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# The interaction of metyrapone and α-naphthoflavone with rat olfactory cytochrome P-450

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Evidence that the olfactory metabolism of xenobiotics is distinctive from hepatic metabolism has become available from autoradiography studies of the distribution of tissue-bound metabolites of *N*-nitrosodiethylamine, which showed the highest concentrations in olfactory epithelium [1]. Conclusive evidence that olfactory epithelium contained high levels of cytochrome P-450 was published by Dahl *et al.* [2]. Olfactory P-450 can demethylate a wide range of compounds including environmental contaminants and odorants [3], and the tissue is capable of hydroxylating progesterone and testosterone in a number of positions on the steriod nucleus [4]. Attempts to induce the olfactory enzyme with classical inducers of hepatic P-450 have so far been unsuccessful. Phenobarbital produced only a 2-fold induction in one study [5], and no induction in another [6]. Similarly Bond [6] could not demonstrate induction using 3-methylcholanthrene, Arochlor 1254®, dioxane, or benzo[a]pyrene, but found a 2-fold induction with TCDD. In contrast, inhibition of olfactory P-450 can be easily demonstrated. Metyrapone can inhibit the deethylation of phenacetin [7], and the production of some of the metabolites of progesterone [8]. In addition Bond [6] has demonstrated inhibition of benzo[*a*]pyrene hydroxylation by metyrapone, α-naphthoflavone, and proadifen. In the present study we have used the 7-methoxy and ethoxy derivatives of coumarin, and the inhibitors metyrapone and α-naphthoflavone, to further characterise olfactory cytochrome P-450. In addition the use of a wide range of inhibitor concentrations has demonstrated apparent activation of the olfactory enzyme.

## *Methods*

Metyrapone and α-naphthoflavone were purchased from Aldrich Chemical Co. (Gillingham, Dorset, U.K.), 7-ethoxycoumarin, glucose-6-phosphate, NADP, and glucose-6-phosphate dehydrogenase (type XII) were from Sigma Chemical Co. (Poole, Dorset, U.K.) and were used as supplied. 7-Methoxycoumarin, purchased from Aldrich, was purified before use. A saturated solution, in diethyl ether, was washed in twice its own volume of glycine–NaOH buffer (0.2 M, pH 2.2). Solid methoxycoumarin was recovered by evaporating the ether under nitrogen. All other reagents were the highest grade available. Male Albino Wistar rats were purchased from Harlan-Olac (Bicester, Oxon., U.K.), kept in the Warwick University animal holding facility, and allowed water and rat chow *ad libitum* between purchase and use (at 200–250 g). Rats were killed by stunning and cervical dislocation. After decapitation the heads were split in sagittal section and the ethmoturbinates placed in (50 mM Tris-HCl, pH 7.4 and 10 mM EDTA). Turbinates from five animals were pooled for each experiment and homogenised with an "Ultra-turrax" homogeniser (Camlabs (Glass) Ltd., Cambridge, U.K.) for 40 sec in 10-sec periods separated by one minute cooling in ice to maintain the temperature below 4°. The homogenate was centrifuged at 100 *g* for 10 min. The pellet was resuspended in fresh buffer, rehomogenised, pooled with the supernatant from the 100 *g* centrifugation, and used as the starting material for fractionation. Livers were homogenised in the same buffer as the olfactory tissue using a glass/Teflon homogeniser (three passes). A first centrifugation at 10,000 *g* for 20 min removed gross debris, nuclei, and mitochondria, and a second centrifugation at 100,000 *g* for 60 min sedimented the microsomes. The microsomes were resuspended in the same buffer as above to give protein concentrations of 1–2 mg/ml for olfactory tissue, and 15–20 mg/ml for liver. Alkoxycoumarin dealkylase assays were carried out by a modification of the method described by Moloney *et al.* [9]. The total volume of incubate (1 ml) contained 0.5 mM NADPH, 6 mM glucose-6-phosphate, 3.5 mM magnesium chloride, 1 unit glucose-6-phosphate dehydrogenase 0.1 M Tris–HCl buffer (pH 7.6), between 0.02 and 0.09 mg microsomal protein and the concentration of inhibitor indicated in the table. In experiments with α-naphthoflavone the cosolvent dimethylformamide was included in both test and control incubations at a final concentration of 0.1% (v/v). Reactions were started after a 10 min preincubation at 37° by the addition of either 0.1 mM 7-ethoxycoumarin or 0.1 mM 7-methoxycoumarin and continued for 20 min. This concentration of substrate assays the high affinity deethylase in liver. The reaction was terminated by the addition of 0.5 ml of 0.2 M glycine–TCA buffer (pH 2.2), and unreacted substrate was extracted with 60–80 hexane (6 ml) for 10 min. 7-Hydroxycoumarin was then extracted into diethylether (4 ml) for 10 min, a 2.5 ml aliquot of ether was back-extracted with 0.2 M glycine–NaOH buffer (pH 10.6) and fluorescence measured in a Perkin–Elmer MPF-3 spectrofluorimeter ($\lambda_{EX} = 370$ nm, $\lambda_{EM} = 450$ nm). It was necessary to wash diethylether twice prior to use with glycine–NaOH buffer (0.2 M, pH 10.6). Standards of hydroxycoumarin (0.5–4 μM final incubate concentration) were carried through the assay procedure concurrently. Protein was assayed by the method of Lowry *et al.* [10].

## *Results*

Results are shown in Table 1. The specific activities of 7-ethoxycoumarin deethylase were six times higher in olfactory tissue than in hepatic tissue. Also shown are the inhibitions of hepatic and olfactory dealkylases. Hepatic 7-ethoxycoumarin deethylase was inhibited by metyrapone between $10^{-3}$ M and $10^{-7}$ M, but hepatic demethylase was inhibited between $10^{-3}$ M and $10^{-5}$ M and had activities higher than control values at $10^{-7}$ M. Hepatic 7-methoxycoumarin demethylase was inhibited by α-naphthoflavone between $10^{-5}$ M and $10^{-6}$ M, whereas hepatic 7-ethoxycoumarin was unaffected at these concentrations. Both olfactory dealkylases were inhibited by metyrapone between $10^{-3}$ M and $10^{-5}$ M, and by α-naphthoflavone between $10^{-5}$ M and $10^{-9}$ M. Olfactory dealkylase activities were greater than control values at $10^{-6}$ M and $10^{-7}$ M metyrapone. Dimethylformamide which was used as a co-solvent in this study reduced the activities of hepatic and olfactory dealkylases to approximately 60% of control values, as shown in Table 1. In a preliminary study (results not shown) it was found that 0.1% (v/v) ethanol, acetone, dimethylsulphoxide, isopropanol, and dimethylformamide all reduced hepatic 7-ethoxycoumarin deethylase by amounts similar to that shown in the table for DMF.

## *Discussion*

The higher specific activities of olfactory 7-ethoxycoumarin deethylase, than the hepatic enzyme, observed in this study is in agreement with previous studies [12]. In contrast the activity of 7-methoxycoumarin demethylase was found to be similar in olfactory and hepatic tissue. This

Table 1. Specific activities of rat olfactory 7-ethoxycoumarin deethylase (7ECD) and 7-methoxycoumarin demethylase (7MCD), and their inhibition by metyrapone and α-naphthoflavone

| Concentration of inhibitor | % Control activity | | | |
|---|---|---|---|---|
| | Hepatic | | Olfactory | |
| | 7ECD | 7MCD | 7ECD | 7MCD |
| Metyrapone | | | | |
| $10^{-3}$ M | 33% (28–38%) | 32% (29–39%) | 3% (2–4%) | 12% (10–14%) |
| $10^{-4}$ M | 69% (56–81%) | 65% (56–71%) | 11% (7–15%) | 41% (33–47%) |
| $10^{-5}$ M | 81% (77–84%) | 85% (82–89%) | 50% (44–58%) | 98% (77–125%) |
| $10^{-6}$ M | 88% (75–99%) | 100% (94–112%) | 109% (95–124%) | 126% (121–133%) |
| $10^{-7}$ M | 99% (100–98%) | 113% (102–131%) | 125% (117–140%) | 132% (121–144%) |
| α-Naphthoflavone | | | | |
| $10^{-5}$ M | 110% (93–124%) | 57% (48–68%) | 21% (15–29%) | 35% (34–37%) |
| $10^{-6}$ M | 107% (98–122%) | 84% (70–92%) | 34% (28–44%) | 46% (41–53%) |
| $10^{-7}$ M | 102% (88–107%) | 102% (81–128%) | 68% (61–75%) | 68% (63–71%) |
| $10^{-8}$ M | 101% (92–118%) | 105% (89–122%) | 74% (61–82%) | 69% (49–87%) |
| $10^{-9}$ M | 106% (92–122%) | 105% (99–116%) | 86% (83–88%) | 78% (68–93%) |
| Specific activities (nmol/min/mg microsomal protein) | | | | |
| Control | 0.66 ± 0.19 | 0.52 ± 0.22 | 3.77 ± 0.62 | 0.40 ± 0.19 |
| DMF (0.1% v/v) | 0.52 (0.49–0.57) | 0.34 (0.19–0.45) | 2.31 (1.96–2.68) | 0.27 (0.12–0.52) |

Inhibitions are expressed as percentage activity of a control containing no inhibitor (mean and range of 3 separate experiments). Native specific activities are mean and 95CL of 6 separate experiments, and those in the presence of dimethylformamide (DMF) the mean and range of 3 separate experiments.

confirms the observations of Dahl and Hadley [13] that not all substrates are metabolised with higher specific activities by olfactory tissue. Reed *et al.* [11] suggested that the higher concentration of cytochrome P-450 reductase in olfactory epithelium was partially responsible for these higher specific activities. If higher reductase levels were the only difference between olfactory and hepatic cytochrome P-450 monooxygenases, all substrates would be equally affected. Since all substrates are not metabolised at a faster rate by olfactory P-450, other factors must be involved, such as different mixtures of isoenzymes, in each tissue. Evidence that this may be the case is provided by our observations that the olfactory enzyme is more sensitive to metyrapone and α-naphthoflavone than the hepatic enzyme, and that some concentrations of metyrapone activate olfactory 7-ethoxycoumarin deethylase. Previous studies on inhibition of olfactory cytochrome P-450 have shown that the enzyme is sensitive to a range of inhibitors [6, 12]. In hepatic tissue 7-ethoxycoumarin deethylase displays biphasic kinetics, indicating the existence of at least two enzymes [14]. One enzyme has a low capacity and a high affinity, whilst the other has a high capacity and a low affinity. Selective inhibition of the high affinity enzyme, would enable the expression of the high capacity enzyme, and would result in apparent activation. This suggestion is not supported by the observations of Reed *et al.* [12]. In the hamster these workers could only demonstrate one olfactory $K_m$ of 7.1 mM, an order of magnitude higher than the highest $K_m$ in liver (53 μM). Further research is necessary to establish whether multiple forms of olfactory P-450 exist.

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