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DIFFERENTIAL EFFECTS OF PHENOBARBITONE AND 3-METHYLCHOLANTHRENE INDUCTION ON THE HEPATIC MICROSOMAL METABOLISM OF THE β-CARBOLINES HARMINE AND HARMOL

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Abstract—The metabolism of the β-carbolines harmine and harmol by C57/BL10 mouse liver microsomes is reported. Marked changes in apparent Michaelis–Menten kinetics and metabolite profiles were induced differentially by phenobarbitone (PB) or 3-methylcholanthrene (MC). With control or PB-induced microsomes harmine was metabolised by both high- and low-affinity reactions (app. K_m = 1 and 29 μM for controls; app. K_m = 1.9 and 21 μM for PB-induced). Only the high-affinity reaction occurred following MC pretreatment, induced 31-fold to V_{max} = 22 nmoles·min^{-1}·mg protein^{-1} (app. K_m = 0.4 μM). With control or PB-induced microsomes harmine was metabolised almost exclusively by *O*-demethylation to harmol (3-fold induction to V_{max} = 9.8 nmoles·min^{-1}·mg protein^{-1}, app. K_m = 73 and 14 μM respectively), which was probably the low-affinity reaction of harmine metabolism. With MC-induced microsomes harmine was metabolised mainly to two unidentified yellow products, with harmol as a minor metabolite formed by a high-affinity reaction (app. K_m = 0.6 μM). Control, PB- and MC-induced microsomes each metabolised harmol by a high-affinity reaction (app. K_m = 0.6–1.9 μM) to unidentified metabolites. Harmol metabolism was not induced by PB, but was induced by MC (22-fold to V_{max} = 11 nmoles·min^{-1}·mg protein^{-1}): this partly explains the relative lack of net harmol production after MC induction. The unidentified MC-induced yellow metabolites of harmine were not formed via harmol. Harmine and harmol metabolism showed the characteristics of cytochrome P-450 catalysed reactions. Harmine gave Type II cytochrome P-450 binding spectra with control and PB-induced microsomes, but a high-affinity Type I spectrum (K_s = 6.5 μM) with MC-induced microsomes. Harmol gave modified Type II spectra in all cases, with again higher-affinity binding to MC-induced microsomes (K_s = 62 μM) than to control ($K_s \gg 500$ μM) or PB-induced microsomes (K_s = 500 μM).

It is now generally accepted that hepatic microsomal cytochrome P-450 (cyt. P-450)† is a family of variant forms, which are selectively inducible by different chemicals and which show differing substrate and reaction specificities [1, 2]. As a result of the discovery of a substrate, ethoxyresorufin, that is apparently highly specific for MC-inducible cyt. P-450 [3], we have begun a study of the structural aspects of condensed heterocyclic substrates that influence their cyt. P-450 specificities. A variety of inducing agents, in particular phenobarbitone (PB) and 3-methylcholanthrene (MC), differentially induce the hepatic microsomal metabolism of several other heterocyclic compounds, including zoxazolamine [4], ethoxycoumarin [5], theophylline [6], ellipticine [7], *N*-methylcarbazole [8] and benzacridine [9]. The implication that this reflects the differing specificities of various induced forms of cyt. P-450 has been confirmed for a few of these substrates using purified cyt. P-450 [1–3, 8]. Harmine (Fig. 1) has been studied here because it is a heterotricyclic compound showing structural relationships with several of the substrates listed earlier. It is metabolised in animals and man by *O*-demethylation followed by glucuronide or sulphate conjugation [10–12]. In mice its hepatic microsomal *O*-demethylation shows the characteristics of a PB-inducible monooxygenase reaction [12] and preliminary studies have indicated large differences between PB and MC in their induction of harmine metabolism [13]. Subsequently it was reported that the microsomal metabolism of certain structurally related γ-carbolines, i.e. tryptophan pyrolysis products, was also induced differentially by PB and MC [14].

Harmine is a member of a class of compounds, the β-carbolines, which, together with the related γ-carbolines, show widespread pharmacological and toxicological actions.

β-Carbolines, which include natural alkaloids and synthetic chemicals, have also been suggested to

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† Abbreviations: cyt. P-450, cytochrome P-450; SKF-525A, β-diethylaminoethyl diphenylpropylacetate; PB, phenobarbitone; MC, 3-methylcholanthrene; ANF, α-naphthoflavone; non-induced microsomes, PB- and MC-microsomes denote, respectively, hepatic microsomes from untreated, PB- and MC-pretreated mice; the term control microsomes refers equally to microsomes from either untreated or olive-oil-treated mice unless otherwise stipulated.

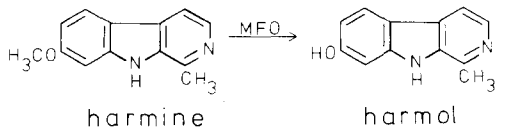

Fig. 1. Structures of harmine and harmol. The *O*-demethylation of harmine to harmol is catalysed by the hepatic microsomal cytochrome P-450 dependent, mixed-function oxidase enzyme system (MFO).

occur naturally in mammals, following their recent discovery in human and rat urine [15, 16], human platelets [17], rat brain and adrenal gland [15, 18–20]. The actions of β-carbolines encompass inhibition and stimulation respectively of the enzymes monoamine oxidase and epoxide hydratase [21–23], a variety of neuropharmacological effects [21, 24, 25], and suggested roles as endogenous ligands [26–29] of the benzodiazepine receptor [30] and mediators of the CNS effects of alcohol [17, 31]. At least one compound recently found to be endogenous, 6-methoxy-tetrahydro-β-carboline [18, 20], has previously been shown to be metabolised in the rat by typically cyt. P-450 mediated reactions [32]. Certain β- and γ-carbolines, that originate in cigarettes and food, can modulate the mutagenicity of other chemicals or u.v.light in bacteria [33, 34] and eukaryotic cells [35] and are themselves mutagenic [36]. The occurrence of endogenous β- or γ-carbolines which are mutagens and carcinogens must be considered a possibility.

MATERIALS AND METHODS

Chemicals. Harmine (harmine hydrochloride hydrate), harmol (harmol hydrochloride monohydrate), harmaline (harmaline hydrochloride dihydrate), harmalol (harmalol hydrochloride), metyrapone, methimazole and ANF were purchased from Aldrich Chemical Co. NADP, isocitric acid and isocitric dehydrogenase were obtained from Sigma Chemical Co. SKF-525A was generously supplied by Smith Kline and French Ltd, U.K.

Animal pretreatment. Adult, male C57/BL10 mice (12–16 weeks old, 25–30 g, bred within the University) were used. They were caged on Lablit mineral bedding (W. P. Usher Ltd, Whetstone, U.K.) for at least 10 days prior to killing and received a commercial diet (Oxoid pasteurised breeding) and tap water *ad lib*. Groups of mice were treated with either sodium phenobarbitone (1 g/l in drinking water for 6 days, with a return to normal drinking water 24 hr prior to death) or MC (80 mg/kg of a 1% solution in olive-oil, once i.p. 3 days before death). Control mice were either untreated or received olive-oil (0.3 ml once i.p.) as indicated.

Liver microsome preparation. The mice were killed by cervical dislocation, the gall bladder excised and the liver removed into ice-cold buffer (0.15 M KCl in 0.01 M phosphate buffer, pH 7.6). Liver microsome fractions were prepared as previously described [37] with the following modifications to prevent degradation of cyt. P-450 to cyt. P-420: (a) homogenizing and washing buffers contained 15% glycerol, and (b) the initial Potter–Elvejhem homogenization was performed at 850 rpm.

Microsomal metabolism of harmine and harmol. The metabolism of harmine by unspecified reactions was measured as the decrease in substrate (harmine), while its concomitant metabolism specifically by *O*-demethylation was measured as the increase in metabolite (harmol). The metabolism of harmol was measured separately as the decrease in substrate (harmol). Reaction mixtures (final volume 2 ml) in 12-ml polypropylene test tubes, containing 0.5 μmoles NADP, 5 μmoles DL-isocitric acid, 0.6 U isocitric dehydrogenase, 10 μmoles magnesium sulphate, 0.1 M phosphate buffer (pH 7.6) and substrate and microsomal protein added in the specified quantities, were incubated at 37° in a shaking water bath at 80 oscillations/min. Reactions were preceded by a 2-min preincubation to generate NADPH and were initiated by addition of either harmine or harmol at concns detailed later. Substrates were stored as 10 mM solutions (in distilled water, at room temp, in the dark) and were diluted immediately prior to use. The reactions were terminated by the addition of 1 ml saturated borate buffer (pH 12.5). Additional incubations of: (a) quantitative external standards of harmine and harmol respectively (borate buffer added before the β-carboline), and (b) background fluorescence (β-carboline omitted) were included in each experiment.

Fluorimetric assay of harmine and harmol. A saturating amount of solid NaCl (approximately 2 g) together with 1.5 ml ethyl acetate were added to each borate-terminated incubate. The sample was rotary-mixed for 15 min and then centrifuged at 2000 rpm for 15 min in a bench centrifuge (MSE Super Minor) to break the solvent–interface emulsion. One millilitre of the upper organic phase was pipetted off and evaporated to dryness at 30° in a vacuum–vortex evaporator (Buchler Instruments Inc., NJ). The residue was stored overnight at −20° and was then redissolved in 6 ml $CHCl_3$ and partitioned against 1.5 ml 0.1 N NaOH by rotary mixing as earlier. The solvent–interface emulsion was broken by steeping the tubes in warm water for 1 min and then centrifuging at 3500 rpm for 15 min. A 1 ml aliquot of the upper, alkali phase was removed into a polypropylene tube for measurement of harmol and the remaining alkali phase and interface aspirated off. A 2-ml portion of the $CHCl_3$ phase, containing harmine, was pipetted off and evaporated to dryness as described earlier.

Immediately prior to fluorescence measurement, the optimal pH of approximately 1 for fluorescence of harmol and harmine was achieved by: (a) acidifying the 1-ml alkali sample (harmol) with 1 ml 0.5 N H_2SO_4, and (b) redissolving the $CHCl_3$ residue (harmine) in 0.1 ml ethanol followed by 1.9 ml H_2SO_4 (pH 1). The harmine solutions were then diluted 10-fold with H_2SO_4 (pH 1) to abrogate the quenching effect of the ethanol. Where necessary the final solutions of harmine and harmol were further diluted with H_2SO_4 (pH 1) to concns that were known to show a linear relationship with fluorescence (see Results). The fluorescence of these solutions was measured in a Perkin–Elmer Model 3000 spectrofluorimeter using wavelengths of 320 nm excitation and 418 nm emission (optimal for both harmine and harmol) and was quantified by comparison with con-

currently extracted harmine and harmol external standards. Standard concns were chosen within the range of substrate concns; when only harmine was used as a substrate the concn of standard harmol was chosen to be half the standard harmine concn.

Estimation of apparent (app.) K_m *and* V_{max}. Harmine metabolism was measured, both as unspecified reactions and as *O*-demethylation to harmol, using two harmine concn ranges: 0.5–5 μM (low [s]) and 10–200 μM (high [s]). Harmol metabolism was measured using the range 0.25–15 μM harmol. Microsomes were pooled from a minimum of five mouse livers. The apparent kinetic parameters of harmine metabolism were calculated from two experiments in the high- and three in the low-concn range, while for harmol metabolism three experiments were performed. Each experiment involved duplicate determinations using five to seven substrate concns over the appropriate range. Unweighted linear regression analysis on Lineweaver–Burk plots was used to determine the app. K_m and V_{max} values.

Inhibition studies. The effects of various inhibitors on the microsomal metabolism of harmine and harmol were examined using the assay outlined earlier. Substrate concns were selected to provide V_{max} reaction rates in non-inhibited incubations. The inhibitors metyrapone, methimazole, ANF, octylamine (all dissolved in acetone) or SKF-525A (in H_2O) were each added at a final incubate concn of 0.5 mM. In addition the effects of CO (atmosphere of $CO:O_2$—4:1) and the dependence on NADPH, NADH and O_2 were measured. Anaerobic conditions were achieved using an O_2-scavenging system [38].

TLC for separation of harmine and harmol metabolites. The ethyl acetate residues from extracted incubates were redissolved in 50 μl ethanol; 25 μl was applied to non-activated, aluminium-backed, 0.5 mm thick silica gel, TLC plates without fluorescent indicator (Kieselgel 60, Merck) and developed in $CHCl_3$:triethylamine:ethanol (60:15:35). Metabolite spots were located by their intrinsic fluorescence under u.v. irradiation (short and long wavelengths) and tentatively identified by their relative R_f, fluorescent colour and colour under white light in comparison with known β-carboline standards (harmine, harmol, harmaline and harmalol). An alternative solvent system of $CHCl_3$:trimethylamine:ethanol (60:1.5:35) was also used.

Cyt. P-450 substrate-binding spectra. Optical absorption difference spectra [39], produced by the interaction of harmine or harmol with either non-induced, PB- or MC-microsomes (approximately 2 mg protein/ml in 0.1 M phosphate buffer, pH 7.6), were measured at room temp in a Varian–Cary 219 spectrophotometer scanning between 500 and 330 nm. Either normal 1-cm cells or tandem cuvettes (2 × 0.45 cm compartments in series in the light path) were used. Harmine or harmol (in H_2O) was added to the sample compartment by microliter syringe, with equivalent vols of water added to the reference compartment: the maximum vol. added was 20 μl per compartment.

Protein and cyt. P-450 assays. Microsomal protein was estimated by the method of Lowry *et al.* [40] and cyt. P-450 as described by Omura and Sato [41].

RESULTS

Extraction and fluorescence measurement

A new assay was developed for the extraction, fluorimetry and thin-layer chromatographic separation of harmine and its metabolites (details in Materials and Methods). Extraction conditions were optimised: (a) for solvent ratios [(i) borate-terminated incubate/ethyl acetate (2:1 v/v), and (ii) $CHCl_3$/0.1 N NaOH (4:1 v/v)], and (b) for extraction pH [(i) pH = 12.5 for combined extraction of harmine and harmol into ethyl acetate from borate, and (ii) pH > 10 for differential extraction of harmine into $CHCl_3$ and harmol into NaOH]. The maximum possible recoveries of harmine and harmol after sampling vol. losses were 22.25 and 44.5% of their respective amounts in the microsomal incubate. After correction for sampling losses, observed recoveries were 78 ± 1% for harmine and 92 ± 1% for harmol (means ± S.E.M., N = 10) and were constant up to 1 mM harmine or harmol. Fluorescence intensity was linear with concns of harmine or harmol up to 5 μM and was maximal at approximately pH 1.

Estimation of apparent K_m *and* V_{max} *for metabolism of harmine and harmol*

Harmine was metabolised both by unspecified reactions (measured as harmine disappearance) and by *O*-demethylation (measured as harmol production), whilst harmol was metabolised only by unspecified reactions (measured as harmol disappearance). Microsomal protein concns and incubation times were selected, on the basis of a series of reaction progress curves, to ensure initial velocities at all the substrate concns used. Initial velocity was generally maintained until either 60% metabolism of harmine or 50% metabolism of harmol had occurred. Consequently incubation times at all substrate concns were restricted to 3 or 5 min, with the exception of harmol metabolism by MC-microsomes at 0.25 and 0.5–2.5 μM substrate, which maintained an initial velocity for only 1 and 1.5 min respectively.

The rates of metabolism of harmine (unspecified or *O*-demethylation) or of harmol were independent of the protein concn of non-induced, PB- or MC-microsomes up to 1 mg/ml.

Table 1 summarises the app. K_m and V_{max} values for the metabolism of harmine (unspecified and *O*-demethylation) by liver microsomes of control (untreated and olive-oil-treated), PB- and MC-induced mice. App. K_m and V_{max} were calculated from linear regression analysis of Lineweaver–Burk plots, 85% of which showed r values greater than 0.9 (r values for the others were between 0.76 and 0.88) over the harmine concn ranges indicated.

Metabolism of harmine by unspecified reactions

The Michaelis–Menten kinetics for the unspecified metabolism of harmine were uniphasic for microsomes from MC-induced mice but were biphasic for microsomes from either control or PB-induced mice (Table 1). At low harmine concns the app. K_m values were 0.4–1.9 μM, being similar for control, PB- and MC-microsomes. At high harmine concns saturation occurred with MC-microsomes but a second, larger

Table 1. App. K_m and V_{max} values for metabolism of harmine (unspecified* and *O*-demethylation)†

Pretreatment	Metabolism	Low [harmine]‡ App. K_m (μM)	Low [harmine]‡ V_{max} (nmoles·min^{-1}·mg protein^{-1})	High [harmine]§ App. K_m (μM)	High [harmine]§ V_{max} (nmoles·min^{-1}·mg protein^{-1})
Untreated	Unspecified	1.0 (0.93–1.10)	1.5 (1.3–1.6)	29 (25, 33)	3.7 (3.2, 4.1)
	O-Demethylation	—‖	—‖	73 (70, 76)	3.4 (3.3, 3.6)
PB	Unspecified	1.9 (1.6–2.2)	4.3 (3.6–5.0)	21 (19, 22)	14.0 (13.0, 15.0)
	O-Demethylation	—‖	—‖	14 (13, 14)	9.8 (9.4, 10.3)
Olive-oil	Unspecified	0.6 (0.48, 0.68)	0.7 (0.64, 0.79)	30 (29, 32)	4.0 (3.9, 4.0)
	O-Demethylation	8.0 (6.9, 9.2)	1.1 (1.1, 1.3)	104 (116, 94)	4.3 (4.5, 4.2)
MC	Unspecified	0.4 (0.24–0.59)	22.0 (19.0–25.0)	—¶	—¶
	O-Demethylation	0.6 (0.46–0.74)	6.0 (4.4–8.1)	—¶	—¶

* Unspecified metabolism was measured as harmine disappearance, *O*-demethylation was measured as harmol production.
† Mean values shown with individual values or ranges of three shown in parentheses.
‡ Values obtained using 0.5–5 μM harmine.
§ Values obtained using 10–200 μM harmine.
‖ Kinetics were uniphasic and showed the app. K_m and V_{max} values listed under high [harmine].
¶ Kinetics were uniphasic and showed the app. K_m and V_{max} values listed under low [harmine].

app. K_m value appeared with control and PB-microsomes, which was similar in each case: 21–30 μM. The differences between the higher and the lower app. K_m values were large (11-, 29- and 50-fold for microsomes of PB-induced, untreated and olive-oil-treated mice respectively) and were greater than the corresponding differences in V_{max} (3.3-, 2.5- and 5.7-fold respectively). The single V_{max} for MC-microsomes (22 nmoles·min^{-1}·mg protein^{-1}) was greater than even the higher V_{max} for control or PB-microsomes (3.7 and 14 nmoles·min^{-1}·mg protein^{-1} respectively). Harmine concns of approximately 5 μM gave the V_{max} reaction rate with MC-microsomes and the lower V_{max} with control or PB-microsomes: at this substrate concn, unspecified metabolism appeared to be induced 30-fold by MC but only 3-fold by PB. Harmine concns > 1 mM gave the higher V_{max} reaction rate with control or PB-microsomes and at these concns the unspecified metabolism appeared to be much less induced by MC (5.5-fold) and similarly induced by PB (4-fold).

Metabolism of harmine by O-demethylation

The kinetics for the *O*-demethylation of harmine were uniphasic for all types of microsomes, except following olive-oil pretreatment when the kinetics were biphasic. With control and PB-microsomes the app. K_m values for *O*-demethylation (the higher app. K_m in the case of olive-oil controls) were of the same order as the higher app. K_m values for unspecified harmine metabolism, whereas with MC-microsomes the app. K_m for *O*-demethylation was more than 20 times lower, being similar to the single app. K_m value for unspecified harmine metabolism by MC-microsomes and the lower app. K_m values for control and PB-microsomes. PB-induction decreased the app. K_m for *O*-demethylation 5-fold but did not markedly alter the higher app. K_m for unspecified harmine metabolism. Similarly, MC-induction reduced the app. K_m for *O*-demethylation 13-fold in comparison to olive-oil controls but did not alter the lower app. K_m for unspecified harmine metabolism. The V_{max} for *O*-demethylation was induced almost 3-fold by PB (V_{max} was approached at >1 mM harmine). MC induced a 6-fold increase in the *O*-demethylation V_{max} at low harmine concns (<5 μM) but had little effect in the high concn range: this was due to app. K_m differences between the olive-oil and MC-microsomes.

At harmine concns high enough (>1 mM) to approach the higher V_{max} reaction rates the conversion of harmine to harmol was approximately 100, 70 and 27% with control, PB- and MC-microsomes respectively; similar percentage conversions occurred at lower harmine concns. Harmol was thus the major metabolite with control and PB-microsomes but only a minor metabolite with MC-microsomes.

Metabolism of harmol

It became apparent that one reason for the seeming lack of *O*-demethylation by MC-microsomes was that the demethylation product, harmol, was itself metabolised much more rapidly by MC-microsomes than by control or PB-microsomes (Table 2). The apparent kinetics for the metabolism of harmol were uniphasic for all four types of microsomes (r values for the Lineweaver–Burk plots were all greater than 0.94). The V_{max} was induced 22-fold by MC (11 nmoles·min^{-1}·mg protein^{-1}) but only 1.6-fold by PB (1.6 nmoles·min^{-1}·mg protein^{-1}). With MC-microsomes the V_{max} for the metabolism of harmol was almost twice the observed V_{max} for its formation from harmine, whereas with control and PB-microsomes the V_{max} for harmol metabolism was lower than for harmol production. In contrast the app. K_m values for harmol metabolism were similar in control, PB- and MC-microsomes (0.6–1.9 μM) and ranked with the lower instead of the higher app. K_m values for the unspecified metabolism of harmine. Consequently, with control and PB-microsomes the app. K_m for harmol metabolism was very much lower than the app. K_m for harmol formation, whereas with MC-microsomes these values were similar.

Inhibition studies

The cofactor dependence and the effects of various inhibitors on the metabolism of harmine and harmol by microsomes of untreated mice are detailed in Table 3. There was an absolute requirement for NADP (presumably in the form of NADPH) and oxygen. Preferential inhibitors of MC-induced forms of cyt. P-450 (ANF) [42, 43], of control and PB-induced forms of cyt. P-450 (metyrapone and SKF-525A) [43–45], of several forms of cyt. P-450 (CO and octylamine) [46–50] and of the non-cyt. P-450 mixed-function amine oxidase (methimazole) [50] were each used at 0.5 mM incubate concn.

There was a possibility, which was investigated experimentally, that the organic inhibitors or their microsomal metabolites could contribute a significant degree of fluorescence in the assays for harmine and harmol. However, with one exception the fluorescence values obtained were at background level. The only measurement that was invalidated by inhibitor fluorescence was that of the metabolism of the lower concn of harmine by unspecified reactions in the presence of ANF (at the higher harmine

Table 2. App. K_m and V_{max} values for metabolism of harmol*†

Pretreatment	App. K_m (μM)	V_{max} (nmoles·min^{-1}·mg protein^{-1})
Untreated	1.85 ± 0.3	1.0 ± 0.05
PB	0.7 ± 0.1	1.6 ± 0.1
Olive-oil	1.9 ± 0.4	0.5 ± 0.1
MC	0.6 ± 0.1	11.0 ± 1.0

* Measured as harmol disappearance using 0.25–15 μM harmol.
† Results are means of three experiments ± S.E.M.

Table 3. Effect of cofactors and inhibitors on the metabolism of harmine (unspecified* and *O*-demethylation) and on the metabolism of harmol†

	Harmine metabolism				Harmol metabolism¶
	Low [harmine]§		High [harmine]‖		
Incubation conditions‡	Unspecified	*O*-Demethylation	Unspecified	*O*-Demethylation	
−NADP	100	97	100	99	100
−NADP + NADH (0.25 mM)	93	83	83	84	80
He**	99	100	99	98	100
$CO:O_2$ (4:1)	39	54	54	55	29
Metyrapone	26	52	21	29	47
SKF-525A	11	53	32	57	43
ANF	—††	87	78	78	48
Octylamine	100	90	49	73	85
Methimazole	43	58	45	64	34
Reaction rate using standard conditions ($nmoles \cdot min^{-1} \cdot mg\ protein^{-1}$)	1.4	0.7	2.8	1.4	0.4

* Unspecified metabolism was measured as harmine disappearance, *O*-demethylation was measured as harmol production.

† Values are % inhibition of reaction rates under standard conditions (see Materials and Methods); means of two experiments using liver microsomes from untreated mice.

‡ Inhibitors present at 0.5 mM concn.

§ Values obtained using 10 μM harmine.

‖ Values obtained using 250 μM harmine.

¶ Values obtained using 20 μM harmol.

** Anaerobic metabolism, He bubbled incubate with O_2-scavenging system [38].

†† Not measurable owing to fluorescence interference from ANF (see text).

concn the fluorescence due to ANF was less than 3% of the fluorescence due to the harmine remaining at the end of an incubation in the absence of inhibitors). With the exception of methimazole, the effects of the inhibitors on harmine metabolism were different at low and high harmine concns respectively. At a low harmine concn (10 μM) ANF or octylamine were the only compounds that gave almost total inhibition of harmine metabolism: octylamine inhibited both the unspecified and *O*-demethylation reactions and ANF inhibited the *O*-demethylation reaction, but owing to the fluorescence interference from ANF its inhibition of the unspecified reactions could not be quantified. At a high harmine concn (250 μM) only ANF produced substantial inhibition (78%) of both types of metabolism, whereas octylamine caused a 73% inhibition of *O*-demethylation but only 49% inhibition of unspecified metabolism. Metyrapone, SKF-525A, methimazole or CO were not potent inhibitors of harmine metabolism, although in each case *O*-demethylation was more sensitive than unspecified metabolism at both high and low harmine concns. Octylamine was the only potent inhibitor of the metabolism of harmol.

TLC

Metabolites extracted from incubations of 40 μM harmine with non-induced, PB- or MC-microsomes were separated by TLC (R_f values in $CHCl_3$:triethylamine:ethanol (60:15:35) are given later). Additional extracts were chromatographed to monitor spontaneous conversion of harmine and the occurrence of any microsomal compounds which might co-chromatograph with putative harmine metabolites. The substrate, harmine (R_f 0.67), and its desmethyl metabolite, harmol (R_f 0.31), were identified by comparison with authentic samples. Visual estimation of the sizes and colour intensities of TLC spots showed that the relative extents of harmine metabolism through unspecified reactions were MC > PB > untreated mice and through *O*-demethylation were PB > untreated > MC. These observations are in accordance with the kinetic data (Table 1). With MC-microsomes four distinct metabolite spots were evident: a yellow compound, designated Y_1 (R_f 0.53), was the major metabolite of harmine; harmol was a minor harmine metabolite and there were two other minor metabolites, designated Y_2 (R_f 0.27) and G (R_f 0.22). Harmol was by contrast the main metabolite of harmine with non-induced or PB-microsomes. However, a small amount of Y_1 was also found with PB-microsomes and there was a trace of Y_1 with microsomes of untreated mice. Harmine and its metabolites were differentiated on the TLC plate not only by their R_f values, but also by their colours under white light and by the colours of their fluorescence. Y_1 and Y_2 were yellow under white light, while harmine, harmol and G were colourless. Under long wavelength u.v. light the fluorescence colours were: harmine—blue, harmol—violet, Y_1—blue-grey, Y_2—blue-grey, G—green: the dihydro-β-carbolines harmaline (R_f 0.51) and harmolol (R_f 0.14) fluoresced light-blue and yellow respectively. In addition, Y_2 changed in colour (as seen under white light) from yellow to red-brown after several days on the TLC plate.

Extracted metabolites from incubations of harmol with non-induced, PB- or MC-microsomes were also separated by TLC using the $CHCl_3$:triethylamine:ethanol (60:15:35) solvent system. This revealed harmol itself but no metabolites.

Cyt. P-450 substrate-binding spectra

The interactions of harmine and harmol with cyt. P-450 were measured by difference spectroscopy (Figs. 2 and 3). Tandem cuvettes (two compartments in series in the light path) were used to balance out the absorption spectra of the substrates themselves (λ_{max} for either harmine or harmol was 340 nm). The literature on the designation of cyt. P-450 binding spectra for organic compounds is confused, but is

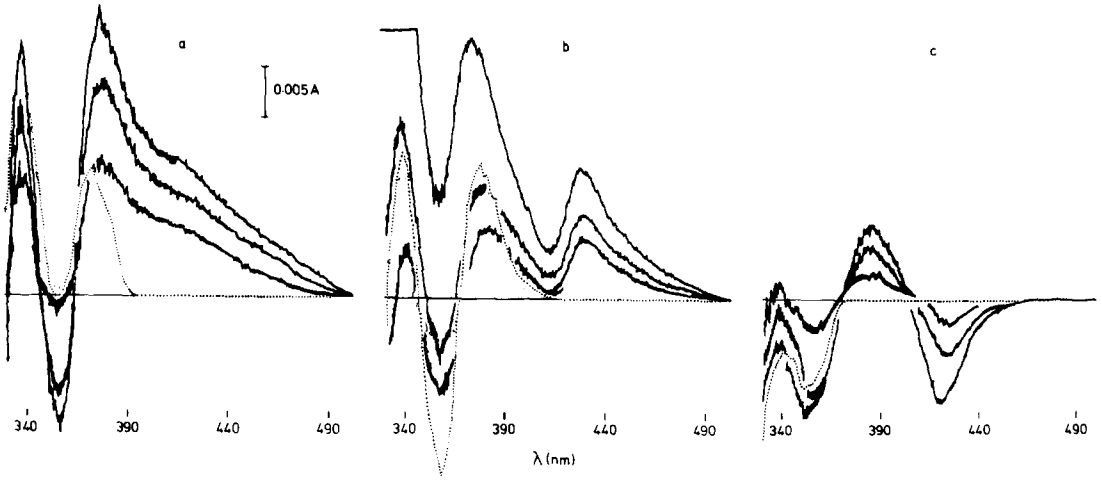

Fig. 2. Microsomal difference spectra of harmine interaction with cytochrome P-450. (—) Native or (. . . .) boiled microsomes of untreated (a), PB-treated (b) and MC-treated (c) mice. The spectral magnitudes were concn-dependent using the following concns of harmine: (a) 30, 70 and 100 μM; (b) 30, 50 and 100 μM; (c) 3, 5 and 10 μM. The spectra for boiled microsomes were obtained using either 100 μM (a and b) or 10 μM (c) harmine. All spectra were obtained using tandem cuvettes and 2 mg microsomal protein/ml as described in Materials and Methods.

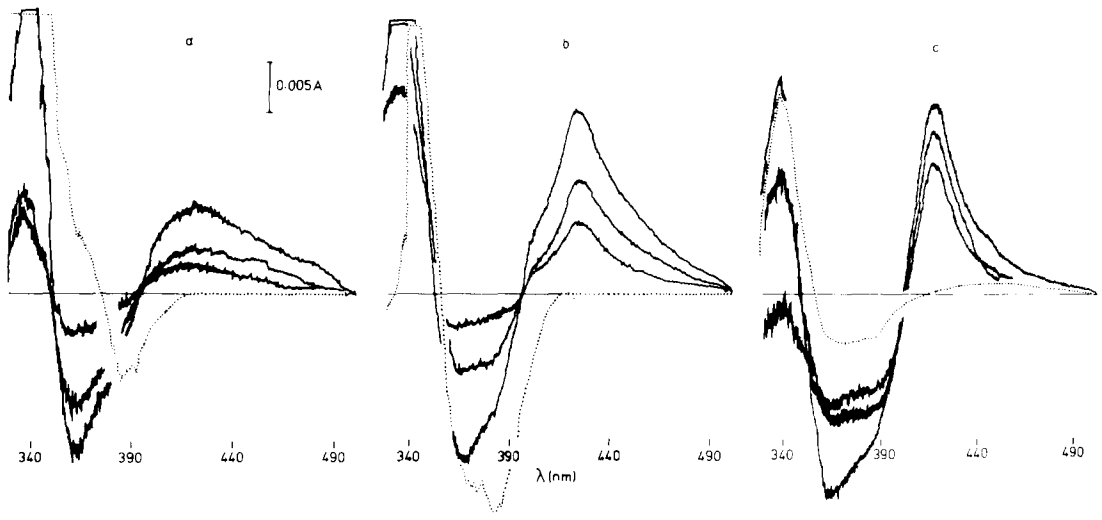

Fig. 3. Microsomal difference spectra of harmol interaction with cytochrome P-450. (—) Native or (. . . .) boiled microsomes of untreated (a), PB-treated (b) and MC-treated (c) mice. The spectral magnitudes were concn-dependent using the following concns of harmol: (a) 20, 50 and 100 μM; (b) 50, 100 and 200 μM; (c) 10, 30 and 70 μM. The spectra for boiled microsomes were obtained using 100 μM harmol. All spectra were obtained using tandem cuvettes and 2 mg microsomal protein/ml as described in Materials and Methods.

generally expressed as follows: Type I spectra (peak at 370–390 nm and a trough at 415–425 nm [39, 51, 52]; Type II spectra (peak at 421–435 nm, a trough (sometimes broad) at 390–410 nm and a transection of the baseline at 410–420 nm [39, 49, 51–54]; modified Type II (or reverse Type I) spectra (peak at 415–421 nm, a sharp trough at 380–390 nm and a baseline transection at 400–410 nm [51, 55–57]).

Harmine (*Fig.* 2). Harmine unexpectedly produced a trio of concn-dependent spectral changes below 400 nm with boiled microsomes: two peaks (λ_{max1} = 340 nm; λ_{max2} = 375 nm, absent with MC-microsomes) and one trough (λ_{min} = 355 nm). Compared to boiled non-induced microsomes, this trio of changes was larger with boiled PB-microsomes and smaller with boiled MC-microsomes.

Addition of harmine to native microsomes produced more intense spectral changes below 400 nm than were observed with boiled microsomes. These spectral features were probably due to non-cyt. P-450 interactions of the microsomes with harmine. (It was shown using [^{3}H]harmine that these interactions did not contribute to the disappearance of harmine measured in the assay for metabolism with either control, PB- or MC-induced microsomes.) In the presence of native non-induced microsomes harmine produced in addition a shoulder on the rising part of the spectrum at approximately 420 nm. This was probably the peak of a very weak Type II interaction, but the expected complementary trough was obscured by the large peak of non-enzymic interaction at 380 nm (Fig. 2a). A much more intense concn-dependent Type II harmine interaction spectrum was seen with native PB-microsomes (λ_{max} = 420 nm; λ_{min} = 412 nm; Fig. 2b). Confirmation that the 412 nm trough was not an artefact due to the tandem cuvettes and that the observed minimum wavelength was not displaced by the proximity of the 380 nm peak, was obtained: (a) by using lower harmine concns (at which the 380 nm peak was smaller relative to the 420 nm peak and 412 nm trough); and (b) by substitution of the tandem cuvettes with normal cuvettes (whereupon, however, the spectra below 400 nm were dominated by the absorbance of harmine itself).

In contrast, addition of harmine to native MC-microsomes produced an entirely different concn-dependent spectral change, indicative of a Type I cyt. P-450 interaction (λ_{max} = 380 nm, λ_{min} = 422 nm; Fig. 2c). Furthermore, unlike the non-induced or PB-induced microsomal modified Type II interactions, which showed concn dependence up to 100 μM, the Type I interaction with MC-microsomes was apparently saturated by 20 μM harmine. A double-reciprocal plot of the magnitude of the Type I spectral change with MC-microsomes [$(\Delta A_{380-422})^{-1}$ vs harmine concn^{-1}] was linear (r = 0.950) for seven concns over the range 1–20 μM, giving a spectral dissociation constant (K_s) of 6.5 μM. A similar plot of the modified Type II interaction with PB-microsomes (5–100 μM harmine) was curvilinear when tandem cuvettes were used, but was linear (r = 0.996) using normal cuvettes (K_s = 39 μM). No K_s was obtained for the very weak interaction with non-induced microsomes.

Harmol (*Fig.* 3). Harmol produced two concn-dependent spectral changes below 400 nm with boiled microsomes: one peak (λ_{max} = 340 nm) and one trough (λ_{min} = 390 nm with non-induced microsomes; 380 nm with a shoulder at 365 nm with PB-microsomes; a broad trough around 370 nm with MC-microsomes). The peak intensity was similar in each case, whereas the trough intensity varied, i.e. boiled PB- > boiled non-induced > boiled MC-microsomes.

Harmol addition to native non-induced, PB- or MC-microsomes produced: (a) a concn-dependent spectral peak at 340 nm, (b) a trough which showed a concn-dependent intensification preferentially at

365 nm relative to 380–390 nm, and (c) a second concn-dependent peak with λ_{max} at either 420 nm (non-induced microsomes, Fig. 3a), 425 nm (PB-microsomes, Fig. 3b) or 418 nm (MC-microsomes, Fig. 3c). As with harmine the 340 nm peak probably reflected a non-cyt. P-450 interaction. There were apparently two components to the trough, with a λ_{min} at 380–390 nm reflecting a non-cyt. P-450 interaction and a λ_{min} at 365 nm representing a modified Type II interaction between harmol and cyt. P-450. The complementary peak to the 365 nm trough was at 418–425 nm (peak wavelength dependent on the induction status of the microsomes) and the relative intensities of these modified Type II spectra were in the order MC- > PB- > non-induced microsomes. The overall shape of the interaction spectrum was similar for all three types of microsomes, except for: (a) notable shifts in λ_{max}; (b) differences in the sharpness of the peak, with the 418 nm peak of MC-microsomes showing much less high wavelength absorption than the 420 or 425 nm peaks of non-induced and PB-microsomes respectively; and (c) the presence with PB-microsomes of a shoulder at 405 nm. Furthermore, at high harmol concns the 390 nm component of the trough was much more evident with MC-microsomes than with non-induced or PB-microsomes. Above 400 nm the harmol interaction spectrum with PB-microsomes appeared to be a composite of the spectra with non-induced and MC-microsomes.

Double-reciprocal plots of the magnitude of the modified Type II spectral change (using tandem cuvettes) were linear over seven harmol concns (20–200 μM), giving K_s values of 500 and 62 μM for PB- and MC-microsomes respectively ($r = 0.952$ and 0.968). The plot of harmol interaction with non-induced microsomes intersected the ordinate very close to the origin, indicating a high K_s value ($\gg 500\ \mu$M), which was not accurately measurable with the substrate concn range used.

The spectral changes occurring above 400 nm upon addition of harmine or harmol to microsomes were verified using normal cuvettes. The involvement of cyt. P-450 in these spectral changes was confirmed by dithionite reduction of the microsomes, which abolished all spectra with the exception of that for harmine with MC-microsomes, which was only slightly diminished.

DISCUSSION

We have shown marked differences between PB and MC in their induction of the murine hepatic microsomal metabolism and cyt. P450 binding of harmine and its *O*-desmethyl metabolite, harmol. The absolute requirements for NADPH and oxygen, together with the inhibitory effects of CO, octylamine and ANF and the inducing effects of PB and MC, strongly suggest that the metabolism of harmine and harmol was catalysed by cyt. P-450. Differences in CO-sensitivity between different metabolic reactions involving the same or different substrates, such as observed here, are not uncommon [47, 48, 58]. The combined measurement of substrate disappearance and *O*-desmethyl metabolite formation showed that *O*-demethylation to harmol was the virtually exclusive route of harmine metabolism in control microsomes and was selectively and moderately induced (3-fold) by PB, but that MC selectively and greatly induced (30-fold) the metabolism of harmine by other routes, so far unidentified. The absence of a large MC-induced increase in harmol formation was compounded by the fact that harmol was itself a substrate for a metabolic reaction that was markedly and selectively induced (20-fold) by MC. The TLC results, supported by our unpublished data for [^{3}H]harmine, showed that the main MC-induced metabolite of harmine was a yellow compound (Y_1) that was, however, not formed via harmol. The identities of Y_1, and Y_2 and G (the minor MC-induced metabolites of harmine), and of the MC-induced metabolites of harmol are currently under investigation. The TLC behaviour of Y_1 in the two alternative solvent systems and its fluorescent colour characteristics indicate that Y_1 was probably a derivative of harmine retaining the β-carboline ring structure and that it was not a product of reduction to the yellow dihydro-β-carboline series (i.e. harmaline derivatives). A structurally related λ-carboline, 3-amino-1-methyl-5*H*-pyrido-[4,3b]-indole (Trp-P-2), is also metabolised by a preferentially MC-inducible reaction to hydroxylamine and nitroso derivatives [14]. Trp-P-2 is a product of tryptophan pyrolysis, and those of its metabolites that are formed preferentially by MC-induced cyt. P-450 are potent mutagens [36, 59]. However, it is unlikely that Y_1 or the unidentified metabolites of harmol were similar hydroxylamines or nitroso compounds, since neither harmine nor harmol possess the 3-amino group at which *N*-hydroxylation and nitrosation occur in Trp-P-2.

There were large kinetic differences between control, PB- and MC-induced microsomes and between the unspecified reactions and the *O*-demethylation reaction of harmine metabolism. The problems of enzyme kinetic interpretation for microsomal reactions have been succinctly discussed elsewhere [5, 60, 61]. Nevertheless, the apparent enzyme kinetics reported here have the practical importance of defining the substrate concns at which harmine and harmol metabolism may be used as valuable tools for measuring PB- and MC-induction of monooxygenation. The effects of the organic inhibitors were problematical. Although the extensive inhibition by octylamine and ANF of the reactions in control microsomes corroborates our conclusion that they were catalysed by cyt. P-450 [42, 43, 49, 50], the marked inhibition by 0.5 mM methimazole, especially of harmine *O*-demethylation, apparently conflicts with this and indicates the involvement of the microsomal mixed-function amine oxidase ('Ziegler's enzyme'). Although according to Prough and Ziegler [50] methimazole is a selective inhibitor of the amine oxidase and at concns up to 1 mM does not inhibit cyt. P-450 in rat liver microsomes, it is nevertheless a substrate for rat liver cyt. P-450 [62] and might reasonably be expected to inhibit it. Metyrapone and SKF-525A are preferential inhibitors of constitutive and PB-induced cyt. P-450, whereas ANF preferentially inhibits cyt. P-450 induced by MC or similar agents [42–45]. The considerably greater sensitivity of harmine metabolism

to ANF than to metyrapone or SKF-525A points to a predominant involvement of MC-inducible forms of cyt. P-450, even in control microsomes. Although the harmine binding spectra for control and PB-induced microsomes were complicated by the effects of apparently non-cyt. P-450 binding, they clearly indicated a Type II ligand interaction of either of the nitrogen atoms in harmine directly with the haem of cyt. P-450 [39, 51, 54, 55]. Pyridine or a pyridinic nitrogen in a larger compound give Type II spectra, while indole has been reported as causing either Type I or Type II spectra [52, 54, 63–65]. It is probable that the Type II interaction of harmine involved its pyridinic nitrogen rather than its bridging nitrogen, since there is a requirement that the nitrogen concerned be unhindered [54, 64]. Moreover, the bridging nitrogen is probably acidic with too high a pK_a value to provide the lone pair of electrons needed for haem liganding. In contrast, with MC-induced microsomes harmine gave a Type I spectrum, indicative of a hydrophobic interaction with the apoprotein of MC-induced cyt. P-450 [51, 52]. The mode of interaction of harmol was apparently similar with control, PB- and MC-induced cyt. P-450 and was different from the mode of interaction of harmine. Harmol gave modified Type II spectra (albeit with abnormally low wavelengths for the trough) indicative of a direct ligand interaction of the C7 oxygen with the cyt. P-450 haem [51, 54, 56]. That harmine and harmol interacted preferentially with MC-induced cyt. P-450 is indicated by the low apparent dissociation constant (K_s) for the harmine interaction (6.5 μM), by the fact that, in contrast to harmine the Type I binding of several other compounds is decreased by MC-induction [43, 57, 66] and by the much lower K_s value for harmol binding with MC-induced microsomes (62 μM) than with control or PB-induced microsomes (> 500 μM). The large disparity between K_s and app. K_m values for harmol could be explained if its metabolism were catalysed by a relatively minor, high-affinity form of cyt. P-450 but the Type II spectra reflected binding mainly to more predominant forms, which had a low affinity for harmol and did not metabolise it.

We should like to explain the large differences in reaction kinetics, the inhibitor responses and the cyt. P-450-binding results by the following suggestion. MC-induced cyt. P-450 had a high affinity for harmine and harmol plus a high reactivity that was directed away from the C7 position of the molecule, whereas control and PB-induced cyt. P-450 had a low affinity together with a relatively low reactivity that was directed toward the C7 position. A consideration of these results together with published reports for other compounds leads to a tentative structure–activity relationship for multiheterocyclic substrates of cyt. P-450. Zoxazolamine [4], ethoxycoumarin [5], ethoxyresorufin [3], ellipticine [7] and harmine and harmol are all linear, condensed multi-ring heterocycles sharing certain structural similarities. In particular, the presence in each of an oxygen or nitrogen atom capable of haem ligand-binding might influence the alignment of the compound in the substrate site of cyt. P-450 and also the position on the substrate molecule at which metabolism occurs. Taken together, the data for these substrates suggest an important influence of substrate size: an increase in the number of rings in the molecule, from two to three or four, increases the substrate selectivity towards MC-induced cyt. P-450, but with three rings providing the greatest metabolic reactivity [3–5, 7, 14].

Finally, in the light of our results and the literature concerning physiological β-carbolines [15–20, 26–29], in particular 6-methoxy-tetrahydro-β-carboline [18, 20, 32], we propose that β-carbolines may be endogenous substrates for cyt. P-450.

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