SUBSTRATE-ELICITED DISSOCIATION OF A COMPLEX OF CYTOCHROME P-450 WITH A METHYLENEDIOXYPHENYL METABOLITE

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SUMMARY The ^{14}C-isosafrole metabolite-rat hepatic cytochrome P-450 complex is stable to dialysis but is readily dissociated by cyclohexane (1 mM) to release free cytochrome P-450 and radioactive ligand. The ratio of cytochrome P-450:isosafrole metabolite in the complex is unity.

INTRODUCTION

Certain methylenedioxyphenyl (MDP)[1] compounds are metabolized by the hepatic mixed function oxygenase system, both *in vivo* and *in vitro*, to intermediates or metabolites which are capable of interacting with the haem of cytochrome P-450 (1-3). These MDP metabolite-cytochrome P-450 complexes are relatively stable as witnessed by their preservation in microsomes isolated from animals pretreated with safrole (4-allyl-1,2-methylenedioxybenzene) or isosafrole (4-propenyl-1,2-methylenedioxybenzene).

After reduction with sodium dithionite, microsomes obtained from rats pretreated with safrole or isosafrole show a difference spectrum with an absorption maximum at 455 nm (3,4). Several workers (1,2,3,5) have related this absorption maximum to the presence of a MDP metabolite-cytochrome P-450 complex.

Addition *in vitro* of certain type I substrates to safrole- or isosafrole-microsomes, produced *in vivo*, results in the time-dependent enhancement of that substrate's own type I binding spectrum (6). It has been suggested on the basis of spectrophotometric studies that this

[1]Abbreviation: MDP, methylenedioxyphenyl.

'displacement phenomenon' may be due to the dissociation of the MDP metabolite-ferricytochrome P-450 complex which is characterized by an absorption maximum at 438 nm (3, and Elcombe, Dickins, Bridges and Nimmo-Smith, in preparation). The present study utilizing ^{14}C-isosafrole confirms that although the metabolite-ferricytochrome P-450 complex is very stable to dialysis it can be readily dissociated by the addition of cyclohexane (1 mM) and furthermore shows the stoichiometry of metabolite to cytochrome P-450 to be 1:1.

MATERIALS AND METHODS

ω-^{14}C-Isosafrole (99% radiochemically pure; diluted with carrier to 1.25 mCi/mmol) was obtained from the Radiochemical Centre (Amersham, UK). Hepatic microsomes were prepared (7) from male Wistar albino rats (150-200 g) which had been dosed intraperitoneally each day for four days with ^{14}C-isosafrole (150 mg/kg) dissolved in corn oil. Spectrophotometric measurements were made with an Aminco DW2 spectrophotometer.

Dissociation of the radioactive isosafrole metabolite-ferricytochrome P-450 complex was also determined by the displacement of radioactivity from the microsomes. ^{14}C-Isosafrole-microsomes were diluted with 66 mM Tris-HCl buffer (pH 7.4) to approximately 1 mg protein/ml and were divided into two 10 ml portions. Samples (3 ml) of each suspension were taken for the determination of protein and of radioactivity. Cyclohexane (1 mM), the displacing substrate, was added to one portion of the isosafrole-microsomes and both portions were incubated for one hour at 37^{o}. The loss of the absorbance at 438 nm was monitored as an indicator of isosafrole metabolite displacement. At one hour displacement appeared to be complete as manifested by loss of 438 nm absorbance in the oxidized spectrum and of 455 nm absorbance in the $Na_2S_2O_4$-reduced spectrum. After this time the microsomal suspensions were each dialysed against 300 ml of 66 mM Tris-HCl buffer (pH 7.4) at 4^{o} for 16 hours.

Protein was then determined by the method of Lowry et al. (8) using bovine serum albumin as standard. The non-diffusible materials were solubilized as described elsewhere (9) and the radioactivities of these solubilized materials and of the diffusates were determined by liquid scintillation counting.

RESULTS AND DISCUSSION

Addition of cyclohexane to ^{14}C-isosafrole-microsomes resulted in the time-dependent intensification of the cyclohexane type I binding spectrum (Fig. 1). Results of a typical parallel experiment in which measurements were made of the displacement of radioactivity from the microsomes are summarized in Table 1. It is clear that a considerable proportion of the

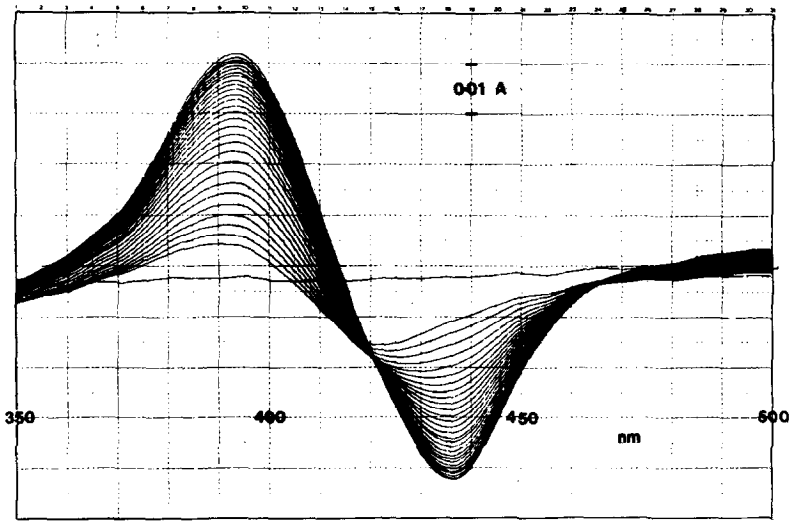

Fig. 1. The Time-dependent Intensification of the Cyclohexane Type I Binding Spectrum in Isosafrole-microsomes. Isosafrole-microsomes were suspended in 66 mM Tris-HCl buffer (pH 7.4) to a protein concentration of approximately 1 mg ml^{-1} and divided between two cuvettes. A baseline of equal light absorbance between 350 and 500 nm was recorded. Cyclohexane (1 mM) was added to the cuvette containing the test suspension and the difference spectrum between 350 and 500 nm repetitively scanned at a speed of 5 nm/sec. Spectra were therefore recorded every minute.

Table 1. An Experiment demonstrating the Displacement of reversibly bound ^{14}C-Isosafrole-related Material from ^{14}C-Isosafrole-microsomes

	Control*	Test*
Original radioactivity (nCi)	29.8	29.8
Radioactivity remaining in microsomes after dialysis (nCi)	24.0	16.9
Loss of radioactivity from microsomes	19.5%	43.3%
Recovery of radioactivity	101.5%	100.6%
Recovery of protein	98.1%	100.1%

* Control: isosafrole-microsomes alone. Test: cyclohexane (1 mM) added as displacer.

^{14}C-isosafrole-related material was displaced from the isosafrole-microsomes during incubation with cyclohexane. It is unlikely that this was a solvent effect since the concentration of cyclohexane was only 1 mM and a similar experiment substituting 25 mM dimethylformamide (which is not a displacer) for the cyclohexane did not lead to displacement of radioactivity. This experiment also demonstrated the high stability of the isosafrole metabolite-cytochrome P-450 complex in the absence of displacing agents. The radioactive material still remaining after displacement appears to be irreversibly bound to microsomal proteins (10).

The loss of ^{14}C-isosafrole-related material from the microsomes due to displacement by cyclohexane (i.e. radioactivity lost from test *minus* radioactivity lost from control) was 7.16 nCi, equivalent to a total of 5.73 nmol isosafrole (i.e. 0.663 nmol/mg microsomal protein).

Previous work (2,5) has indicated that the MDP metabolite competes with carbon monoxide for binding to ferrocytochrome P-450. Hence the concentrations of cytochrome P-450, as measured by the method of Omura and Sato (11), were apparently different before and after the displacement phenomenon. Assuming an extinction coefficient of 91 $mMcm^{-1}$ (11) for the carbon monoxide-ferrocytochrome P-450 complex, the apparent haemoprotein concentration had increased by 0.611 nmol/mg microsomal protein after the displacement reaction. It therefore appears that the 'displaceable isosafrole-related material' and the haemoprotein of the isosafrole metabolite-cytochrome P-450 complex were in a ratio of 0.663:0.611 (1.08:1.00).

Hence in summary the present work has demonstrated that the isosafrole metabolite-cytochrome P-450 complex produced *in vivo* is equimolar in isosafrole-related material and in cytochrome P-450; it can be dissociated by some type I substrates into free cytochrome P-450 and a soluble isosafrole-related material.

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