (Koch-Light, Puriss) and added to the cuvette in μl quantities. *N*,*N*-dimethylformamide had no effect on the measured parameters at concentrations of 5 μl or less per 2.5 ml microsomal suspension (about 25 mM). Specific experimental details are described in the figure legends.

The effect of temperature on the initial rate of displacement was investigated over two separate temperature ranges; 17–36° (microsomal protein concentration = 1 mg/ml and displacer concentration = 250 μM) and 11–28° (protein concentration, 2 mg/ml and displacer concentration, 400 μM). 2-*n*-Heptylbenzimidazole was used as the displacing agent.

For the anaerobic experiment, microsomes (1 mg protein/ml) were bubbled with oxygen-free nitrogen for five minutes prior to the addition of a deoxygenating system consisting of glucose (10 mM), glucose oxidase (50 units/ml) and catalase (600 units/ml). The mixture was incubated at 37° for 10 min in the thermostatted cuvette holder of the spectrophotometer before addition of the displacer (2-*n*-heptylbenzimidazole, 200 μM). Control experiments were carried out by omitting the oxygen-depleting reagents.

## RESULTS

Figure 1 illustrates the spectral changes concurred on the addition of 7-ethoxycoumarin to isosafrole-microsomes. Before addition of displacer to the sample cuvette a baseline of equal light absorbance was recorded, since both the reference and sample cuvettes contained the same concentration of the 438 nm absorbing complex. On the addition of a displacer to the sample cuvette an immediate binding spectrum (in this case Type I) was observed; then with increasing time and dissociation of the 438 nm absorbing complex, the spectral minimum shifted from 420 to 438 nm and increased in size. This is due to the 438 complex being dissociated in the sample cuvette but not in the reference cuvette. In the present work we have estimated the displacing ability of various compounds by determining the rate of decrease in absorbance at 438 nm with respect to an isosbestic point (475–490 nm) depending on the compound examined).

The validity of this measurement is supported by the following evidence. The addition of safrole to isosafrole-microsomes resulted in spectral changes similar to those shown in Fig. 1. Using different concentrations of safrole as a displacer the rate of change of absorbance at 438 nm was determined. Figure 2 demonstrates that the rate of dissociation of the complex was dependent upon the concentration of the displacer utilized. By replotting these data as a double reciprocal plot (Fig. 3) the maximal size of the 438 nm trough may be obtained from the reciprocal of the y axis ($\Delta A_{438-475}$ nm) intercept. It may be seen in this plot that the maximal change of absorbance at 438 nm is constant irrespective of the concentration of the displacer. Furthermore, the maximal absorbance change at 438 nm was always a constant value for a given preparation of isosafrole-microsomes (which thus contained a constant amount of the isosafrole metabolite–cytochrome P-450 complex) regardless of the displacing agent or concentration of that displacer used to effect the dissociation of the complex.

Hence, it was deemed valid to utilize measurements at 438 nm as an estimate of the rate of dissociation of the isosafrole metabolite–ferricytochrome P-450 complex. It should be noted here that previously [14] $\Delta A^{-1}$ *versus* $t^{-1}$ plots were used for similar purposes, however, the ΔA measurements in this case were $\Delta A_{(peak-trough)}$ values of the intensified binding spectrum, and the maximal ΔA values were dependent on the concentration of the displacer employed. This was because the $\Delta A_{(peak-trough)}$ values are a combination

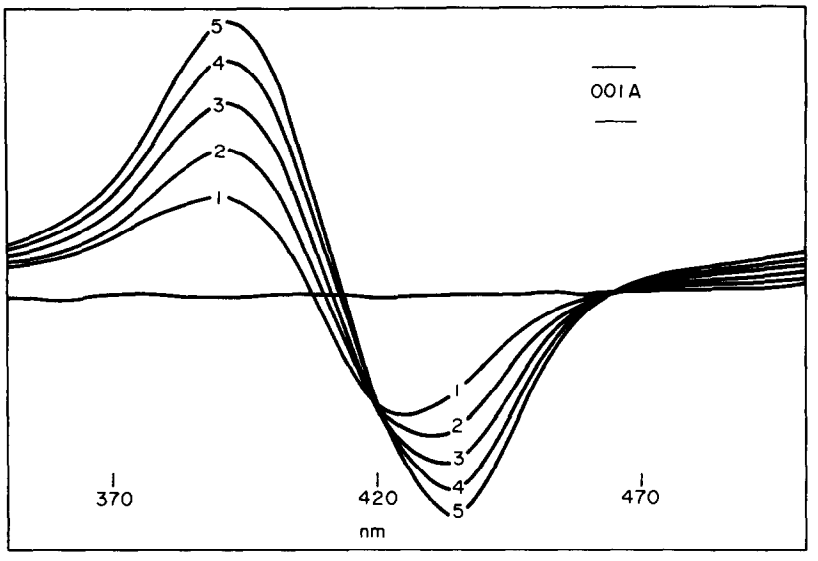



Fig. 1. Spectral changes which occurred on the addition of 7-ethoxycoumarin to isosafrole-microsomes. Isosafrole-microsomes (1 mg microsomal protein/ml) were divided between two cuvettes and a baseline of equal light absorbance obtained. 7-Ethoxycoumarin (250 $\mu$M) was added to the sample cuvette and the spectra between 350 and 500 nm recorded every minute.

of the spectra due to binding of displacer and dissociation of complex.

A series of carboxylic acids were used to dissociate the isosafrole metabolite–cytochrome P-450 complex. Butyric acid possessed detectable displacing ability and higher analogues were increasingly active until undecanoic acid (log P = 4.65, where log P = log octanol/water partition coefficient) the optimal displacer (Fig. 5) was reached. Carboxylic acids with longer alkyl chains than this compound elicited slower rates of displacement. All the fatty acids tested between hexanoic acid and myristic acid produced Type I binding spectra with oxidized cytochrome P-450 (Table 1).

A series of *n*-alkylcarbamates were also studied. The addition of methyl- or ethylcarbamate to isosafrole-microsomes resulted in a very weak Type RI binding spectrum. These compounds were unable to

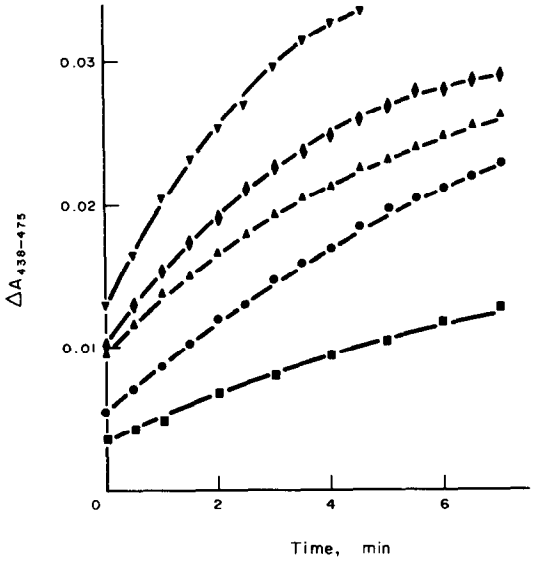



Fig. 2. Plot of optical change *versus* time for different displacer concentrations. Isosafrole-microsomes (1 mg protein/ml) were divided between two cuvettes. Various displacer (safrole) concentrations were added to the sample cuvette and the spectrum between 350 and 500 nm was recorded every minute. $\Delta A_{438-475}$ was then measured and plotted against time. Safrole (displacer) concentrations: ▼, 833 $\mu$M; ◆, 417 $\mu$M; ▲, 125 $\mu$M; ●, 83.3 $\mu$M; ■, 41.7 $\mu$M.

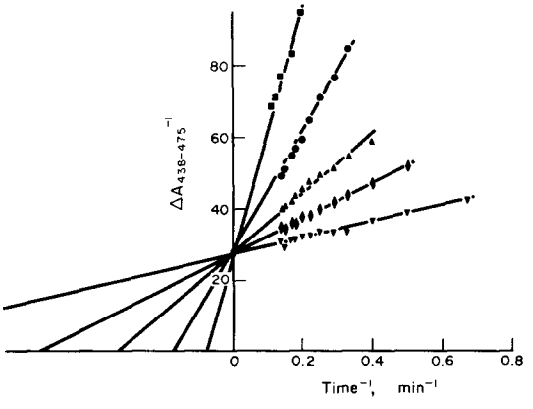



Fig. 3. Double reciprocal plot of displacer concentration *versus* the rate of dissociation of the isosafrole metabolite–cytochrome P-450 complex. Conditions as in Fig. 2.

elicit the dissociation of the isosafrole metabolite–ferricytochrome P-450 complex. Higher carbamates (pentyl-, hexyl-, octyl-, decyl- and dodecyl-) interacted with isosafrole-microsomes to give Type I spectra (Table 1). These higher analogues were capable of eliciting the dissociation of the complex. The rate of dissociation of the complex at a fixed displacer concentration (1.0 mM) increased with increasing length of the alkyl chain until decylcarbamate and then declined again. Figure 6 demonstrates the relationship between log P and rate of displacement for the *n*-alkylcarbamates.

When alcohols were tested as displacers, *n*-decanol (log P = 4.03) was the optimal displacer (Table 2). Alcohols with longer or shorter side-chains possessed lesser displacing ability. The three alkanes tested (all Type I substrates) were good displacers of the isosafrole metabolite, with hexane showing the greatest activity (Table 2). Several known substrates of cytochrome P-450-mediated metabolism all produced similar initial rates of dissociation. MDP compounds were very active displacers; however, the loss of the side-chain from the ring system (as in methylenedioxybenzene) produced a decrease in displacing ability (Table 2).

Several endogenous steroids were also studied. It was found that progesterone and testosterone elicited considerably greater rates of displacement than cortisol and oestradiol-17$\beta$ (Table 2). Cholesterol was unable to dissociate the isosafrole metabolite–cytochrome P-450 complex.

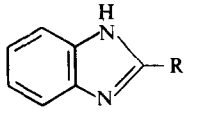
The greatest rates of displacement obtained so far were elicited by members of a series of 2-*n*-alkyl-substituted benzimidazoles (Fig. 7 and Table 2). These compounds have been shown [21, 22] to interact with hepatic microsomal cytochrome P-450 in a variety of modes. Type I, RI (reverse Type I) and mixed Type I/RI spectra may be obtained depending on the state of induction of the microsomes used, the specific alkylbenzimidazole used and its concentration.

The type of spectra observed on the addition of 2-*n*-alkylbenzimidazoles to isosafrole-microsomes was dependent upon the length of the alkyl substituent. The short chain 2-alkylbenzimidazoles (methyl-, ethyl- and propyl-) initially elicited Type RI binding spectra, which became modified with time (Fig. 4) resulting in a Type RI spectrum with an extra absorption minimum at 438 nm (due to dissociation of the 438 nm absorbing complex in the sample, but not the reference, cuvette). The higher analogues used (hexyl-, heptyl-, octyl- and nonyl-) were Type I compounds and the only time-dependent changes observed were the shift in the absorption minimum from about 420 nm to 438 nm and an increase in magnitude of the spectra. This was similar to the change indicated in Fig. 1 for 7-ethoxycoumarin.

As the length of the 2-alkyl substituent was increased the rate of decrease in absorbance at 438 nm (i.e. the rate of dissociation of the complex) increased to a maximum at 7 carbon atoms (log P = 4.96). With substituents longer than this optimal chain length the rate of displacement declined. Figure 7 illustrates the relationship between log P and rate of dissociation of the 438 nm absorbing complex by 2-alkylbenzimidazoles.

Oxygen is not involved in the displacement reaction as shown by experiments carried out under anaerobic conditions. The initial rate of dissociation elicited by 2-*n*-heptylbenzimidazole was identical for both aerobic and anaerobic incubations. The rate of the displacement reaction increased linearly over a temperature range of 11°–36°. No break in the Arrhenius plot was detected, and a value of 8.25 kJ/mol was calculated for the activation energy of the displacement process.

Table 1. $K_s$ values and log P values for the binding of 2-*n*-alkylbenzimidazoles, *n*-alkylcarbamates and carboxylic acids to cytochrome P-450



2-*n*-alkylbenzimidazoles

| R = , | Spectrum | $K_s$ ($\mu$M) | Log P† |
|---|---|---|---|
| methyl | RI | 420 | 1.96 |
| ethyl | RI | 710 | 2.48 |
| propyl | RI | 1000 | 2.96 |
| butyl | RI | 2500 | 3.48 |
| pentyl | RI | 380 | 3.96 |
| hexyl | I | 0.40 | 4.48 |
| heptyl | I | 0.33 | 4.96 |
| octyl | I | 0.37 | 5.48 |
| nonyl | I | 0.48 | 5.96 |

$R—O—CO—NH_2$ *n*-alkylcarbamates

| R = , | Type of Spectrum | $K_s$ ($\mu$M) | Log P |
|---|---|---|---|
| butyl | I | 1900 | 0.74 |
| pentyl | I | 930 | 1.29 |
| hexyl | I | 500 | 1.74 |
| heptyl | I | 170 | 2.29 |
| octyl | I | 71 | 2.74 |
| decyl | I | 35 | 3.74 |
| dodecyl | I | N.A.‡ | 4.74 |
| tetradecyl | RI | N.A.‡ | 5.74 |

R—COOH Carboxylic acids

| R = , | Type of spectrum | $K_s$ ($\mu$M) | Log P |
|---|---|---|---|
| propyl | N.A. | N.A. | 0.79 |
| butyl | N.A. | N.A. | 1.35 |
| pentyl | I | 500 | 1.90 |
| hexyl | I | 2100 | 2.45 |
| heptyl | I | 345 | 3.00 |
| octyl | I | 161 | 3.55 |
| nonyl | I | 128 | 4.09 |
| decyl | I | 78 | 4.65 |
| undecyl | I | 79 | 5.20 |
| tridecyl | I | 67 | 6.30 |
| pentadecyl | N.A. | N.A. | N.A. |
| heptadecyl | N.A. | N.A. | N.A. |

* $K_s$ values are the apparent spectral dissociation constants of compounds binding to cytochrome P-450.

† log P values are the $\log_{10}$ of the octanol/water partition coefficients.

‡ NA = not available.

Table 2. Rates of displacement of various alkanes, alcohols, methylenedioxyphenyl compounds, cytochrome P-450 model substrates and steroids

| (i) Displacer concentration = 200 $\mu$M. Displacer | Initial rate of displacement ($\Delta E_{438-490}$/min/mg protein) | Type of spectrum with isosafrole microsomes | log P |
|---|---|---|---|
| 2-*n*-Heptylbenzimidazole | 25.8 | I | 4.96 |
| *n*-Decylcarbamate | 2.8 | I | 3.74 |
| Cytochrome P-450 (model) substrates | | | |
| Biphenyl | 2.6 | I | 4.09 |
| 7-Ethoxycoumarin | 2.4 | I | N.A. |
| Cyclohexane | 2.5 | I | 2.73 |
| 4-Nitroanisole | 2.0 | I | N.A. |
| Methylenedioxyphenyl compounds | | | |
| Safrole | 8.5 | I | 3.18 |
| Isosafrole | 6.3 | I | 3.38 |
| Dihydrosafrole | 9.0 | I | 3.58 |
| Methylenedioxybenzene | 3.5 | I | 2.08 |
| Alkanes | | | |
| Hexane | 6.6 | I | 3.0 |
| Heptane | 3.7 | I | 3.5 |
| Steroids | | | |
| Progesterone | 3.2 | I | N.A. |
| Testosterone | 2.8 | I | N.A. |

| (ii) Displacer concentration = 1.0 $\mu$M Displacer | Initial rate of displacement ($\Delta_{438-490}$/min/2 mg protein) | Type of spectrum with isosafrole microsomes | log P |
|---|---|---|---|
| Alkanes | | | |
| Pentane | 19.0 | I | 2.50 |
| Hexane | 25.4 | I | 3.00 |
| Heptane | 13.4 | I | 3.50 |
| Alcohols | | | |
| Octanol | 8.4 | RI/I | 3.03 |
| Decanol | 10.0 | RI/I | 4.03 |
| Undecanol | 7.1 | RI/I | 4.53 |
| Methylenedioxyphenyl compound | | | |
| Piperonyl butoxide | 6.3 | I | 5.20 |
| Steroids | | | |
| Cortisol | 2.7 | RI | N.A. |
| Oestradiol-17-$\beta$ | 2.7 | I | N.A. |
| Testosterone | 21.9 | I | N.A. |
| Progesterone | 27.1 | I | N.A. |
| Cholesterol | 0 | N.D. | N.A. |

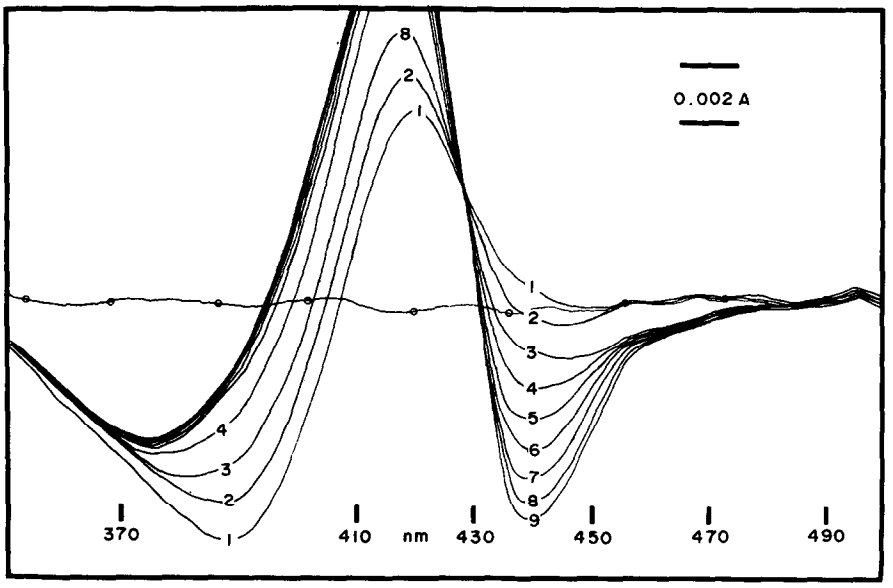



Fig. 4. 2-Ethylbenzimidazole as a displacer. Isosafrole-microsomes (1 mg protein/ml) were divided between two cuvettes and a baseline of equal light absorbance obtained (○—○) at 37°. 2-Ethylbenzimidazole (1 mM) was added to the sample cuvette and the initial binding spectrum between 350 and 500 nm recorded (Spectrum 1). Spectra 2–9 were obtained at successive 2.5 min intervals after the addition of 2-ethylbenzimidazole.

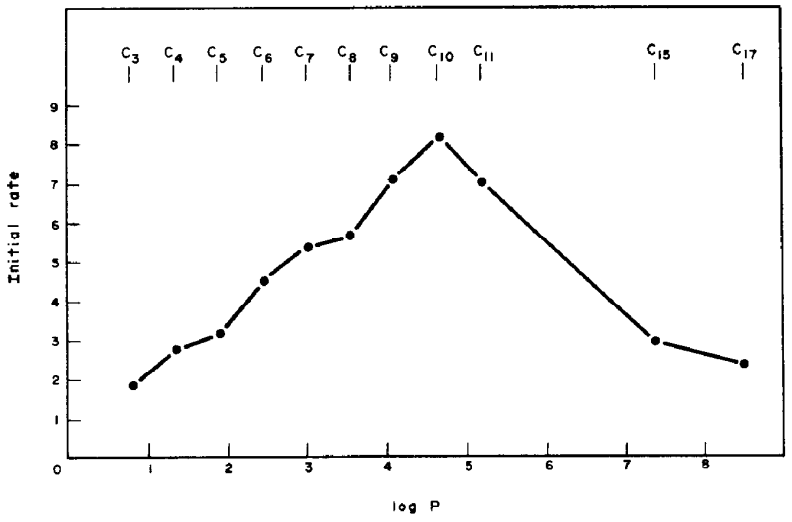



Fig. 5. Carboxylic acids as displacers. Isosafrole-microsomes (2 mg protein/ml) were added to a cuvette and a baseline was established. The carboxylic acid (1 mM) was added and the initial rate of displacement (expressed as $\Delta A_{438-490}$/min/2 mg microsomal protein) was recorded. The spectra were recorded in the dual wavelength mode ($\lambda_1$ = 438 nm, $\lambda_2$ = 490 nm) at 37°. Points represent the mean of two or more determinations. $C_3$, $C_4$, $C_5$ etc. refers to the number of carbon atoms in the substituent alkyl chain.

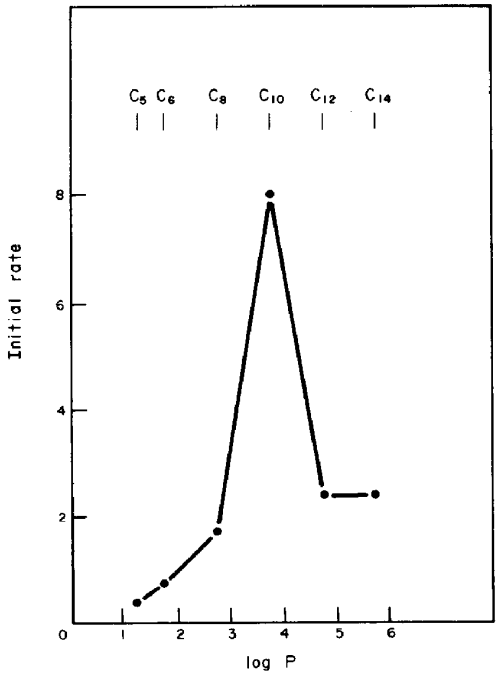



Fig. 6. *n*-Alkylcarbamates as displacers. Conditions as in Fig. 5 except that *n*-alkylcarbamates (1 mM) were used to elicit the displacement process.

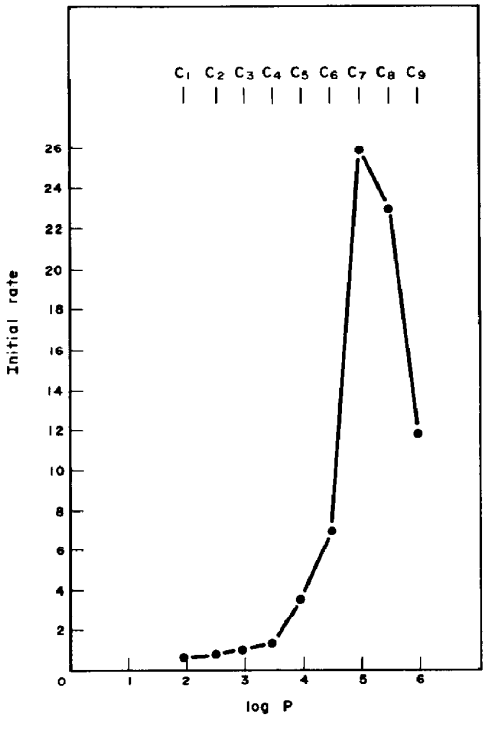



Fig. 7. 2-*n*-Alkylbenzimidazoles as displacers. Conditions as in Fig. 5 except that 2-*n*-alkylbenzimidazoles (200 $\mu$M) were used as displacers and the microsomal protein concentration was 1 mg/ml. The initial rate of displacement is expressed as $\Delta A_{438-490}$/min/mg microsomal protein.

## DISCUSSION

Previous studies have shown that the oxidized isosafrole metabolite–cytochrome P-450 complex absorbs light at 438 nm [4] and that during the displacement process the complex is dissociated with the release of an isosafrole-related material into solution [16]. The resultant displacement spectrum is a summation of three spectral changes: (a) the initial binding spectrum to uncomplexed cytochrome, (b) the absorption minimum due to dissociation of the 438 nm absorbing complex, and (c) the spectrum due to the displacer binding to 'released' cytochrome.

Various series of compounds have been tested for their ability to dissociate the *in vivo* generated rat hepatic microsomal isosafrole metabolite–cytochrome P-450 complex. The *n*-alkyl-substituted carbamates, alcohols and carboxylic acids all displaced the metabolite. Carbamates and carboxylic acids produced Type I binding spectra with oxidized cytochrome P-450 [23] whereas lower alcohols gave RI spectra and higher alcohols i.e. pentanol, hexanol, etc., gave additive Type I and RI binding spectra [24]. The lowest effective log P necessary for displacement to occur was about 1.0 and the optimal log P was between 3 and 5. The $K_s$ values for the carbamates and fatty acids used in this study supported the hypothesis that lipophilicity was important for displacement. For these compounds, a correlation has been obtained between log P and the log $K_s$ values for the substrate–cytochrome P-450 interaction [23]. As the alkyl chain length was increased, the $K_s$ value decreased and the initial rates of displacement of the isosafrole metabolite increased. For each series, an optimal carbon chain length for displacement was observed. Further increase in chain length beyond this optimum produced a decrease in displacement rate despite the fact that the $K_s$ values for the higher members of each series do not decrease appreciably.

An optimal length of 10 carbon units was obtained for the carbamates, fatty acids and alcohols tested. This $C_{10}$ unit seemed to be independent of the properties of the functional group (—COOH. —OH or —O.CO.$NH_2$). The $C_{10}$ carbamates (log P = 3.74), alcohol (log P = 4.03) and fatty acids (log P = 4.65) showed very similar displacement rates. It is notable that the straight chain alkanes pentane, hexane and heptane were even better displacers than the $C_{10}$ carbamate, alcohol and fatty acid. Thus the presence of a functional group capable of binding through an ionic interaction or through hydrogen bonding is not essential for displacement and may even retard the rate of dissociation of the cytochrome P-450–metabolite complex.

In the various series of alkyl compounds we have investigated, three factors appear to be important for effective displacement to occur: (a) the nature of any functional group attached to the *n*-alkyl chain; (b) the overall dimensions of the displacing molecule; (c) its lipophilicity.

The series of 2-*n*-alkyl-substituted benzimidazoles include the compounds which gave the greatest initial rates of displacement of the isosafrole metabolite–cytochrome P-450 complex. The optimal displacing compound was 2-*n*-heptyl-benzimidazole (log P = 4.96). The rate of dissociation decreased when the alkyl chain length was increased beyond this $C_7$ unit. This compound had a very low $K_s$ value for Type I binding to cytochrome P-450 (0.3 $\mu$M) (cf. decylcarbamate (35 $\mu$M) and undecanoic acid (78 $\mu$M).

Since most of the compounds mentioned above are not naturally occurring in liver, various endogenous sterols and steroids were also tested for their ability to dissociate the isosafrole metabolite–cytochrome P-450 complex because the cyclopentanoperhydrophenanthrene nucleus possesses similar overall dimensions to the optimal displacers in each of the series tested above. Cholesterol produced no detectable displacement but the other steroids tested all caused dissociation of the complex. However, progesterone and testosterone were far more active than oestradiol and cortisol, showing that subtle changes in the substituents on the steroid ring system can produce large differences in displacing ability. These findings cannot be entirely explained as due to differing affinities of these steroids for the hepatic microsomal cytochrome P-450 of male rats [25].

How does one envisage the displacement reactions? The isosafrole metabolite appears to be bound to the haem moiety of cytochrome P-450 and Type I and RI compounds are displacers whereas Type II compounds are not [14].

It seems improbable that the displacement mechanism is one of simple competition for binding sites since the carbene group of the isosafrole metabolite is almost certainly bound to the haem iron of cytochrome P-450, i.e. the Type II site. However, it is plausible that the isosafrole metabolite is a bidentate ligand, binding to both the Type I site (protein) and the Type II site. In the $Fe^{2+}$ state a highly stable Fe–carbene (iron–carbon) $\sigma$-bond would be produced which would be further stabilized by $\pi$ back-bonding (a donation of electrons from the iron atom to the carbon) and would be very difficult to displace. In the $Fe^{3+}$ state the complex would be less stable, since the $\pi$ back-bonding would not be a preferred configurational state. Hence dissociation of the Fe–C bond and concomitant displacement of the metabolite can occur. The methylene carbene of the putative isosafrole metabolite may interact with the haem moiety while the propenyl side-chain is embedded in the hydrophobic Type I binding site of the cytochrome P-450. The cytochrome P-450 species from which the isosafrole metabolite is displaced is possibly a novel haemoprotein induced by isosafrole administered *in vivo* [26]. The oxidized state of this haemoprotein binds the isosafrole metabolite less avidly than the haemoprotein induced by phenobarbitone which explains the lack of ready substrate-elicited dissociation of the isosafrole metabolite–ferricytochrome P-450 complex generated *in vitro*. Although relatively lipophilic substances are capable of dissociating the novel ferrihaemoprotein–isosafrole metabolite complex, access to the 'displacer site' may be limited as evidenced by the lack of, or decreased displacing ability of large lipophilic molecules. As no typical Type II compounds (aniline, imidazole, *n*-octylamine) elicit displacement even if they possess an *n*-alkyl chain as does octylamine, it appears that for the displacement of the isosafrole metabolite to occur, the breaking of a link between

the metabolite and the apoprotein portion of cytochrome P-450 is the initiating reaction.

No requirement for oxygen was found for the displacement reaction and the initial rate of dissociation of the isosafrole metabolite–cytochrome P-450 complex proceeded linearly over a temperature range of 11°–36°. Unlike the Arrhenius curves constructed for various mono-oxygenase reactions [27, 28] no break in the curve at temperatures of about 20° was detected for the displacement process. The activation energy calculated from the Arrhenius plots of the displacement reaction (8.25 kJ/mol) was somewhat higher than the values obtained for the metabolism of 4-nitroanisole, 7-ethoxycoumarin [27], ethylmorphine, aminopyrine and benzphetamine [28]. Thus it appears that changes in the fluidity of the lipid in the membrane caused by temperature changes [29] do not affect the rate of displacement.

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