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Electron transfer in P450 mechanisms. Microsomal metabolism of cyclopropylbenzene and *p*-cyclopropylanisole

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1. The metabolism of cyclopropylbenzene (**1a**) and 4-cyclopropylanisole (**1b**) was studied using liver microsomal preparations from control, phenobarbital- and β-naphthoflavone treated rats.

2. With all three types of microsomes **1a** was metabolized by benzylic hydroxylation to give 1-phenylcyclopropanol and by aromatic hydroxylation at C-4; the former predominated by a factor of 2–4. BNF-induced microsomes also formed 2-cyclopropylphenol. No cyclopropyl ring-opened metabolites of **1a**, including benzoic acid, were detected in any of the incubations.

3. With PB-induced microsomes **1b** underwent *O*-demethylation (90%) and benzylic hydroxylation; no other metabolites were detected.

4. Progress curves for metabolism of **1a** are markedly nonlinear after only limited conversion of substrate, suggesting the possibility that **1a**, like other cyclopropyl compounds, could be a suicide substrate for one or more isozymes of P450.

5. For both **1a** and **b**, metabolite formation and enzyme inactivation can be explained by conventional P450 reaction mechanisms not involving electron abstraction.

Introduction

P450s oxidize most substrates by one of three mechanistic pathways which differ beginning at the initial step of enzyme–substrate interaction (Guengerich 1990, White 1991). Aliphatic hydroxylation, including many examples α to a heteroatom halogen, or pseudohalogen such as –CN, or $-NO_2$ (i.e. dealkylation pathways), is initiated by the ferryl oxygen abstracting a hydrogen atom from the substrate. Hydroxyl transfer from the iron to the transient carbon radical of the substrate then forms the alcohol product. With substrates having a relatively low oxidation potential, e.g. certain amines (Miwa *et al.* 1983) dihydropyridines (Lee *et al.* 1988), polycyclic aromatic hydrocarbons (Cavalieri and Rogan 1985), or highly strained aliphatic hydrocarbons (Stearns and Ortiz de Montellano 1985), electron transfer from the substrate to the ferryl or oxo-haem moiety appears to initiate metabolism. On the other hand, olefin epoxidation (Hanzlik and Shearer 1978), aromatic hydroxylation (Korzekwa *et al.* 1989) and heteroatom (N, P, S) oxygenation involves direct addition of the ferryl oxygen to a π-bond or lone pair, although an electron transfer step preceding bond formation is not ruled out.

P450-catalysed benzylic hydroxylation is a particularly favourable pathway of metabolism for many drugs, natural products and organic chemicals. In terms of the general P450 mechanisms mentioned above, some cases of benzylic hydroxylation present an interesting potential ambiguity. For example, benzylic hydroxylation could logically be regarded as an aliphatic hydroxylation proceeding by a hydrogen atom transfer/hydroxyl radical recombination (HAT) mechanism (scheme I, see below); toluene metabolism appears to provide a simple example of

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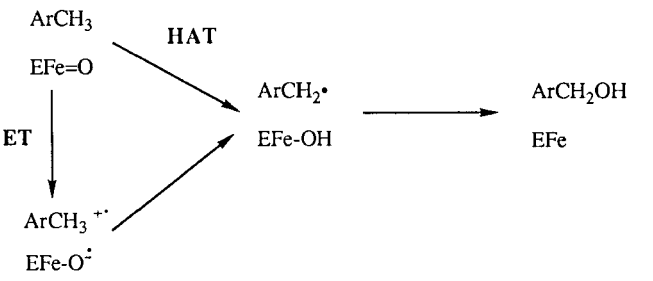

Scheme 1.

this (Ling and Hanzlik 1989). However, if the oxidation potential of the aromatic system were low enough, electron transfer (ET) followed by deprotonation, a well-documented (Eberson 1967, 1983, Schlesener and Kochi 1984, Baciocchi *et al.* 1991a, b) means of chemical oxidation at benzylic positions, might occur.

One widely used method for detecting radical or cation-radical intermediates involves introducing a cyclopropyl substituent into the substrate and searching for rearranged (i.e. cyclopropyl ring-opened) products (for a review see Suckling 1988). Other recent examples include the oxidation of *trans* 2-phenyl-1-methylcyclopropane by the non-haem monooxygenase of *Pseudomonas oleovorans* (Fu *et al.* 1991), and the use of *trans*-1,2-dimethylcyclopropane to determine the rate of hydroxyl rebound within the active site of P450 (Bowry and Ingold 1991). We recently demonstrated that arylcyclopropanes containing a benzylic hydrogen make suitable probes for differentiating HAT versus ET mechanisms in chemical systems which carry out benzylic oxidation (Riley and Hanzlik 1989). Specifically, free radical chlorination of cyclopropylbenzene (**1a**) leads to 1-chloro-1-phenylcyclopropane in yields up to 56%, indicating that if it is formed, the 1-phenylcyclopropyl radical can react to form cyclopropyl ring-intact products. On the other hand, 4-cyclopropylanisole (**1b**) reacts with the one-electron oxidant manganese(III)acetate to form exclusively the ring-opened diacetate corresponding to diol **9b** (Riley and Hanzlik 1989).

Our objective in the present work was to make use of this relatively clean dichotomous behaviour of arylcyclopropanes to probe the mechanism(s) by which P450 catalyses benzylic hydroxylation. An additional purpose was to verify an intriguing earlier report that the major metabolite of cyclopropylbenzene with phenobarbital-induced rat liver microsomes was benzoic acid (Suckling *et al.* 1982). To this end we investigated the P450-catalysed oxidation of cyclopropylbenzene (**1a**) and 4-cyclopropylanisole (**1b**). Both substrates were found to undergo efficient benzylic hydroxylation to 1-arylcyclopropanol metabolites; *O*-demethylation of **1b** and aromatic hydroxylation of **1a** were also observed. However, no evidence of ring-opened metabolites, including benzoic acid, was found. The kinetics of product formation suggest that cyclopropylbenzene is a suicide substrate for one or more isozymes of P450. Enzymatic mechanisms accounting for these observations are proposed.

Materials and methods

Cyclopropylbenzene, propiophenone, cinnamyl alcohol and benzoic acid were obtained from Aldrich (Milwaukee, WI, USA) and used as received. All solvents were obtained from Fisher Scientific (Fairlawn, NJ, USA). Solid-phase extraction cartridges (SPECs; 100 mg, C18) were obtained from

Varian (Harbor City, CA, USA). Glucose 6-phosphate, glucose 6-phosphate dehydrogenase, and β-nicotinamide adenine dinucleotide phosphate ($NADP^+$) were obtained from Sigma Chemical Co. (St Louis, MO, USA). Glucose 6-phosphate and $NADP^+$ were stored at −10°C as dry powders and reconstituted in buffer (pH 7·4, 0·1 M phosphate containing 1 mM EDTA) immediately prior to use. Glucose 6-phosphate dehydrogenase was reconstituted in buffer (500 IU/ml) and stored in a refrigerator until use. Cofactor-generating solutions were prepared immediately prior to microsomal incubations and contained 28·4 mg glucose 6-phosphate, 8·4 mg $NADP^+$ and 20 μl glucose 6-phosphate dehydrogenase (500 IU/ml) per ml of phosphate buffer. The incubations were started by addition of 50 μl cofactor solution/mg of microsomal protein.

Microsomal preparation

Microsomes were prepared from rat pretreated with sodium phenobarbital (PB; 50 mg/kg × 3 days i.p. in saline) or β-naphthoflavone (BNF; 50 mg/kg × 4 days i.p. in corn oil) using the method of Narasimhan *et al.* (1988) with the exception that KCl was omitted from the buffer. Control microsomes were prepared in a similar manner from untreated animals. Protein concentrations were measured using the Bradford assay (Bradford 1976) and microsomes were stored −78°C at concentrations of 10–15 mg protein/ml.

Microsomal incubations

Incubations were conducted in 13 × 100 mm culture tubes with Teflon-lined screw caps and typically contained 4·0 mg microsomal protein and 3·4 μmol substrate (added neat) in a volume of 2·0 ml. After preincubation at 35°C for 3 min, 0·2 ml NADPH regenerating cofactor was added and the sealed tubes were then incubated for 0–60 min.

Workup method A. The reaction was quenched by the addition of 30 μl 12 N HCl, vortexed and then centrifuged at 2000 *g* for 5 min to sediment protein. The supernatant was then applied to a preconditioned (methanol then water) C18 SPEC and unretained material was eluted (2 ml/min) with gentle suction. The SPEC was then washed with water (2 ml). Metabolites were eluted with diethyl ether (2 ml), and derivatized with ethereal diazomethane; 2,4,6-trimethylphenol (35 μl, 1·08 mg/ml, 309 nmol) was added as an internal standard prior to analysis by GC-MS.

Workup method B. The reaction was quenched by the addition of 1·0 ml ether: pentane (1 : 3), vortexed, and the organic layer was transferred using a pipette into a clean, dry test tube containing sodium sulphate. Further extractions were carried out (2 × 0·5 ml ether : pentane (1 : 3)) and the organic layers for each incubation pooled. *n*-Decane (63·4 nmol in 100 μl pentane) was added as an internal standard prior to concentration to approximately 20 μl using a microconcentrator (Slaughter *et al.* 1993). The resulting sample was analysed by glc or GC-MS after addition of 50 μl heptane.

Analytical procedures

Extracts of metabolites prepared by method A, along with various authentic standards, were examined by glc using a DB-5 column (5% vinyl methyl silicone, 15 m × 0·53 mm id, 1·5 μ film thickness; J&W, Rancho Cordova, CA, USA) using a 'ballistic' nonlinear temperature programme at low heater power. Peak detection was by flame ionization detection and quantitation was by electronic integration. In other cases analyses were performed on a Hewlett Packard GC-MSD (model 5890 series 2 GC, model 5971A mass selective detector) operating in the electron impact mode at 70 eV. The gas chromatograph used a capillary DB-5 column (30 m × 0·23 mm id, 1·5 μ film thickness; J&W). Oven temperature programming is reported as: initial temperature (°C), hold-time (min), ramp (°C min^{-1}), final temperature (°C), and hold time (min). Peak detection and quantitation were carried out in either full spectrum or selected ion modes. Metabolite quantitation by GC-MS was based on plots of detector response ratio versus mole ratio (analyte/internal standard) of known solutions. Reported detection limits are the lowest concentrations of analyte actually used and reflect a signal-to-noise ratio of ≥ 5; in most cases the actual limit of detection would be considerably less than that reported. Silica gel was used for all preparative chromatography.

Synthesis of standards

1-Phenylcyclopropanol (**2a**) was prepared by a modification of the synthesis of 1-methylcyclopropanol reported by DePuy *et al.* (1964). Magnesium turnings (0·48 g, 0·02 mol) were added to a three-necked flask containing freshly distilled ether. Bromobenzene (3·14 g, 0·02 mol) in ether was added slowly via an addition funnel. The reaction was kept under a nitrogen atmosphere and stirred at room temperature until all the magnesium had been consumed and the solution was grey. 1,3-Dichloroacetone (2·54 g, 0·02 mol) in ether was slowly dripped onto the Grignard reagent and the reaction stirred for 30 min. Next, ethylmagnesium bromide (100 ml, 2 M solution in THF, 0·02 mol) and an ethereal solution of anhydrous ferric chloride (250 mg/50 ml) were added simultaneously from separate addition funnels over the course of 1 h; copious evolution of gas (ethane) occurred. The reaction was left to stir at room temperature overnight during which time a black sludge formed. The supernatant was decanted into

2 N HCl (15 ml) saturated with NH_4Cl over crushed ice (100 g). The organic layer was separated, washed with saturated sodium bicarbonate, dried over sodium sulphate and concentrated *in vacuo* without heating to remove the solvents. Column chromatography (ethyl acetate : hexanes, 1 : 3) yielded a colourless oil (440 mg, 16%) with the following characteristics:

^{1}H-nmr (500 MHz, $CDCl_3$): δ 7·3 (m, 3 H), 7·2 (m, 2 H), 2·3 (s, 1 H), 1·3 (m, 2 H), 1·1 (m, 2 H).
^{13}C-nmr (75 MHz, $CDCl_3$): δ 144·3, 128·4, 126·4, 124·4, 56·6, 17·9.
EI-MIS m/z (%): 134 (M^+, 32), 133 (100), 105 (71), 77 (46).

1-(4′-Methoxyphenyl)cyclopropanol (**2b**) was synthesized from 4-bromoanisole in 15% yield using the same method as for **2a**; it had the following characteristics:

^{1}H-nmr (300 MHz, $CDCl_3$): δ 7·2 (dd, 2 H), 6·8 (dd, 2 H) 3·75 (s, 3 H), 1·1 (m, 2 H), 0·9 (m, 2 H).
^{13}C-nmr (75 MHz, $CDCl_3$): δ 159, 136, 131, 127, 114, 56, 17.
GC-MS (DB-5, 60-5-10-200-0, R_t 14·8 min) m/z (%): 164 (M^+, 47), 163 (61), 149 (17), 135 (100), 133 (57), 77 (55).

4-Cyclopropylphenol (**3**) was synthesized from **1a** using a sequence of acetylation, oxidation and hydrolysis.

4′-Acetylcyclopropylbenzene was prepared as described by Hart *et al.* (1968). Vacuum distillation (190°C, 10 mmHg) afforded a 64% yield of a colourless oil which solidified on standing in the refrigerator, it had the following characteristics:

^{1}H-nmr ($CDCl_3$, 300 MHz): δ 7·84 (d, 2 H), 7·09 (d, 2 H), 2·54 (s, 3 H), 1·88 (m, 1 H), 1·08 (m, 2 H), 0·78 (m, 2 H).
^{13}C-nmr ($CDCl_3$, 125 MHz): δ 197·0, 150·8, 135·0, 128·9, 125·8, 26·9, 16·1, 10·8.
EI-MS m/z (%): 160 (M^+, 21), 145 (100), 115 (44).

4′-Cyclopropylphenyl acetate. 4′-Acetylcyclopropylbenzene (450 mg, 2·81 mmol) was dissolved in 10 ml methylene chloride in a culture tube cooled in ice. *m*-Chloroperbenzoic acid (MCPBA, 85%, 1 g, 6·8 mmol) was added to the tube and stirring begun. Trifluoroacetic acid catalyst (Canan Koch and Chamberlain 1989) (200 μl) was added in 25-μl aliquots while the tube was standing on ice. The MCPBA dissolved completely giving a colourless solution. The tube was capped tightly, covered with foil and left to stir at room temperature for 3 days. During this time the reaction was monitored by tlc (5% ethyl acetate/hexane); a white precipitate formed and the supernatent changed from colourless to yellow. The precipitate was removed and discarded. The filtrate was washed successively with 10% sodium sulphate solution, saturated sodium bicarbonate solution and distilled water and dried over over sodium sulphate. After concentration *in vacuo*, 301 mg of a yellow oil was obtained (61% crude yield). Further purification by column chromatography using 5% (v/v) ether/hexane as eluent gave a colourless oil (151 mg, 31% yield) with the following characteristics:

^{1}H-nmr ($CDCl_3$, 300 MHz): δ 7·1 (d, 2 H), 6·9 (d, 2 H), 2·25 (s, 3 H), 1·90 (m, 1 H), 0·95 (m, 2 H) 0·65 (m, 2 H).
^{13}C-nmr ($CDCl_3$, 125 MHz): δ 169·7, 148·4, 141·5, 126·6, 121·3, 21·1, 14·9, 9·1.
EI-MS m/z (%): 176 (M^+, 10), 135 (53), 134 (92), 133 (79), 77 (42), 43 (100).

4-Cyclopropylphenol (**3**). 4′-Cyclopropylphenyl acetate (57·1 mg, 0·32 mmol) was added to 5 ml tetrahydrofuran (THF; freshly distilled from Na/benzophenone) in a culture tube. Lithium aluminum hydride (LAH, 40 mg, 1 mmol) was added to the tube which was then capped and left to stir at room temperature. Excess LAH was quenched by the addition of ethyl acetate and the organic layer separated from the precipitate by centrifugation and decantation. After evaporation, purification by column chromatography using $CHCl_3$ as eluent gave 8·6 mg (20%) of the desired phenol having the following characteristics:

^{1}H-nmr (CDCl, 300 MHz): δ 6·8 (d, 2 H), 6·6 (d, 2 H), 4·55 (s, 1 H), 1·7 (m, 1 H), 0·8 (m, 2 H), 0·5 (m, 2 H).
^{13}C-nmr ($CDCl_3$, 125 MHz): 153·4, 136·0, 127·0, 115·1, 14·6, 8·5.
EI-MS m/z (%): 134 (M^+, 87), 133 (100), 107 (50), 105 (37), 77 (36).

2-Cyclopropylphenol (**4**) was synthesized from **1a** using a sequence of nitration, reduction, separation of the isomeric anilines, diazotization and hydrolysis. Nitration of cyclopropylbenzene as described by Hahn *et al.* (1968) gave an oil whose nmr spectrum and GC-MS analysis were consistent with a 2 : 1 mixture of 2- and 4-nitrophenylcyclopropane. GC-MS (DB-5, 60-5-10-200-0). Para isomer, R_t 16·7 min; m/z (%): 163 (M^+, 97), 115 (100), 91 (52). Ortho isomer, R_t 15·2 min; m/z (%): 163 (M^+, 3), 135 (100), 91 (92), 79 (82), 77 (81).

The above mixture of 2- and 4-nitrophenylcyclopropane (0·77 g, 4·7 mmol) was transferred to a Parr flask using 25 ml absolute ethanol and Adams catalyst (platinum oxide, 25 mg) was added. The flask was evacuated and flushed with hydrogen three times and then shaken in a hydrogen atmosphere for 6 h at 45 p.s.i. After 6 h the catalyst was removed by filtration and the solvent evaporated yielding 0·75 g of a yellow oil. Purification by column chromatography (5% ether/hexanes) yielded two major fractions. The first-eluting was 2-cyclopropylaniline (274 mg, 36%) and the second was 4-cyclopropylaniline (154 mg, 20%); they had the following characteristics:

2-Cyclopropylaniline. ^{1}H-nmr ($CDCl_3$, 500 MHz): δ 7·1 (t, 2 H), 6·75 (m, 2 H), 4·0 (s, 2 H), 1·7 (m, 1 H), 0·95 (m, 2 H), 0·65 (m, 2 H).
^{13}C-nmr ($CDCl_3$, 125 MHz) δ 146·3, 128·5, 127·2, 126·9, 118·2, 114·5, 11·4, 5·0.
EI-MS *m/z* (%) 133 (M^+, 100), 132 (83), 118 (79).

4-Cyclopropylaniline. ^{1}H-nmr ($CDCl_3$, 500 MHz): δ 6·93 (d, 2 h), 6·64 (d, 2 H), 3·42 (s, 2 H), 1·83 (m, 1 H), 0·88 (m, 2 H), 0·62 (m, 2 H).
^{13}C-nmr ($CDCl_3$, 125 MHz): δ 144·0, 133·9, 126·8, 115·3, 14·6, 8·3.
EI-MS *m/z* (%): 134 (8), 133 (M^+, 81), 132 (100), 106 (87).

2-Cyclopropylphenol (**4**). 2-Cyclopropylaniline (200 mg, 1·5 mmol) was added to 16% (v/v) aqueous sulphuric acid at 0°C. A white precipitate formed which dissolved upon addition of a cold solution of sodium nitrite (106 mg, 1·5 mmol) in water (2·5 ml). The solution was left to stir at 0°C for 1 h to ensure complete formation of the diazonium salt. The cold diazonium salt was added dropwise to 5 ml boiling 10% (v/v) sulphuric acid in a Claisen distillation apparatus at a rate sufficient to continue distillation. The distillate changed from colourless to yellow indicating that product was being collected. Distillation was stopped when the distillate was colourless again. On cooling a brown oil separated from the aqueous phase of the distillate and was extracted into ether. After drying over sodium sulphate the ether was removed by rotary evaporation to yield a brown tar. Further purification by column chromatography using chloroform as the eluent resulted in the isolation of several bands of coloured material. The major product, 2-cyclopropylphenol (9·6 mg), had the following characteristics:

^{1}H-nmr (500 MHz), $CDCl_3$): δ 7·2 (m, 2 H), 6·9 (m, 2 H) 5·45 (s, 1 H), 1·9 (m, 1 H), 1·0 (m, 2 H), 0·7 (m, 2 H).
^{13}C-nmr (125 MHz, $CDCl_3$): δ 155·4, 128·7, 127·7, 127·4, 120·4, 114·6, 9·2, 5·3.
IR ($CDCl_3$) cm^{-1}: 3020, 1215, 910, 760, 735.
GC-MS (DB-5, 50-0-10-250-0, R_t 7·5 min) *m/z* (%): 134 (M^+, 100), 133 (48), 119 (42), 115 (34).

1-Phenyl-2-propen-1-ol (**6**). Vinyl magnesium bromide (100 mmol, 10 ml 1·0 M solution in THF) was carefully added via a syringe to freshly distilled THF in a dry 100 ml three-necked round-bottom flask fitted with a condenser and maintained under a nitrogen atmosphere. A solution of benzaldehyde (1·06 g, 100 mmol) in THF (10 ml) was dripped slowly into one of the necks of the flask via a pressure equalizing dropping funnel. Upon addition of benzaldehyde the solution changed from a deep red to a yellow colour with evolution of gas. The reaction mixture was then heated to reflux for 4 h and quenched by pouring onto a mixture of ice (50 g) and glacial acetic acid (10 ml). The resulting mixture was then brought to pH7 with sodium bicarbonate, extracted with ether, and the ether dried over sodium sulphate. Concentration *in vacuo* gave 0·31 g yellow oil. Column chromatography of this fraction yielded 0·21 g yellow oil, which was further purified by sublimation giving 170 mg colourless oil with the following characteristics:

^{1}H-nmr (300 MHz, $CDCl_3$): δ 7·30–7·15 (m, 5 H) 6·0–5·9 (m, 1 H) 5·25 (dt, 1 H) 5·1 (d, 1 H) 4·6 (s, 1 H).
GC-MS (DB-5, 60-5-10-200-0, R_t 10·9 min) *m/z* (%): 134 (M^+, 64), 133 (100), 105 (74), 92 (61), 77 (75).

1-Phenyl-1,3-propanediol (**9a**). Cyclopropylbenzene (500 mg, 4·24 mmol) was added to a round-bottom flask containing a suspension of lead tetraacetate (Ouellette and Shaw 1964) (4·24 g, 10 mmol) in glacial acetic acid (25 ml). The mixture was then warmed to 70°C and left stirring overnight. There was a gradual dissolution of the lead tetraacetate to give an almost clear solution. The reaction mixture was cooled to room temperature, transferred to a large Erlenmeyer flask standing in an ice bath, and neutralized by addition of 10 ml water and solid sodium bicarbonate until effervescence had ceased and pH was around 7. The solution was then extracted with ether, and the ether dried over sodium sulphate and concentrated *in vacuo* to yield 1·07 g of a faint yellow-coloured oil. Tlc (30% ether/hexane, v/v) showed the presence of two spots the first (R_f 0·72) of which was isolated by column chromatography on a small scale (100 mg) and shown by GC-MS to contain diacetate product(s) (DB-5 60-5-10-200 R_t 14·7, 15·0 min). This fraction was then reduced with LAH by refluxing in THF overnight. Excess LAH was quenched with saturated tartaric acid solution and filtered. The filtrate was dried over sodium sulphate and the THF removed *in vacuo*. Column chromatography (50% ether/hexane, v/v) yielded three fractions; the desired diol (0·76 g, 70%) eluted last and had the following characteristics:

^{1}H-nmr (300 MHz, $CDCl_3$): δ 7·3–7·15 (m, 5 H), 4·85 (dd, 1 H) 3·75 (t, 2 H), 1·88 (m, 1 H).
^{13}C-nmr (75 MHz, $CDCl_3$): δ 144·7, 129·0, 128·9, 128·0, 126·1, 74·7, 61·8, 40·8.
GC-MS (DB-5, 60-5-10-200-0, R_t 14·6 min) *m/z* (%): 152 (M^+, 24), 134 (10), 107 (100), 79 (86), 77 (50).

1-(4′-Methoxyphenyl)-1,3-propane diol (**9b**) was synthesized in 36% yield from 4-cyclopropylanisole using the same method as for **8a**. It had the following characteristics:

^{1}H-nmr (300 MHz, $CDCl_3$): δ 7·20 (d, 2 H), 6·80 (d, 2 H), 4·85 (q, 1 H), 3·75 (m, 5 H), 3·3 (bs, 1 H), 2·3 (bs, 1 H), 1·85 (m, 2 H).
^{13}C-nmr (75 MHz, $CDCl_3$): δ 144·7, 128·9, 127·9, 126·2, 74·7, 61·8, 55·7, 40·8.
GC-MS (DB-5, 60-5-10-200-10, R_t 18·2 min) *m/z* (%): 182 (M^+, 9), 137 (100).

2-Phenyl-1,3-propane diol (**10a**). Diethyl phenylmalonate (0·69 g, 2·9 mmol) was weighed into a clean, dry 25 ml round-bottom flask. Ether (freshly distilled from CaH_2, 20 ml) was added and the resulting solution cooled over crushed ice while lithium aluminum hydride (LAH, 200 mg, 5·2 mmol) was added. After the addition of LAH was complete the flask was allowed to warm to room temperature and reflux was initiated. After 3 h heat was removed and the reaction was left to stir at room temperature overnight. Excess LAH was quenched with saturated tartaric acid solution and filtered. The filtrate was dried over sodium sulphate and the THF removed *in vacuo*. The resulting oil was dissolved in ether to which hexane was added to give a cloudy appearance. Upon standing overnight, colourless needles (0·37 g, 84%) formed. The product had the following characteristics:

Mp 50°C; lit. (Searles *et al.* 1960) mp 49·5–50·5°C.
^{1}H-nmr (300 MHz, $CDCl_3$): δ 7·36–7·18 (m, 5 H), 3·95 (m, 4 H), 3·10 (m, 1 H), 2·6 (bs, 2 H).
^{13}C-nmr (75 MHz): δ 140·0, 129·2, 128·5, 127·2, 66·5, 50·1.
GC-MS (DB-5, 60-5-10-200-0, R_t 15·0 min) m/z (%): 152 (M^+, 1), 134 (1), 104 (100), 103 (43).

Results

Separation and quantitation of metabolic standards

Potential metabolites of cyclopropylbenzene (**1a**, figure 1) were purchased or synthesized and used to evaluate glc-based analytical methods for their detection, identification and quantitation. Figure 2A shows a typical glc separation of several authentic standards of potential mono-hydroxylated derivatives of **1a**, together with 2,4,6-trimethylphenol as an internal standard and methyl benzoate (a derivative of a reported metabolite of **1a**). Additional retention time data are given in table 1, along with information on the efficiency with which the standards could be recovered from microsomal incubations to which they were added, and an indication of their detectability with signal-to-noise ratio ≥ 5.

The observation (table 1) that propiophenone (**5a**) has the same retention time on a DB-5 column as its isomer 1-phenylcyclopropanol (**2a**) was initially of some concern, since **2a** decomposes to **5a** under acidic, basic and thermal conditions, but several control experiments (not shown) established that the latter does not isomerize to the former under the analytical conditions employed and that **5a** is absent from incubations of **1a**. For example, authentic standards of propiophenone and 1-phenylcyclopropanol were added to microsomal incubations which were then extracted using method A. The samples were then analysed by glc using a DB-17 (50% phenyl methyl silicone) column (80°C) which gave good separation of **2a** and **5a**. The retention time of the peaks were then compared with authentic standards which were analysed directly. Unfortunately routine analysis of microsomal incubations could not be made using this column as other compounds of interest were not adequately resolved, so the DB-5 column was chosen for general use.

Microsomal oxidation of cyclopropylbenzene (**1a**)

Direct glc examination of extracts of incubations of cyclopropylbenzene (**1a**) with PB-microsomes revealed the presence of two metabolite peaks with retention times consistent with a mono-oxygenated cyclopropylbenzene structure (figure 2B, peaks 2 and 3). Glc comparison of these two metabolites to various authentic standards of potential metabolites of **1a** (figure 2A, table 1) as well as comparisons of their EI-MS to those of the standards (not shown) revealed that the microsomal metabolites were 1-phenylcyclopropanol (**2a**) and 4-cyclopropylphenol (**3**), respectively. Other $C_9H_{10}O$ isomers which might have been formed as metabolites (e.g. compounds **5a–8a**) were not detected. 2-Cyclopropylphenol (**4**) was observed only with BNF-induced microsomes.

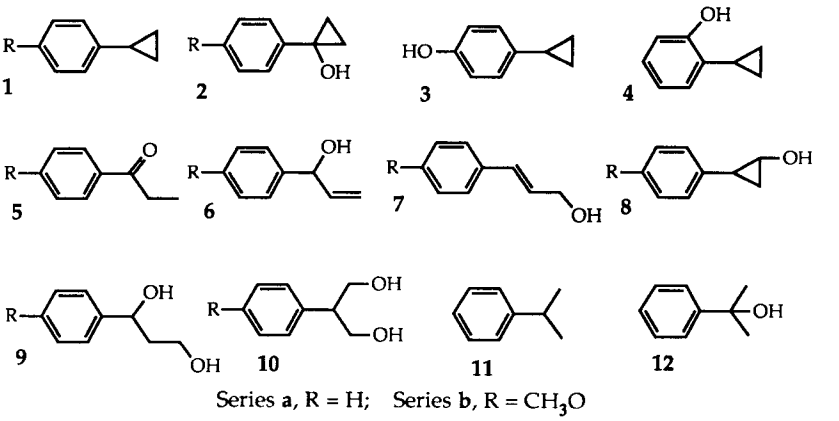

Figure 1. Structures of possible metabolites of cyclopropylbenzene (**1a**) and 4-cyclopropylanisole (**1b**). Compounds are numbered to correspond to the text.

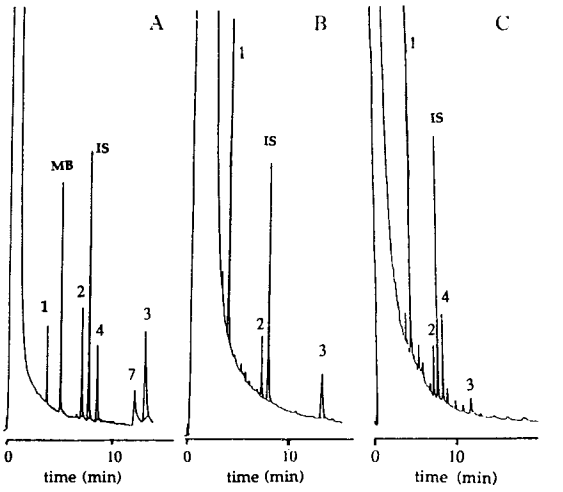

Figure 2. Glc profiles of synthetic standards and actual metabolites of cyclopropylbenzene (**1a**). Peaks are numbered to correspond to structures in figure 1 (series a). (A) Authentic standards (MB = methyl benzoate; (B) and (C) metabolites of **1a** formed by PB-induced or BNF-induced rat liver microsomes, respectively. Incubations contained 4 mg microsomal protein, 0·2 ml NADPH regenerating system and 3·4 μmol cyclopropylbenzene in a total volume of 2·2 ml. After 30 min the incubations were extracted and derivatized using method A and analysed by glc (DB-5 megabore, 60–100°C using a nonlinear 'ballistic' thermal gradient).

Since it has been reported that the major metabolite of cyclopropylbenzene with phenobarbital-induced rat liver microsomes is benzoic acid (Suckling *et al.* 1982), a specific search of our incubation mixtures was made for this compound. Control experiments in which benzoic acid (50 nmol) was added to a microsomal incubation, extracted (method A), derivatized with diazomethane and analysed by glc (DB-5, 60–110°C) showed that benzoic acid would easily have been detected (as its methyl ester; table 1) had it been formed as a metabolite of **1a** under typical microsomal incubation conditions. However, within the limit of detection (approximately 1% of total metabolites), none was observed. We conclude, therefore, that contrary to earlier reports, benzoic acid is not formed as a metabolite of cyclopropylbenzene by either control, PB- or BNF-induced rat liver microsomes.

One-electron oxidation of arylcyclopropanes is known to lead to fragmentation of the cyclopropane ring and the production of 1-aryl-1,3-disubstituted propane derivatives (Riley and Hanzlik 1989). Thus a search was made for diols **9a** and **10a** as potential metabolites of **1a**. As shown in figure 3B, these materials are easily

Table 1. Retention times, limits of detection and recoveries of potential cyclopropylbenzene metabolites.

Compound	Retention time[a] (min)	Recovery efficiency[b] (%)	Detectability[c] (nmol/incubation)
Methyl benzoate	5·4	93	< 13
1-Phenylcyclopropanol, **2a**	7·2	92	< 88
2-Cyclopropylphenol, **4**	9·2	100	< 64
4-Cyclopropylphenol, **3**	14·8	103	< 42
Cinnamyl alcohol, **7a**	13·4	95	< 35
Propiophenone, **5a**	7·2	nd	< 32

[a] GC conditions: DB-5 column, 15 m × 0·53 mm, oven temperature programmed 60–110°C using a nonlinear 'ballistic' gradient. See figure 2A for typical chromatogram.

[b] Workup method A (see Materials and methods).

[c] Numbers present the lowest amounts actually detected in control experiments; limits of detection were not pursued but would have been much lower.

nd, Not detected.

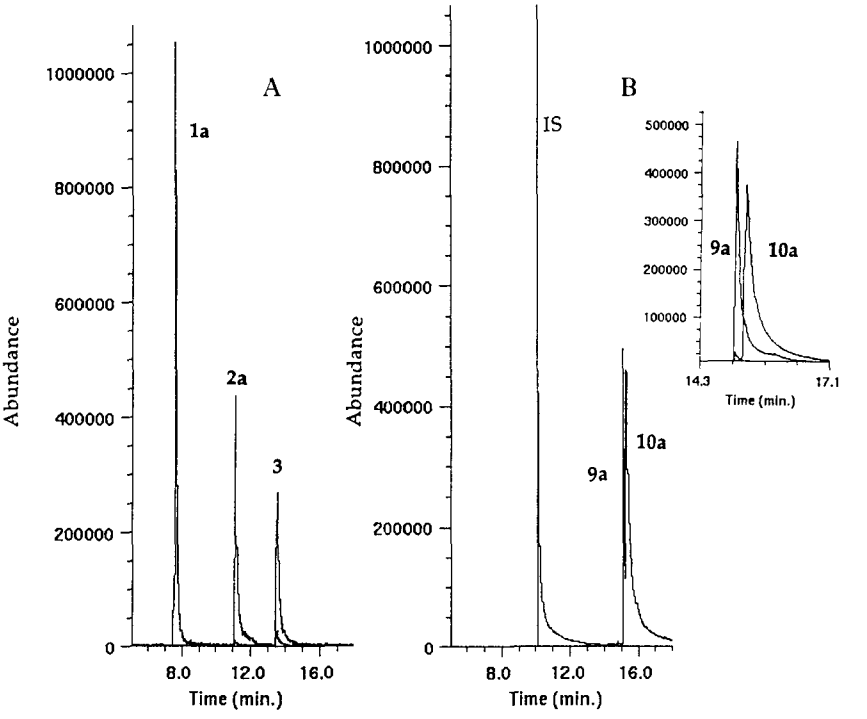

Figure 3. GC-MS comparison of the diol region of chromatograms from (A) extract (method A) of an incubation of cyclopropylbenzene with PB-induced rat liver microsomes (conditions similar to figure 2B); and (B) the same extract of these microsomes spiked with diol standards **9a** (62 nmol/incubation) and **10a** (76 nmol/incubation). The MSD was operated in selected ion monitor mode (SIM) and chromatograms integrated for the ion current under the base peak of each metabolite (*m*/*z* 133, 133, 107 and 104 for **2a**, **3**, **9a** and **10a**, respectively). IS refers to the internal standard (4-methylbenzyl alcohol) used to determine the recovery of the diols from microsomal incubations. The inset details the separation of diol isomers under these chromatographic conditions.

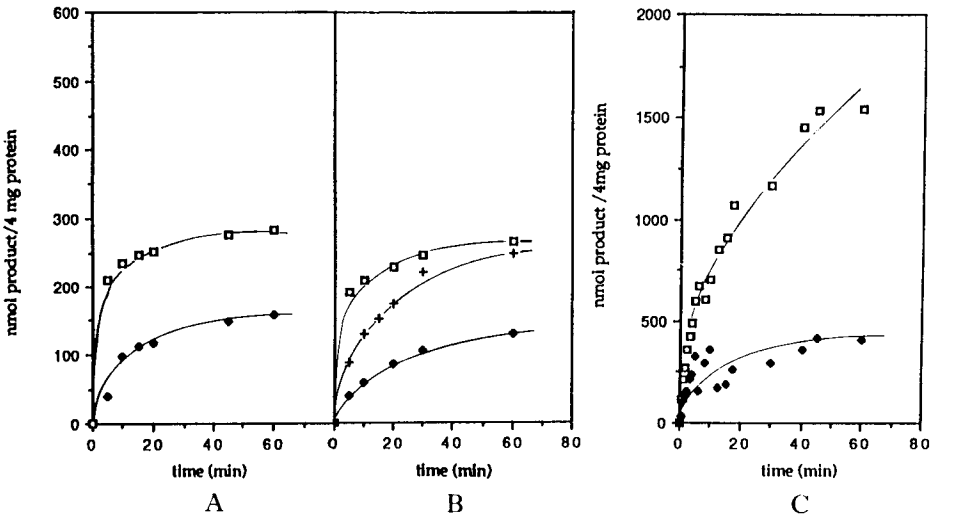

Figure 4. Formation of 1-phenylcyclopropanol (□), 4-cyclopropylphenol (♦) and 2-cyclopropylphenol (+) from cyclopropylbenzene by liver microsomes from (A) untreated rats, (B) BNF-induced rats, and (C) PB-induced rats. Each incubation contained 4 mg microsomal protein, 0·2 ml NADPH regenerating system and 3·4 μmol cyclopropylbenzene in 2·2 ml total volume. After extraction (method A) samples were analysed by glc (DB-5 megabore, 60–110°C using a non-linear 'ballistic' thermal gradient). Each symbol represents a single determination and each time course depicts a single representative example.

detected in microsomal extracts if they are added as standards, but they were not detected as metabolites of **1a** (figure 3A). This result is also consistent with the absence of **6a** and **7a** as microsomal metabolites of **1a** as noted above.

Effect of induction status on microsomal oxidation of cyclopropylbenzene

Oxidation of **1a** by microsomes from untreated animals gave **2a** and **3** as the only detectable products. As shown in figure 4A, formation of both **2a** and **3** decelerated and stopped, in parallel and unusually rapidly, after consumption of only 10–12% of the available substrate, suggesting that enzyme inactivation by a reactive intermediate formed during metabolism of **1a** might be taking place. Induction of P450 with phenobarbital showed a six-fold overall increase in the formation of **2a** from **1a**, while formation of phenol **3** increased only about four-fold compared with control microsomes (figure 4C). The rate of formation of both **2a** and **3** were both markedly nonlinear, but formation of phenol **3** decelerated and stopped much more rapidly than formation of **2a**, indicating that more than one isozyme of P450 is responsible for the overall metabolism of **1a** in microsomes.

Formation of **2a** and **3** from **1a** was also seen (figure 4B) with microsomes from BNF-induced rats at concentrations similar to those of control microsomes. In addition, a third metabolite, 2-cyclopropylphenol (**4**), which was not detected with control and PB-microsomes was also observed, which is in agreement with other reports of 2-hydroxylation of mono-substituted aromatic compounds by BNF-inducible isozymes (Lau and Zannoni 1979, 1981, Nakajima *et al.* 1991). Formation of all three metabolites decelerated rapidly, as in the case of both control and PB-microsomes (figure 4A and C).

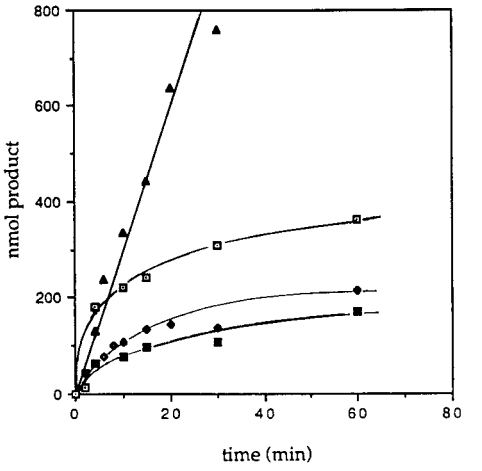

Figure 5. Formation of cumyl alcohol from cumene in the absence (△) and presence (□) of phenylcyclopropane. Also depicted is the formation of 1-phenylcyclopropanol from cyclopropylbenzene in the absence (◇) and presence (■) of cumene. Incubations contained 4 mg PB-induced microsomal protein, 0·2 ml NADPH regenerating system, and either 3·4 μmol of cyclopropylbenzene, 3·4 μmol of isopropylbenzene or 1·7 μmol of each, in a total volume of 2·2 ml. Incubations were quenched and extracted using method B and analysed by GC-MS (DB-5, 60-5-10-200) operating in full scan mode. Each symbol represents a single determination and each time course depicts a single representative example.

Time course of metabolism of cyclopropyl- versus isopropylbenzene

Several cyclopropane derivatives other than **1a** are known to act as metabolism-dependent inactivators (i.e. suicide substrates) of P450 enzymes (Hanzlik *et al.* 1979, Guengerich *et al.* 1984, Tullman and Hanzlik 1984). The marked non-linearity of the metabolism of **1a** by all types of microsomes (figure 4) could be one indication of suicide substrate activity on the part of **1a**. To investigate this, an analogue of **1a** was sought which would lack the cyclopropyl group but otherwise have a similar size and shape (so that it would likely be a potential substrate for the same isozymes of P450 which oxidize **1a**); isopropylbenzene (i.e. cumene, **11**) was chosen for this purpose.

As shown in figure 5, the metabolism of cumene to cumyl alcohol (**12**, essentially the only detectable metabolite from cumene with PB-microsomes) is very rapid and very nearly linear for at least 20 min. The slight slowing may result from substrate depletion, which amounts to 46% after 30 min. In contrast, the formation of **2a** from **1a** decelerates markedly during this interval, even though much less consumption of substrate has occurred. However, when the metabolism of cumene is monitored in the presence of **1a**, the initial rate of formation of cumyl alcohol is decreased slightly, but rather than remaining essentially constant over 20 min, it too decelerates rapidly, just like that of **1a** oxidation. These results are consistent with the idea that compound **1a** (but not cumene) is a suicide substrate for one or more isozymes of P450 which contribute significantly to the benzylic hydroxylation of both **1a** and cumene; other possible interpretations are discussed below.

Microsomal metabolism of 4-cyclopropylanisole (**1b**)

The incorporation of the methoxy substituent in **1b** makes it more easily subject to one-electron oxidation than **1a** (Shono and Matsumura 1970). Thus the

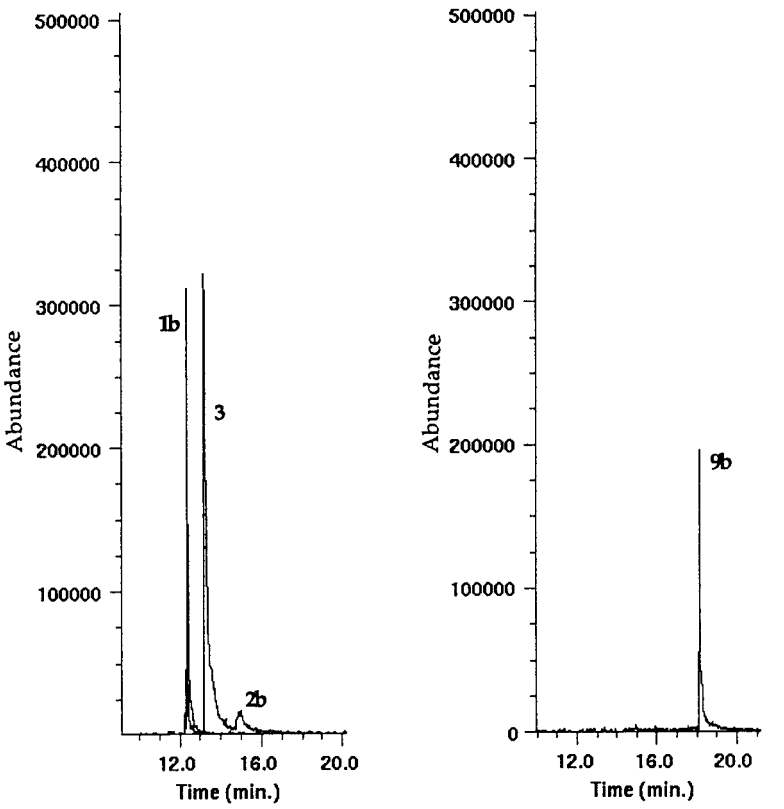

Figure 6. Chromatographic profiles of incubation of 4-cyclopropyl-anisole with PB-induced microsomes (left) and authentic standard of 1-(4'-methoxyphenyl)-1,3-propane diol **9b** (right). The incubations contained 4 mg microsomal protein, 0·2 ml NADPH regenerating system, and 2·5 μmol 4-cyclopropylanisole in 2·2 ml total volume. After incubation for 30 min the incubation was quenched and extracted using method A and analysed by GC-MS (DB5, 60-5-10-200-10). The MSD was run in SIM mode and data integrated for base peaks of each metabolite (m/z 133, 135 and 137 for **3**, **2b** and **9b**, respectively).

metabolism of **1b** was also examined for the possible formation of ring-opened products such as **5b–10b**. PB-induced rat liver microsomes were found to metabolize **1b** very efficiently. However, the primary (> 90%) route of metabolism was *O*-demethylation to produce **3**, along with a smaller amount of benzylic hydroxylation to produce **2b**, both of which were identified by GC-MS comparison to authentic standards (figure 6). No evidence for the formation of ring-opened products **5b–10b** was observed, even with the aid of several authentic standards (figure 1) and the sensitivity of the GC-MS.

Discussion

Cyclopropylarenes **1a** and **b** are efficiently oxidized by P450 enzymes from several different types of rat liver microsomes. The metabolites formed are those expected by analogy to known metabolic pathways for simple alkylbenzene derivatives, i.e. phenols from *O*-demethylation of **1b** or aromatic hydroxylation of **1a**, and benzylic alcohols from both **1a** and **b**. No evidence was found for ring-opened (i.e. phenylpropane-type) metabolites such as compounds **5–10**. Previous reports (Suckling *et al.* 1982, 1985) have claimed that the major metabolites of **1a**

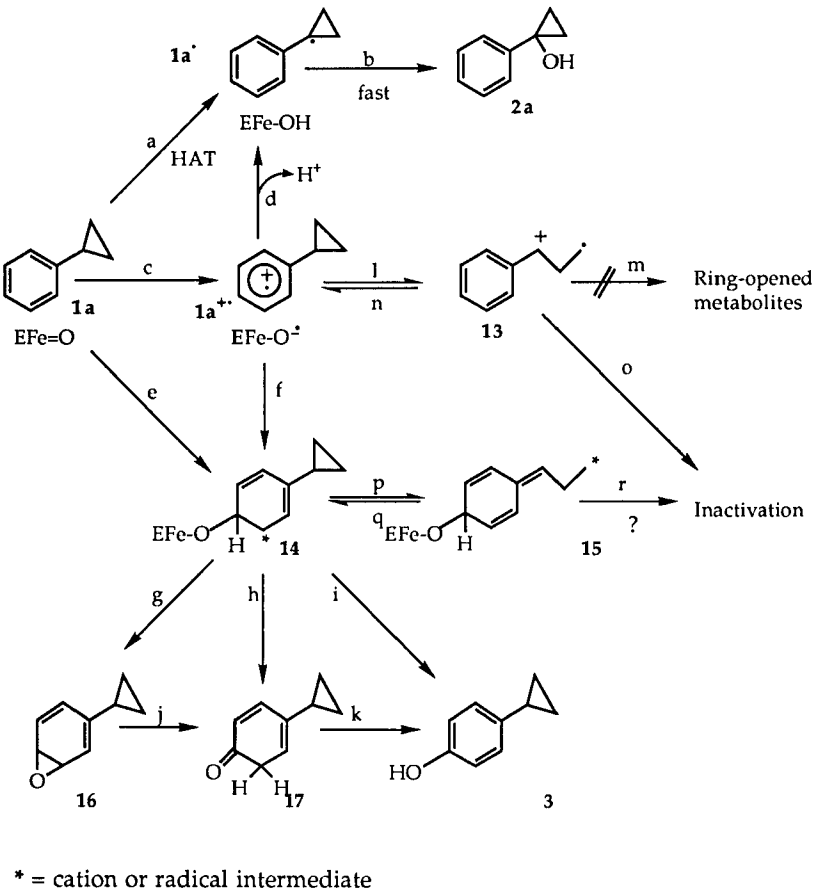

Figure 7. Suggested pathways for P450-catalysed product formation and enzyme inactivation by cyclopropylbenzene.

with PB-induced rat liver microsomes were benzoic acid (a six-electron oxidation product) and phenol **4**. However, in these reports product identification was apparently based solely on inferences drawn from hplc retention times; no spectroscopic characterization of metabolites was reported and except for benzoic acid, no authentic standards of potential metabolites were apparently available. The work described above clearly shows that control and PB-induced rat liver microsomes form neither benzoic acid nor phenol **4** as metabolites of **1a**, although phenol **4** is formed by BNF-induced microsomes.

O-demethylation of **1b** to **3**, like that of most other simple methyl ethers, probably involves a typical α-hydroxylation mechanism, while formation of phenols **3** and **4** from **1a** probably involves aromatic hydroxylation via classical mechanisms (Tomaszewski *et al.* 1975, Hanzlik *et al.* 1984, Korzekwa *et al.* 1989) as illustrated in figure 7 (i.e. steps e and g–k). The efficient formation of ring-intact cyclopropanol metabolites from both **1a** and **b**, together with the absence of ring-opened metabolites, contrasts with the behaviour of **1b** toward one-electron oxidants, which form exclusively ring-opened products related to diol **9** (Riley and Hanzlik 1989). On the surface, these P450-catalysed benzylic hydroxylations are

inconsistent with an ET mechanism (figure 7, steps c, d, b), but consistent with a hydrogen atom abstraction/recombination mechanism f (figure 7, steps a and b). However, one caveat (Bowry and Ingold 1991) which must be acknowledged is that while π cation-radicals of cyclopropylarenes undergo rapid effectively irreversible ring-opening reactions in solution (i.e. figure 7, step l), this process may not be feasible (or the reverse reaction, step n, may be accelerated) because of steric constraints imposed by the structure of the enzyme's active site. In this instance the absence of ring-opened metabolites is not necessarily inconsistent with an ET mechanism. We shall return to this point later (see below).

The induction of various P450 isoforms by pretreatment with either PB (which induces CYP2B1/2) or BNF (which induces CYP1A1) had differing but typical effects on the product profile of the enzymatic oxidation of **1a**. Microsomes induced with PB-showed a four to six-fold increase in the formation of **2a** and **3** compared with microsomes from untreated control rat. On the other hand induction with BNF did not appear to affect the conversion of **1a** to **2a** and **3**, but it did lead to the formation of a new metabolite (2-cyclopropylphenol, **4**) which was not observed in incubations using either control or PB-induced microsomes. Neither control nor induced microsomes formed any of the ring-opened metabolites **5a–10a**.

Perhaps the most remarkable feature of the enzymatic oxidation of **1a** is the markedly decelerating kinetics of formation of both **2a** and **3** by both control and induced microsomes. Other compounds containing cyclopropane rings have been reported to be mechanism-based inhibitors (suicide substrates) of P450 (Hanzlik *et al.* 1979, Guengerich *et al.* 1984, Tullman and Hanzlik 1984). It is possible that cyclopropylbenzene acts in an analogous fashion, i.e. beginning with electron transfer. To investigate this further we compared the kinetics of oxidation of **1a** with that of a 'simple' alkylbenzene which lacked the potentially reactive cyclopropane moiety. Isopropylbenzene (cumene, **11**), a saturated analogue of **1a**, lacks the reactivity of the cyclopropane ring but has otherwise very similar steric and lipophilic properties to **1a**; hence, it can reasonably be assumed to be metabolized by the same P450 isozymes as **1a**.

Incubation of cumene showed a rapid but constant rate of formation of 2-phenyl-propan-2-ol (cumyl alcohol, **12**) over the first 20 min of incubation, and only a slow decline in rate over the next 40 min, possibly due to substrate depletion, which amounted to almost 45% after 30 min (figure 5). However, in the presence of **1a**, the rate of formation of cumyl alcohol decelerated rapidly compared with incubations of cumene in which **1a** was not present. Neither substrate depletion nor product inhibition are likely to account for such a striking deceleration of metabolism. Nor is there any reason to expect that **1a** should be markedly more efficient than cumene at uncoupling the oxidations and/or leading to auto-oxidative destruction of P450. In our view, the dramatic deceleration of metabolism of **1a** (and of cumene when **1a** is present) is most readily explained by postulating the oxidation of **1a** to a reactive intermediate which partitions either to product formation or enzyme inactivation; the latter event would then account for the marked decelerations of the metabolism.

The apparent activity of cyclopropylbenzene but not isopropylbenzene as a suicide substrate suggests that the cyclopropyl moiety *per se* is intimately involved in the enzyme inactivation process. Several possible mechanisms for this are suggested in figure 7, along with pathways for formation of the known hydroxylated metabolites of **1a** (and **b**). As noted above, formation of benzylic alcohol **2a** is most

reasonably accounted for by a classical abstraction/recombination mechanism depicted as steps a and b in figure 7. The alternative electron abstraction route (steps c, d and b) is unlikely, based on the high oxidation potential of **1a** (Shono and Matsumura 1970) and the absence of small ring-opened metabolites such as **5a–10a**.

Formation of phenol **3**, the only other soluble metabolite of **1a**, must involve addition of the ferryl oxygen of P450 to C-4 of the aromatic ring to give an intermediate such as **14**. Whether this occurs as a single step process (step e) as suggested above, or via a preliminary electron-abstraction (i.e. steps c and f) can not be ascertained, but again, the latter seems rather unlikely based on the high oxidation potential of **1a**. Once the C-O bond is established at C-4, classical pathways of aromatic hydroxylation (Tomaszewski *et al.* 1975, Hanzlik *et al.* 1984, Korzekwa *et al.* 1989), with or without an arene oxide intermediate (**16**), with or without a NIH-shift (steps j or h versus i, respectively), can account for the formation of phenol **3**. In this context there are two conceivable ways to involve the cyclopropyl moiety of **1a** in reactive intermediate formation and enzyme inactivation. The first would involve steps c, l and o of figure 7, but as already mentioned the high oxidation potential of **1a** renders step c unlikely and the absence of ring-opened metabolites **5a–10a** fails to support **13** as a viable intermediate. A second and potentially more attractive mechanism occurs via steps p and r of figure 7, i.e. as a branch of the aromatic hydroxylation pathway rather than the benzylic hydroxylation pathway as originally anticipated. Direct formation of **14** via step e avoids the need to invoke formation of $\mathbf{1a}^{+\cdot}$ by an unlikely electron abstraction (step c), but still allows utilization of the strain energy of the cyclopropyl moiety to drive reactive metabolite formation via ring opening. The ortho-isomer variants of **14** and **15** could also explain the apparent suicide substrate behaviour of **1a** with the BNF-induced isozyme of P450 which carries out ortho hydroxylation (cf. figures 4A and B).

In conclusion, P450 catalysed oxidation of probes **1a** and **b** gives only cyclopropyl ring-intact products, the formation of which is most readily explained by a classical hydrogen atom abstraction/recombination mechanism or via arene oxidation mechanisms. An electron abstraction mechanism cannot be entirely ruled out, since the lack of ring-opened products could also be due to steric constraints of the enzyme active site as compared with free solution; however, in the case of **1a** electron abstraction would appear to be unlikely because of its high oxidation potential. The kinetics of product formation from **1a**, and the effect of **1a** on the metabolism of cumene, strongly implicate **1a** as a suicide substrate for one or more isozymes of P450. The reactive intermediate has not been identified but an argument can be made for its formation via the aromatic hydroxylation pathway rather than the benzylic hydroxylation pathway.

Acknowledgement

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