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THE METABOLISM OF 4-METHOXY-β-CHLORO STYRENE BY LIVER MICROSOMAL MONOOXYGENASES

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Abstract—The compound 4 methoxy-β chloro styrene has been chosen as a model substrate for microsomal monooxygenases to study the relative rates of *O*-demethylation, ring hydroxylation and covalent binding by the suggested epoxide intermediate. The phenolic products were identified by g.c.–m.s. and the covalent binding was measured as non-extractable ^{14}C-labeled metabolites. The monooxygenation by rat liver microsomes resulted mainly in an *O*-demethylation reaction and the relative extent of covalent binding was dependent on the pretreatment of the animals with either phenobarbital or 3-methylcholanthrene. Ring hydroxylation occurred in position 3 and was measurable only after pretreatment with 3-methylcholanthrene. It was concluded that each cytochrome P450 has an individual pattern of detoxifying vs toxifying pathways. The species of cytochrome P450 mainly responsible for covalent binding was very little affected by metyrapone and had a rather low affinity for the substrate.

Drugs and foreign compounds undergo oxidative degradation reactions in the body, which are catalyzed by an unspecific monooxygenase system localized mainly in liver, but also in lung, small intestine or skin. It is now believed that the monooxygenase system consists of a flavoprotein and various cytochrome P450 enzymes with different but overlapping substrate specificities [1–3]. This enables the system to attack almost any lipophilic organic compound by insertion of an oxygen atom. This leads to an increase in water solubility and hence a more rapid excretion from the body. The position into which the oxygen atom is incorporated is determined by chemical and stereochemical factors of both the substrate and the enzyme and the reactivity of the enzymic active oxygen species. In general the monooxygenation proceeds strongly electrophilic with preference for the weakest CH-bonds [4]. Alcohols and phenols are the main stable products of the reaction, which can be either directly excreted or after conjugation or further metabolic conversion.

From some substrates unstable and reactive products may be formed which can stabilize by secondary reactions in the cell [5]. *N*-Hydroxy compounds, arene oxides or carbenes can be candidates for such intermediates, which show an electrophilic behavior and therefore can further react with water or glutathione or may even bind covalently to macromolecules of the cell. The latter event has been suggested as the direct or indirect cause of cell necrosis or chemical carcinogenesis [6, 7]. In view of the many drugs and chemicals in our environment it seems desirable to make predictions on the potential metabolities and hence the toxicity of a given compound. It seemed reasonable to study model compounds first, for which all possible metabolities could be identified and quantized. If one metabolite was reactive towards macromolecules, it would be of interest to compare the relative rates of formation of toxic and nontoxic metabolites in different animals or in the same species of different genetic outfit or exposed to different environmental conditions. As such a model drug we chose 4-methoxy-β-chloro styrene which can undergo *O*-demethylation as well as ring hydroxylation in the 2 and 3-position. In addition, since this compound is an analogue of vinyl chloride and contains a double bond, it may be epoxidized to a reactive epoxide as has been suggested for vinyl chloride [8, 9] or trichloroethylene [9, 10].

MATERIALS AND METHODS

Chemicals. [^{14}C]Malonic acid was obtained from New England Nuclear. Non-radioactive malonic acid, 4-methoxy cinnamic acid, 2,4-dimethoxy cinnamic acid, 3,4-dimethoxy cinnamic acid and 3,3,3-trichloropropene oxide were purchased from EGA-Chemie. Metyrapone (2-methyl-1,2-bis-(3-pyridyl)-1-propanone) was a gift of Ciba Geigy, Chemical Company, Basle, Switzerland. L-Glutathione and L-cysteine were products from Boehringer Mannheim, GmbH, Germany. Thin layer chromatography (t.l.c. was performed on pre-coated plates (silica gel, 60 F-254, Merck, Darmstadt).

Analytical methods. Gas chromatographic analyses (g.l.c.) were performed in a 5700 A Hewlett–Packard gas chromatograph with a hydrogen flame ionization detector and a steel column (3 m × 3 mm) packed with 5% DEGS on Chromosorb G.

Abbreviations: t.l.c., thin layer chromatography; g.l.c., gas liquid chromatography; DEGS, diethyleneglycolsuccinate; n.m.r., nuclear magnetic resonance; TMS, tetramethylsilane; 3-MC, 3-methylcholanthrene.

For combined gas chromatography–mass spectrometry (g.c.–m.s.) a 1400 Varian Aerograph gaschromatograph (equipped with OV1 or OV17 glass columns) was coupled with a MAT 311 Varian mass spectrometer and a SS 111 MS Varian Computer.

Nuclear magnetic resonance (n.m.r.) spectra were recorded on a Varian spectrometer (EM-390, 90 Mhz) and optical spectra on a Shimadzu UV 300 spectrophotometer.

For optical difference spectra an Aminco DW-2 spectrophotometer and 10 mm cuvettes containing 2.5 ml of a microsomal suspension in 0.1 M Tris–HCl buffer 7.6 were used. (Details of the experimental conditions are given in the legends to the figure.)

Synthesis of 4-methoxy-β-chloro styrene. 4-Methoxy-β-chloro styrene was obtained from 4-methoxy cinnamic acid after treatment with an aqueous solution of potassium hypochlorite by 0° [11], m.p. = 32°, m.p. (lit.) = 32–33°, $\varepsilon_{265\,nm} = 2.25 \times 10^4\ M^{-1}$. The stereochemistry was clearly indicated by analysis of the n.m.r. spectrum ($CDCl_3$, TMS) which revealed peaks at 3.80 (*S*, 3H, methyl of the methoxy group), 6.45 (*D*, 1H, $J = 14$ Hz, olefinic proton), 6.77 (*D*, 1H, $J = 14$ Hz, olefinic proton), 6.83 (*D*, 2H, $J = 9$ Hz, aromatic protons) and 7.22 p.p.m. (*D*, 2H, $J = 9$ HZ, aromatic protons). Nevertheless small quantities of the *cis*-isomer (3%) could be detected by gas chromatography–mass spectroscopy.

The synthesis of the ^{14}C-labeled compound was based on a Knoevenagel synthesis with malonic acid and 4-anisaldehyde [12] in order to get [^{14}C]-4-methoxy cinnamic acid. [^{14}C]-4-Methoxy-β-chloro styrene was obtained from 4-methoxy cinnamic acid as described above.

The product was purified by column chromatography(Kieselgel 60, 35–70 mesh ASTM, Merck, Germany) and was shown to be more than 99 per cent pure by the following methods: (i) t.l.c. with cyclohexane–methylene chloride (3:1, v/v) as a solvent followed by scanning on a Berthold LB 2721 radiochromatogram scanner, and (ii) g.l.c. on a 5% DEGS column as described above. The specific activity was 1.0 mCi/mmole.

The possible metabolite 4-hydroxy-β-chloro styrene was obtained from the 4-methoxy compound by treatment with boron tribromide [13]. Characteristic ions in the mass spectrum of this phenol were found at $m/e = 156$, 154 and 119.

Preparation of microsomes. Male Sprague–Dawley rats (140–180 g) were used. Sodium phenobarbital was given i.p. in a daily dose of 80 mg/kg body weight for three days. 3-Methylcholanthrene was dissolved in corn oil and applied i.p. in a dose of 20 mg/kg for two days. Microsomes were prepared as described previously [14] and protein was determined by the Biuret reaction according to Gornall *et al.* [15].

Covalent binding experiments. Microsomal incubations were performed in reaction vessels containing in a final volume of 1.0 ml: 5 mM $MgCl_2$, 8 mM D,L-Na-isocitrate, 5 μl/ml isocitrate dehydrogenase (from pig heart) and 0.20 nM [^{14}C]-4-methoxy-β-chloro styrene (sp. act. 1 mCi/m-mole) in 0.1 M Tris–HCl buffer pH 7.6. The microsomal protein content was 2 mg/ml.

After 5 min of preincubation, the microsomal enzyme reaction was started by addition of NADPH (1 mM) and the vessels were shaken at 37° under air for 30 min.

When the time dependence was studied the total incubation volume was increased to 6 ml and 0.5 ml aliquots were withdrawn at intervals and added to 1 ml of ethanol in a 1.5 ml Eppendorf reaction vessel.

The mixture was centrifuged, the supernatant discarded and 0.5 ml 70% ethanol was added to the protein pellet. The precipitate was resuspended in 70% ethanol, using a Teflon pestle adapted to the Eppendorf tube. The protein was centrifuged again and the extraction procedure repeated using 0.5 ml of different solvents. The following extraction sequence was used: twice with 70% ethanol, once with 0.1 M Tris–HCl buffer (pH = 7.6), once with 70% ethanol, once with ethanol–ethyl ether (4·1), once with acetone–ethyl ether (4·1) and finally once with 50% ethanol.

Each supernatant was tested for radioactivity. After the last extraction no further radioactivity could be removed from the protein, even if other solvents were used. The radioactivity bound to the microsomal protein remained constant after these extraction procedures. It was therefore, assumed, that this extraction method removes the original 4-methoxy-β-chloro styrene as well as all metabolites reversibly bound to protein.

For counting the microsomal protein was dissolved in Soluene-350 (Packard) and transferred to a counting vial. Radioactivity was determined in 10 ml of Dimilune-30 (Packard) and measured in a Packard Tri-Carb liquid scintillation counter. All values were corrected for quenching by internal and external standardization.

Microsomal metabolism of 4-methoxy-β-chloro styrene. Microsomal incubations were performed in reaction vessels containing in a final volume of 50 ml: 5 mM $MgCl_2$, 1 mM glucose-6-phosphate, 0.9 U glucose-6-phosphate dehydrogenase and 0.5 mM 4-methoxy-β-chloro styrene in 0.1 M Tris–HCl buffer pH 7.6. The microsomal protein content was 2 mg/ml. After 5 min of preincubation, the microsomal enzyme reaction was started by addition of NADPH (0.3 mM) and the vessels were shaken under air for 20 min at 30°.

Sodium chloride was then added until saturation. This was followed by extraction with 100 ml of ethyl ether. After evaporation of the ether phase the residue was applied to 0.25 mm thin layer plates (silica gel 60-F_{254}, Merck, Germany) and chromatographed using methylene chloride–methanol (99:1,v/v) as a solvent. Two zones, which reacted positive with Folin's phenol reagent were eluted with ethyl ether and prepared for g.c.–m.s. analysis.

RESULTS

Substrate binding spectra. A first indication that an organic compound can serve as a substrate for the microsomal monooxygenase system is provided by the formation of an enzyme–substrate complex. Since this is accompanied by a large blue shift of the Soret absorption band from 418 to 388 nm of oxidized cytochrome P450 it can be conveniantly followed by optical difference spectrophotometry of a microsomal suspension [16]. When a methanolic solution of 4-methoxy-β-chloro styrene was added to liver microsomes from phenobarbital-pretreated rats a difference spectrum was observed with a peak at 388 nm and a trough at 426 nm (Fig. 1).

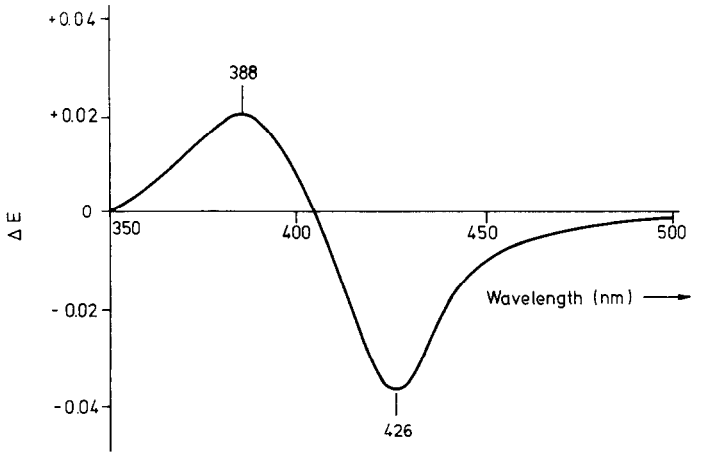


Fig. 1. Difference spectrum of 4-methoxy-β-chloro styrene (saturation) in microsomes from phenobarbital pretreated rats. Each cuvette contained 5 mg of microsomal protein (1.8 nmoles cytochrome P450/mg protein) in 2.5 ml of 0.1 M Tris–HCl buffer pH 7.6. Eight μl of 0.1 M 4-methoxy-β-chloro styrene (in methanol) were added to the sample cuvette.

A Lineweaver–Burk plot (not shown) of the concentration dependence of the magnitude of spectral change revealed an apparent dissociation constant (K_s) of 2×10^{-5} M, which points to a relative high affinity of the compound to cytochrome P450. Microsomes from untreated control rats gave a similar binding spectrum but with only half of the maximal spectral heights. The mere existence of a substrate difference spectrum does not prove that the compound is also monooxygenated, since an uncoupled reaction may have been possible [17]. Therefore, the potential metabolites of this substrate were synthesized and looked for in a microsomal incubation assay.

Phenolic metabolites of 4-methoxy-β-chloro styrene. Lipophilic compounds with methoxy groups at an aromatic ring can be cleaved by microsomal monooxygenases in the presence of NADPH and dioxygen to yield formaldehyde and the corresponding phenol [18]. This was also the case with 4-methoxy-β-chloro styrene as judged from the formaldehyde determination with the Nash reagent [19]. In microsomes from untreated controls 78 nmoles formaldehyde/mg microsomal protein were formed in 20 min, whereas the corresponding values were 53 and 142 nmoles for microsomes from 3-methylcholanthrene- and phenobarbital-induced rats, respectively (see also Table 2). The separation of an ethyl ether extract of the incubation mixtures on thin layer plates revealed a metabolite, which by its R_f-value and mass spectrum could be identified as 4-hydroxy-β-chloro styrene. Methylation with diazomethane of the extracted material resulted in the formation of the original substrate.

The t.l.-chromatograms from assays with microsomes from 3-methylcholanthrene-induced rats contained a second metabolite which was indentified as 3-hydroxy-4-methoxy-β-chloro styrene by the following criteria: the mass spectrum exhibited peaks at 186, 184, 171 and 169, treatment with diazomethane yielded the 3,4-dimethoxy derivative which was synthesized from 3,4-dimethoxy cinnamic acid by treatment with an

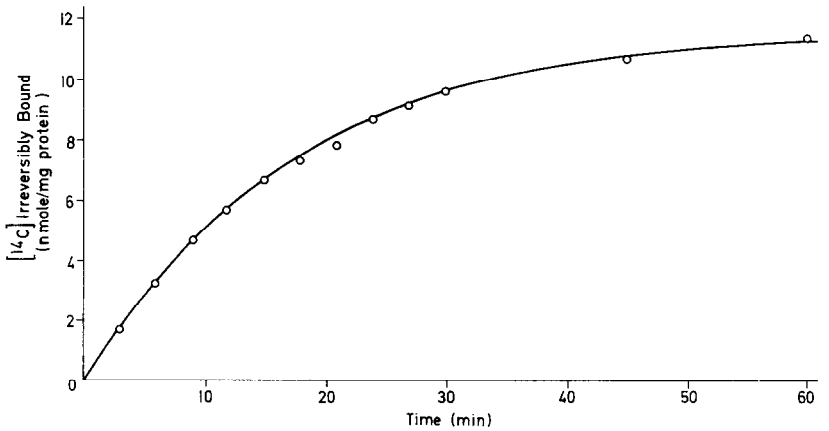


Fig. 2. Time dependence of the irreversible protein binding of [^{14}C]-4-methoxy-β-chloro styrene (0.20 mM) catalyzed by liver microsomes from phenobarbital-pretreated rats in the presence of an NADPH regenerating system.

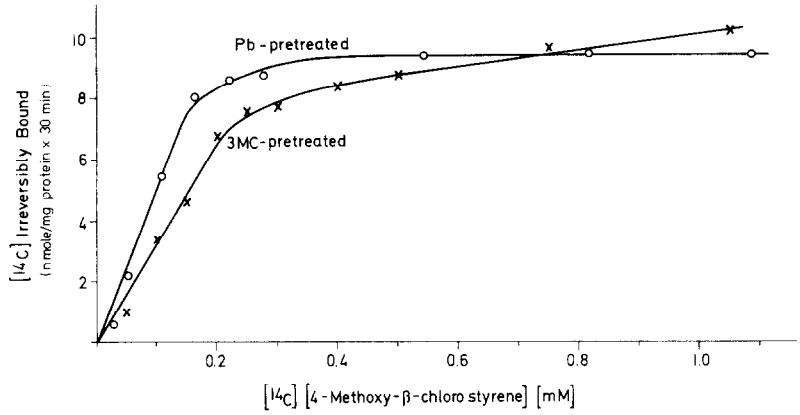


Fig. 3. Irreversible protein binding of [^{14}C]-4-methoxy-β-chloro styrene. Dependence on substrate concentration. Microsomes from phenobarbital- or 3-methylcholanthrene-pretreated rats were incubated with an NADPH-regenerating system and increasing substrate concentrations. After 30 min the assay was tested for protein binding as described in Materials and Methods.

aqueous solution of potassium hypochlorite [11]. Both compounds showed the characteristic ions at m/e = 200, 198, 185 and 183.

The total amount of the 3-hydroxylated metabolite was about 5% of the *O*-demethylated product and hence only plays a minor role in the metabolite pattern. Incubations with phenobarbital-induced microsomes did not yield measurable amounts of the 3-hydroxy metabolite. In no case, was a metabolite corresponding to 2,4-dimethoxy-β-chloro styrene which was synthesized from 2,4-dimethoxy cinnamic by the usual procedure [11], detected in gas chromatograms of the methylated extracts. It was therefore assumed that 2-hydroxylation of the ring did not occur to an appreciable extent in microsomal preparations from either control or pretreated rats.

Covalent binding of [^{14}C]*4-methoxy-β-chloro styrene.* A fourth possible metabolite could be formed by an attack of the active oxygen of cytochrome P450 at the double bond in the side chain. The resulting chloro epoxide would be an extremely reactive compound. In agreement with this assumption all our attempts failed to synthesize this derivative by the conventional techniques known for epoxide formation. It was very likely, however, that in view of the strongly electrophilic properties of the active oxygen complex [4] an intermediate formation of the epoxide would occur, followed by a secondary stabilization with nucleophiles in the medium. If a reaction with functional groups of proteins would occur this would result in covalent binding, which was tested by the use of the ^{14}C-labeled substrate. A time-dependent increase in irreversibly bound ^{14}C-activity was observed in the presence of NADPH and dioxygen (Fig. 2). The ^{14}C-binding showed a saturation above 3 mM substrate concentrations which was very similar in microsomes from phenobarbital- or 3-MC-treated rats (Fig. 3). A rough estimate of the concentration at half maximal binding gives a value of about 2×10^{-4} M and therefore is about an order of magnitude higher than the K_s-value.

The results of Table 1 clearly indicate that the covalent binding process is dependent on NADPH and dioxygen. Carbon monoxide shows the expected inhibition, but metyrapone has a surprisingly small effect.

Trichloropropene oxide as a potent inhibitor of epoxide hydrase [20] did not increase the covalent binding, which would have been the case if the hydrase had converted major amounts of the proposed epoxide intermediate to the dihydrodiol. In contrast sulfhydryl compounds like cysteine and glutathione showed a

Table 1. Irreversible protein binding of [^{14}C]-4-methoxy-β-chloro styrene (0.20 mM) catalyzed by liver microsomes from phenobarbital-pretreated rats in the presence of a NADH-regenerating system after 30 min incubation

		Percentage
+NADPH (control) under air	9.0 ± 1.0	100
−NADPH under air	0.1 ± 0.05	1
+NADPH under argon	0.3 + 0.1	3
+NADPH under 80% CO, 20% O_2	0.8 ± 0.1	9
+NADPH +5 × 10^{-5} M Metyrapone	5.9 ± 0.5	66
+NADPH +2 × 10^{-5} M TCPO†	9.0 ± 1.0	100
+NADPH +10^{-3} M L-cysteine	1.3 ± 0.2	15
+NADPH +10^{-3} M L-glutathione	0.7 ± 0.1	8

* Protein binding is expressed as nanomoles of total metabolites irreversibly bound to 1.0 mg of microsomal protein.

† Trichloropropene oxide.

Table 2. Covalent binding and formaldehyde formation from 4-methoxy-β-chloro styrene in liver microsomes from phenobarbital (PB), 3-methylcholanthrene (3-MC) treated and control rats

	Controls	PB	3-MC
		nmoles/mg protein	
Covalent binding	5.2 ± 0.5	5.9 ± 0.6	5.2 ± 0.5
Formaldehyde	78 ± 11	142 ± 15	53 ± 11

The incubation was carried out 20 min at 30° with a protein concentration of 2 mg/ml. Substrate concentration 0.6 mM. Each value is the average of three determinations.

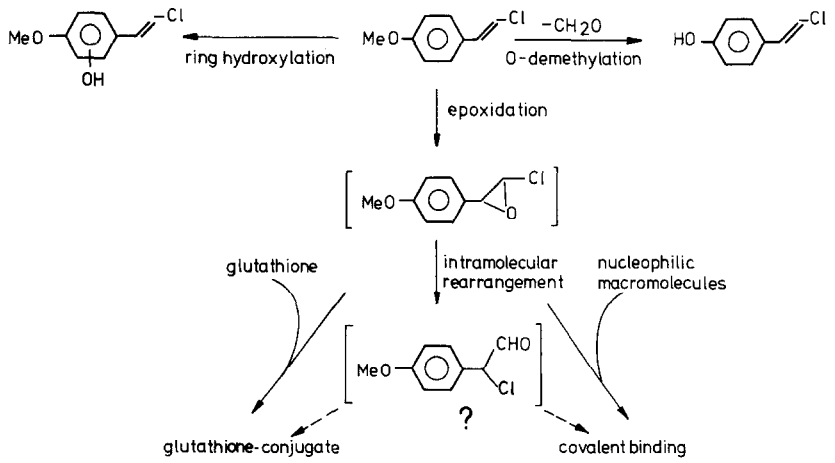
marked inhibition, suggesting that a competition of these compounds with a reactive intermediate occurs [21].

In view of the heterogeneity of the various cytochrome P450 forms in microsomes it was interesting to investigate the ratios of *O*-demethylation to covalent binding, since this can be taken as a measure for the detoxification versus toxification capacity of the liver for this given compound. Table 2 shows the interesting fact that these ratios are different when control animals are compared with animals pretreated with phenobarbital and 3-MC.

A near doubling of the demethylation activity by phenobarbital pretreatment is accompanied by only a slight and not significant increase in covalent binding. 3-MC pretreatment rather decreased the demethylation reaction without affecting or even increasing the covalent binding to microsomal proteins.

DISCUSSION

The model drug 4-methoxy-β-chloro styrene can be metabolized by cytochrome P450-dependent monooxygenases to principally four metabolites as summarized in the following scheme:



The demethylated and the 3-hydroxy derivatives could be clearly identified whereas the 2-hydroxy compound was not detected, probably because of the considerable steric hindrance in the ortho-position of the vinyl chloride group. Due to its high reactivity the chloroepoxide varied with the preparation of microsomes. Different would not be expected to accumulate since they are known as reactive electrophilic species which, as an example, can react with secondary amines already at room temperature[22]. They also could be trapped by SH-compounds and this would explain the inhibitory effect of glutathione or cysteine on the covalent binding. The lack of a stimulatory effect of TCPO as an inhibitor of epoxide hydrase on the covalent binding does not disprove this hypothesis since *trans*-disubstituted epoxides are known not to be substrates for the hydrase [23]. We therefore suggest that the epoxide of 4-methoxy-β-chloro styrene is the metabolite responsible for the covalent binding of the substrate, but its chemical identification requires further study. However, it cannot be excluded, that further metabolism of 4-hydroxy-β-chloro styrene might involve reactive intermediates as it has recently been shown in the case of benzene [24]. Nevertheless, this possibility seems to us unlikely since phenobarbital pretreatment does not significantly increase the covalent binding although the *O*-demethylation is increased almost two-fold. For the aim of our study the exact nature of the reactive intermediate was only of secondary importance. It was more relevant to establish that the relative amounts of covalent binding, ring hydroxylation and *O*-demethylation

pretreatments cause the formation of different cytochrome P450 patterns [1, 2] which must result in different metabolite patterns. Obviously, this becomes important if one of the metabolites exhibits toxic properties. In a forthcoming study we will show that 4-methoxy-β-chloro styrene indeed can cause liver necrosis under conditions where the cellular glutathione level is decreased (B. Mansour, H. v Seebach and V. Ullrich, manuscript in preparation).

The ultimate goal would be to identify the species of cytochrome P450 which contributes mostly to the formation of the toxic metabolite. It is unlikely that the main form present in microsomes from phenobarbital-pretreated rats causes the covalent binding since pretreatment by this inducer does not increase the covalent binding although the cytochrome P450 content is more than doubled and the *O*-demethylation is increased almost two-fold. Metyrapone which blocks the monooxygenation of substrates of the phenobarbital induced cytochrome P450(s) almost completely also has very little effect on the covalent binding at a concentration of 5×10^{-5} M. The half-maximal affinity for the ^{14}C-binding is estimated about one magnitude higher than the K_s-value determined from the optical difference spectra. Since these spectra are much more intense after phenobarbital induction they rather reflect binding of the substrate to one or more phenobarbital-induced forms.

On the other hand the "toxifying" species of cytochrome P450 is also not identical with the form or forms induced by 3-methylcholanthrene, since only a slight increase in the covalent binding was observed, whereas the monooxygenase activity towards substrates like 7-ethoxycoumarin increased nearly fivefold [25]. It must be concluded, however, that cytochrome P448 exhibits a weak activity for covalent binding, because it is the major form of cytochrome P450 in these microsomes and therefore must contribute to the observed *O*-demethylating, ring-hydroxylating and covalent binding activities.

In conclusion we suggest that the main toxifying species of cytochrome P450 occurs in liver microsomes of untreated animals and is not induced by phenobarbital or 3-methylcholanthrene. This applies however only for the substrate 4-methoxy-β-chloro styrene and has to be reinvestigated for each toxic compound separately.

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REFERENCES

1. V. Ullrich and P. Kremers, *Archs Tox.* **39**, 41 (1977).
2. S. S. Thorgeirsson and D. W. Nebert, *Adv. Cancer Res.* **25**, 149 (1976).
3. D. A. Haugen, M. J. Coon and D. W. Nebert, *J. biol. Chem.* **251**, 1817 (1976).
4. V. Ullrich, *Angew. Chem. internat. Edit.* **11**, 701 (1972).
5. F. Oesch, *Arzneimittel. Forsch./Drug. Res.* **27**, 1832 (1977).
6. S. D. Nelson, M. R. Boyd and J. R. Mitchell, in *ACS Symposium Series*, No. 44, *Drug Metabolism Concepts* (Ed. D. M. Jerina) p. 155 (1977).
7. J. R. Gillette, in *Handbook of Physiology*, (Eds D. H. K. Lee, H. L. Falk, S. D. Murphy and S. R. Geiger) *Am. Physiol. Soc, Bethesda*, p. 577 (1977).
8. H. Kappus, H. M. Bolt, A. Buchter and W. Bolt, *Toxic. appl. Pharmac.* **37**, 461 (1976).
9. D. Henschler and G. Bonse, *Archs Tox.* **39**, 7 (1977).
10. B. L. Van Duuren and S. Banerjee, *Cancer Res.* **36**, 2419 (1976).
11. G. Borsche and J. Heimburger, *Ber. dtsch. chem. Ges.* **48**, 456 (1915).
12. J. R. Johnson, in *Organic Reactions*, Vol. I (Ed. R. Adams) p. 249. John Wiley & Sons, New York (1947).
13. J. F. W. McOmie, M. L. Watts and D. E. West, *Tetrahedron* **24**, 2289 (1068).
14. V. Ullrich, *Hoppe-Seyler's Z. Physiol. Chem.* **350**, 357 (1969).
15. A. G. Gornall, C. J. Bardawill and M. M. David, *J. biol. Chem.* **177**, 751 (1949).
16. J. B. Schenkman, H. Remmer and R. W. Estabrook, *Molec. Pharmac.* **3**, 113 (1967).
17. H. Staudt, F. Lichtenberger and V. Ullrich, *Eur. J. Biochem.* **46**, 99 (1974).
18. J. Axelrod, *Biochem. J.* **63**, 634 (1956).
19. T. Nash, *Biochem. J.* **55**, 416 (1953).
20. F. Oesch, *Xenobiotica* **3**, 305 (1973).
21. E. Boyland, in *Handbook of Experimental Pharmacology, Concepts in Biochemical Pharmacology*, Part 2 (Eds B. B. Brodie and J. R. Gillette) p. 584. Springer-Verlag, Berlin (1971).
22. A. Kirrmann and R. Nouri-Bimorghi, *Bull. Soc. chim. Fr.* 3213.
23. F. Oesch, *Biochemistry* **10**, 4858 (1971).
24. A. Tanek, K. L. Platt, P. Bentley and F. Oesch, *Molec. Pharmac.* **14**, 920 (1978).
25. V. Ullrich, U. Frommer and P. Weber, *Hoppe Seyler's Z. physiol. Chem.* **354**, 514 (1973).