A Direct, Highly Sensitive Fluorometric Assay for a Microsomal Cytochrome P450-Mediated O-Demethylation Using a Novel Coumarin Analog as Substrate

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Direct Fluorometric Assay, Cytochrome P450, Demethylation of 3-Chloro-7-methoxy-4-methylcoumarin

A highly sensitive fluorometric assay for the determination of monooxygenase activity in liver microsomes is described. The assay is based on the use of 3-chloro-7-methoxy-4-methylcoumarin which is demethylated to 3-chloro-7-hydroxy-4-methylcoumarin. The rate of formation of 3-chloro-7-hydroxy-4-methylcoumarin was recorded as an increase of fluorescence (λ_A = 380 nm, λ_F = 480 nm) with time. When 3-chloro-7-methoxy-4-methylcoumarin was incubated in the presence of $MgCl_2$ and NADPH with rat liver microsomes, a continuous increase of the fluorescence could be measured. The reaction proceeded linearly for about 10 min and at least up to a concentration of 0.1 mg/ml of microsomal protein. Besides 3-chloro-7-hydroxy-4-methylcoumarin a hydroxylated derivative of the substrate was formed as a second metabolite during the incubation. Using an excitation wavelength of 380 nm and a fluorescence/emission wavelength of 480 nm, the fluorescence of this substance (λ_A = 338 nm, λ_F = 422 nm) amounted only to about 1% of the fluorescence of the main product. The use of 3-chloro-7-methoxy-4-methylcoumarin as substrate enables the fluorometric determination of the O-dealkylation activity of a cytochrome P450-dependent monooxygenase system in rat liver which is inducible by phenobarbital but not by 3-methylcholanthrene.

Introduction

The microsomal monooxygenase system of liver catalyses the metabolism of a wide variety of endogenous compounds including drugs, insecticides and carcinogens. To investigate monooxygenase systems, sensitive methods for the determination of enzyme activity are necessary.

DeLuca *et al.* (1986) reported the development of a direct fluorometric assay for a cytochrome P450 catalyzed reaction (O-deethylation), which is based on the use of 7-ethoxy-4-trifluoromethylcoumarin (EFC), an analog of the widely employed substrate 7-ethoxycoumarin (7-EC) (Ullrich and Weber, 1972). According to DeLuca *et al.* the use of 7-EC suffers from the drawback that the excitation and emission maxima of this substrate (340 and 450 nm respectively) correspond to NADPH, limiting the sensitivity of the direct 7-EC-assay. The assay using EFC as substrate does not have this disadvantage but the the low solubility of EFC in buffer requires the addition of 0.2% DMSO, which inhibits the reaction by about 10% (Buters *et al.*, 1993).

We report here the development and characterisation of a direct fluorometric assay for a cytochrome P450 catalyzed O-demethylation based on the use of 3-chloro-7-methoxy-4-methylcoumarin (CMMC) (Fig. 1). This assay exhibits the advantages of the direct EFC-test. Moreover, the substrate used in our assay does not require the addition of DMSO due to a better solubility in buffer.

Abbreviations: EFC, 7-ethoxy-4-trifluoromethylcoumarin; 7-EC, 7-ethoxycoumarin; PB, phenobarbital; MC, 3-methylcholanthrene; DMSO, dimethyl sulfoxide; CHMC, 3-chloro-7-hydroxy-4-methylcoumarin; CMMC, 3-chloro-7-methoxy-4-methylcoumarin.

Materials and Methods

Materials

3-chloro-7-hydroxy-4-methylcoumarin (CHMC) was obtained from Sigma-Aldrich Chemie GmbH (Steinheim, Germany). 3-chloro-7-methoxy-4-methylcoumarin was synthesized from CHMC with the aid of dimethylsulfate. 3-methylcholanthrene (MC) and metyrapone (2-methyl-1,2-di-

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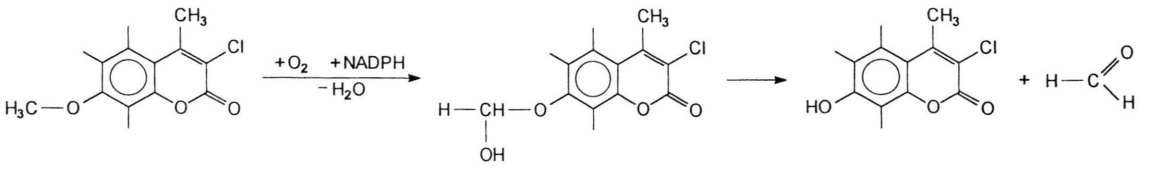

I II

Fig. 1. O-demethylation of 3-chloro-7-methoxy-4-methylcoumarin (I) to 3-chloro-7-hydroxy-4-methylcoumarin (II) by the cytochrome P-450 dependent monooxygenase enzyme system.

3-pyridyl-1-propanone), chlorzoxazone (5-chloro-2-hydroxybenzoxazole) and α-naphthoflavone (7,8-benzoflavone) were obtained from Sigma (Deisenhofen, Germany), phenobarbital (Luminal^R) (PB) from Bayer (Leverkusen, Germany). The sodium salts of NADH and NADPH were purchased from Boehringer Mannheim (Mannheim, Germany). The other commercial products were from Merck, (Darmstadt, Germany). All chemicals and biochemical used were of the highest grade available.

Chromatography

Thin-layer chromatography was performed on precoated TLC silica gel 60 plates (20 × 20 cm, thickness 0.25 mm) from Merck (Darmstadt, Germany) using the solvent system toluene/acetone (9:1, v/v). Developed plates were examined for fluorescence under the longwave ultraviolet light (366 nm) of an UV lamp (MinUVIS 13100) from Desaga (Heidelberg, Germany).

High-performance liquid chromatography was carried out on a HPLC system consisting of a Pharmacia LKB pump 2249 (Pharmacia LKB Biotechnology, Freiburg, Germany), a model 7125 syringe loading sample injector (Rheodyne, Cotati, California, USA) and a 250 × 8 mm column prepacked with nucleosil C_{18}, 5 μm particle size (Grom, Herrenberg-Kayth, Germany). The elution was performed with a mobile phase methanol/water (6:4, v/v). Detection was carried out with a Model RF-535 fluorescence HPLC monitor (Shimadzu Corporation, Kyoto, Japan). The excitation wavelength was 380 nm, the detection wavelength was 480 nm. The fluorescence detector was connected with a data processor, Chromatopac C-R6A (Shimadzu, Corporation, Kyoto, Japan).

Fluorometry

Fluorescence measurements were performed with a „Fluorolog F212" spectrofluorometer (Spex Industries Inc., Grasbrunn, Germany).

The fluorescence activation and emission spectra were recorded in a quartz cuvette with a 10 mm light path, Type 104 F-QS; the enzyme activities were assayed using a quartz cuvette with a 10 mm light path Type 119 000 F QS (Hellma GmbH & Co, Mülheim, Germany).

Mass spectrometry

Mass spectrometry was performed using a mass spectrometer „SSQ 7000" (Finnigan MAT). Fragmentation patterns resulting from electron impact of the main metabolite and the second minor metabolite have been determined.

Animals and enzyme induction

Male Sprague-Dawley rats, weighing 250–300 g, were purchased from Charles River (Sulzfeld, Germany). The animals were kept under standardized conditions with free access to pellet need and tap water.

Effects of pretreatment with phenobarbital or 3-methylcholanthrene in liver microsomes: One group of animals was induced with PB by replacing the drinking water by a solution of PB (0.1% in tap water) for 6 days prior to sacrifice. A second group of rats was given MC (20 mg/kg body weight in corn oil) intraperitoneally once each day for 3 days.

Preparation of microsomes

Rats were sacrificed by placing them into a carbon dioxide atmosphere. Immediately after death livers were excised.

All steps to prepare the microsomal fraction were carried out at 0–4 °C. Livers were homogenized in 0.25 M sucrose (4 ml/g liver) in a Waring blendor model 32 BL 80 (Waring products division, Dynamics Corporation of America, New Hartfort, USA). The homogenate was centrifuged at 13000×*g* for 30 min. The microsomal fraction was obtained by centrifuging the 13000×*g* supernatant for 60 min at 100000×*g*. The pellet was resuspended in 0.25 M sucrose and centrifuged again for 60 min at 100000×*g*. The pellet was resuspended in 0.25 M sucrose and stored as 1.2 ml aliquots at −70 °C.

Protein concentration was determined according to Lowry *et al.* (1951) using bovine serum albumin as a standard.

Test procedure

For measuring microsomal CMMC O-demethylase activity a standard incubation mixture was prepared by adding 2.79 ml of 3-chloro-7-methoxy-4-methylcoumarin ($2 \cdot 10^{-5}$ M in 0.05 M tris (hydroxymethyl)aminomethane/HCl (Tris/HCl), pH 7.6, dissolved with the aid of an ultrasonic bath), 30 µl of $MgCl_2$ (10^{-1} M), 90 µl of rat liver microsomes (corresponding to about 90 µg protein, in 0.25 M saccharose) in a fluorometer cuvette, thermostated to 30 °C. After preincubation for 5 min, the reaction was initiated by addition of 90 µl of NADPH (10^{-3} M in 0.05 M Tris/HCl, pH 7.6). The rate of formation of CHMC was recorded as the increase in fluorescence (λ_A = 380 nm, λ_F = 480 nm) versus time. To calibrate each measurement, 90 µl of a solution of 3-chloro-7-hydroxy-4-methylcoumarin ($2 \cdot 10^{-5}$ M in 0.05 M Tris/HCl buffer, pH 7.6) were added to the incubation mixture at the end of the experiment.

Product identification

For product identification by means of thin-layer chromatography and high-performance liquid chromatography the following incubation mixture was used: 95.0 ml of CMMC ($2 \cdot 10^{-5}$ M in 0.05 M Tris/HCl buffer, pH 7.6), 1.0 ml of $MgCl_2$ (10^{-1} M), 1.5 ml of liver microsomes from PB-induced rats (corresponding to about 1.5 mg protein, in 0.25 M saccharose), 3.3 ml of NADPH (10^{-3} M in 0.05 M Tris/HCl).

Six incubation mixtures were incubated, one of which was drawn up as a blank. To the latter incubation mixture 50 ml of chloroform was added and stirred for 30 min before the addition of NADPH. The procedure of the following steps was identical for all incubation mixtures. The incubation mixtures were incubated by shaking for 30 min in a water bath at 30°. With the exception of the blank, the reaction of each incubation mixture (sample) was terminated by the addition of 50 ml of chloroform. The samples were extracted 3 times with 50-ml portions of chloroform. The extract of the blank was reduced by evaporation to about 0.5 ml, the combined extracts of the other samples („probes") were also restricted to about 0.5 ml. The solutions were applied in lines (samples: 10.5 cm; blank: 2.5 cm in length) to a thin layer plate and chromatographed 2 times in the solvent system toluene/acetone (9:1, v/v). In the case of the „samples", examination of the TLC plate under UV-light revealed in addition to the substrate band a second band, which comigrated with an authentic sample of CHMC.

A silica gel band, 1.5 cm in width, was scraped off the plate from the dotting line to the solvent front in the direction of the solvent flow ("cross section band"). The rest of the band, which comigrated with CHMC („product band") was also scraped off in order to determine by HPLC whether this band represents a single substance. The silica gel portions were extracted twice with 2.5-ml portions of chloroform; the extracts were filtered through a glass fibre disc (APFF-Type F, Millipore, Bedford, Mass., USA). Before subjecting the samples to separation by HPLC the filtrates were evaporated, and the residues were redissolved each in 1 ml of methanol. The solutions were injected in 100 µl portions and chromatographed at a flow rate of 2 ml/min.

Concerning the HPLC chromatograms of the „product band", the fractions with the retention time of an authentic sample 3-chloro-7-hydroxy-4-methylcoumarin as well as the fractions of another metabolite with a retention time of 8.8 min were combined and evaporated to dryness. The residues were dissolved in 0.05 M Tris/HCl (pH 7.6) and the solutions were subjected to fluorescence spectroscopy.

With an other set of incubations chromatographic separations were carried out in the same

way. The HPLC-fractions of the two substances were also combined and evaporated to dryness. The residues were analysed by mass spectrometry.

Results

Fluorescence spectroscopy

The excitation spectra and the fluorescence emission spectra of CMMC and of CHMC (10^{-5} M in 0.05 M Tris/HCl buffer, p.H. 7.6) are shown in Fig. 2. The emission spectra were recorded at an excitation wavelength of 330 nm. If the excitation wavelength is adjusted to 380 nm the fluorescence of CHMC, measured at 480 nm is – compared with that of CMMC – enhanced by the factor of $3 \cdot 10^3$.

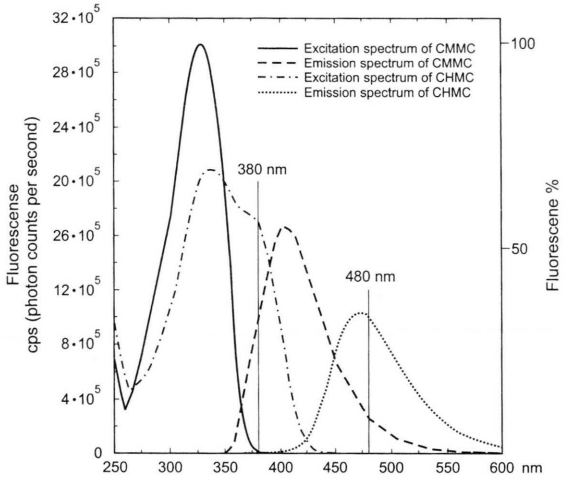

Fig. 2. Uncorrected fluorescence activation and emission spectra of 3-chloro-7-methoxy-4-methylcoumarin (CMMC) and 3-chloro-7-hydroxy-4-methylcoumarin (CHMC) (10^{-5} M solutions in Tris/HCl, pH 7.6). The emission spectra were recorded at an excitation wave length of 330 nm. If the excitation wave length is adjusted to 380 nm the fluorescence of 3-chloro-7-hydroxy-4-methylcoumarin, measured at 480 nm, is – compared to that of 3-chloro-7-methoxy-4-methylcoumarin – enhanced by the factor of $3 \cdot 10^3$. Key: (—) Excitation spectrum of 3-chloro-7-methoxy-4-methylcoumarin; (– – –) fluorescence emission spectrum of 3-chloro-7-methoxy-4-methylcoumarin; (–·–·–·) excitation spectrum of 3-chloro-7-hydroxy-4-methylcoumarin; (···) fluorescence emission spectrum of 3-chloro-7-hydroxy-4-methylcoumarin. The maximum of the excitation curve of CMMC was taken as 100%.

The only cofactor which might, regarding its excitation and emission maximum (380 nm and 470 nm, respectively) influence the measurement of fluorescence in the test is NADPH. Compared with the substrate, NADPH was added to the incubation mixture in a molar ratio of 1 : 1.5. At the wavelengths of 380 nm and 480 nm the interfering fluorescence of NADPH is in this concentration about 1000-fold lower than that of CHMC.

Incubation experiments with rat liver microsomes

Identification of the products: For product identification by means of chromatography, CMMC was incubated as described above with liver microsomes from phenobarbital induced rats. The extracts of the incubation mixtures were analysed by TLC using the mobile phase toluene/acetone (9:1, v/v). In comparison with the controls, a new fluorescent line with the R_F-value of authentic CHMC (R_F = 0.45) was seen under 366 nm illumination. This band contained – as shown by HPLC – besides the main product a further metabolite. The identification of the main product (t_R = 11.2–11.3) as CHMC was carried out by fluorescence spectroscopy and mass spectrometry: The main metabolite showed the same excitation and fluorescence spectra as CHMC exhibiting maxima at λ_A = 340 nm and λ_F = 475 nm.

The mass spectrum was identical with that of the authentic substance. As in the spectrum of authentic CHMC the molecular ion (*m/z* 210) forms the base peak and a strong $[M\text{-}CO]^+$ ion, 28 mass units lower, is also present (*m/z* 182). An ion at *m/z* 154 and another ion at *m/z* 147 is formed from the $[M\text{-}CO]^+$ ion by loss of CO and Cl respectively. A loss of Cl from the ion at *m/z* 154 forms an ion peak at *m/z* 119 and a loss of CO from this ion forms a peak at *m/z* 91.

The minor metabolite (t_R = 8.7–8.9) exhibited (in 0.05 M Tris/HCl buffer, pH 7.6) an excitation maximum λ_A at 338 nm and a fluorescence emission maximum λ_F at 422 nm. The mass spectrum of the substance showed that this metabolite is a hydroxylated product of the substrate CMMC. The molecular ion (*m/z* 240) forms the base peak in the spectrum of this minor metabolite and a $[M\text{-}CO]^+$ ion (*m/z* 212) is also present. Loss of Cl from this ion intervenes to provide an ion at *m/z* 177. Otherwise, loss of Cl from the molecular ion forms an ion at *m/z* 205. Loss of CO from this ion also forms the ion at *m/z* 177.

In order to estimate the fluorescence intensity of the minor metabolite in comparison with that of

CHMC, we collected both metabolites, employing three different separations on the HPLC-column.The fractions were evaporated, the residues dissolved in 0.05 M Tris/HCl, pH 7.6. Exited at 380 nm, the fluorescence intensity of the minor metabolite, measured at 480 nm, amounted only to 1.21 ± 0.28% to that of CHMC.

Direct fluorometric assay: When CMMC was incubated with liver microsomes of untreated rats, a continuous increase of fluorescence could be measured as soon as the reaction has been initiated by the addition of NADPH. The O-demethylation of the substrate by liver microsomes from untreated, PB- and MC-pretreated rats displayed a pH-optimum between 7.4 and 7.6. The reaction proceeded linearly at least up to 0.2 mg/ml of microsomal protein and for about 10 min (see Fig. 3). Under the conditions described above, the mean O-demethylase activity was calculated to be 1.5 ± 0.13 nmol CHMC/min · mg protein. The specific activities found in liver microsomes of rats pretreated with PB and MC were 1.9 ±0.52 and 1.3 ± 0.11 nmol CHMC/min · mg protein. Hence, pretreatment of rats with phenobarbital increased slightly the specific demethylation activity. But, the metabolic rate using microsomes of rats pretreated with MC were less than those observed for constitutive microsomal activities.

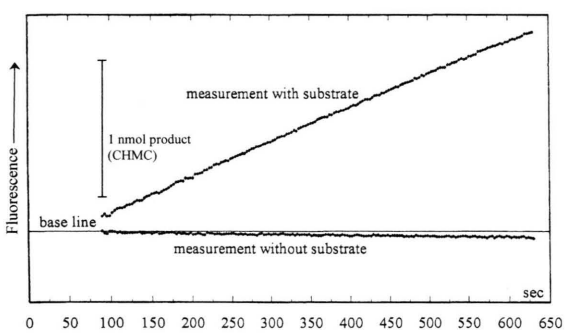

Fig. 3. Recording of 3-chloro-7-hydroxy-4-methylcoumarin (CHMC) formation by liver microsomes from rats pretreated with phenobarbital.
The standard incubation was prepared by mixing 2.79 ml of 3-chloro-7-methoxy-4-methylcoumarin ($2 \cdot 10^{-5}$ M in 0.05 M Tris/HCl, pH 7.6), 30 µl of $MgCl_2$ (10^{-1} M), 90 µl of rat liver microsomes (corresponding to 90 µg protein, in 0.25 M saccharose) in a fluorometer cuvette, thermostated at 30 °C. After preincubation for 5 min the reaction was initiated by addition of 90 µl of NADPH (10^{-3} M in 0.05 M Tris/HCl, pH 7.6). The rate of formation of 3-chloro-7-hydroxy-4-methylcoumarin was recorded as the increase in fluorescence (λ_A = 380 nm, λ_F = 480 nm). The back ground rate of change with time (incubation without substrate) was very small.

Cofactor requirements: The enzymatic generation of CHMC was oxygen-dependant: This could be shown by gassing the incubation mixture (-NADPH) with argon prior to the addition of NADPH. There was a strict requirement for NADPH. The addition of NADH instead of NADPH as electron donor resulted in a loss of enzyme activity which was found to be only 7% (PB-pretreated rats) and 12% (MC-pretreated rats). Also Mg^{2+} had a significant effect on the O-dealkylation activity (Table I). The fluorescence of NADPH did not interfere with the test.

Effect of various inhibitors on the O-demethylation of CMMC by rat liver microsomes: CMMC O-demethylation responded to PB but not to MC induction. To gain more insight into the cytochrome P450 isoenzymes catalysing O-demethylation of CMMC, inhibition studies were performed in liver microsomes of untreated rats and rats pretreated with PB (see Table II). Metyrapone (2-methyl-1,2-di-3-pyridyl-1-propanone) inhibited the 3-chloro-7-methoxy-4-methylcoumarin O-demethylation in PB induced microsomes (62%) to a greater extend than in uninduced microsomes (28%). Chlorzoxazone inhibited the metabolic generation of 3-chloro-7-hydroxy-4-methylcoumarin in microsomes of PB-induced rats by 61% and in microsomes of uninduced rats by 28%. The classic inhibitor of the cytochrome 1A family (P450 1A1 and 1A2), α-naphthoflavone, (Murray and Reidy, 1990; Boobis *et al.*, 1990; Guengerich, 1991) inhibited the reaction neither in PB induced nor in uninduced microsomes.

Assay kinetics: The enzyme kinetics of CMMC O-demethylation were determined using liver microsomes from-untreated rats and PB- induced rats. Representative examples of Hanes and Eadie-Hofstee transformation are shown in Fig. 4. In the Hanes and Eadie-Hofstee plots of the CMMC O-demethylation by microsomes of PB-treated rats, more than one phase became apparent. We conclude that more than one isoenzyme metabolized the substrate. V_{max} and K_m were calculated from a linear regression equation fitted to the linear part of the Hanes transformed data. As in the case of deethylation of EFC (Buters *et al.*, 1993) this approach yields hybrid parameters. Linear regression analysis of the data presented gives

Table I. Cofactor requirement for the O-demethylation of 3-chloro-7-methoxy-4-methylcoumarin by liver microsomes of untreated rats and rats pretreated with phenobarbital and 3-methylcholanthrene, respectively.

Cofactor omitted or added	Untreated rats	Rats pretreated with	
	Relative activity (%)	Phenobarbital Relative activity (%)	3-Methylcholanthrene Relative activity (%)
Complete system[a]	100 (1.5 nmol·min^{-1}·mg prot.$^{-1}$)	100 (1.9 nmol·min^{-1}·mg prot.$^{-1}$)	100 (1.3 nmol·min^{-1}·mg prot.$^{-1}$)
- NADPH[b]	0	0	0
- NADPH + NADH[c]	10	7	12
- $MgCl_2$[d]	59	75	74
- O_2 + Ar[e]	0	0	0

[a] Complete reaction mixture was prepared by adding 2.79 ml 3-chloro-7-methoxy-4-methylcoumarin ($2 \cdot 10^{-5}$ M) in 0.05 M Tris / HCl (pH 7.6), 30 µl of $MgCl_2$ (10^{-1} M), 90 µl of rat liver microsomes (corresponding to about 90 µg protein, in 0.25 M saccharose) in a fluorometer cuvette, thermostated to 30 °C. After preincubation for 5 min, the reaction was initiated by addition of 90 µl of NADPH (10^{-3} M in 0.05 M Tris / HCl, pH 7.6).
[b] NADPH was replaced by 0.05 M Tris / HCl
[c] NADPH was replaced by 10^{-3} M NADH
[d] $MgCl_2$ was replaced by 0.05 M Tris / HCl
[e] Complete reaction mixture (- NADPH) was gassed with oxygen-free argon for 20 min. NADPH solution was gassed with oxygen-free argon as well prior to addition to the reaction mixture.
The activity is expressed on the basis of nmol 3-chloro-7-hydroxy-4-methylcoumarin generated per mg microsomal protein and min.

Table II. Effect of various inhibitors on the O-demethylation of 3-chloro-7-methoxy-4-methylcoumarin by rat liver microsomes of untreated rats and rats pretreated with phenobarbital.
The inhibitors were coincubated with 3-chloro-7-methoxy-4-methylcoumarin, rat liver microsomes, $MgCl_2$ and NADPH.

	Inhibitor concentration	Untreated rats	Rats pretreated with phenobarbital
		Inhibition (%)	Inhibition (%)
Complete system		0	0
Metyrapone	10^{-4} M	28	62
Chlorzoxazone	10^{-3} M	28	61
α-Naphthoflavone	10^{-5} M	0	0

the following parameters (v_{max} is given in nanomol per min per milligram protein and apparent K_m in micromolar): v_{max} = 4.41, K_m = 14.96 for PB-induced microsomes; v_{max} = 3.78, K_m = 4.27 for microsomes of untreated rats.

Discussion

We have examined the utility of the coumarin derivative, CMMC, in the determination of monooxygenase activities in liver microsomes of untreated, PB- and MC-treated rats. We were able to describe a direct test. Cofactor requirements and inhibitors point to the involvement of cytochrome P450 as terminal oxidase.

Besides the demethylation product CHMC only one further metabolite was found to be formed during the incubations. Under assay conditions (pH 7.6), the fluorescence of this substance was only about 1% of the fluorescence of the main product. We found a high fluorescence yield of CHMC relative to CMMC at an exitation wave length and a fluorescence/emission wave length of

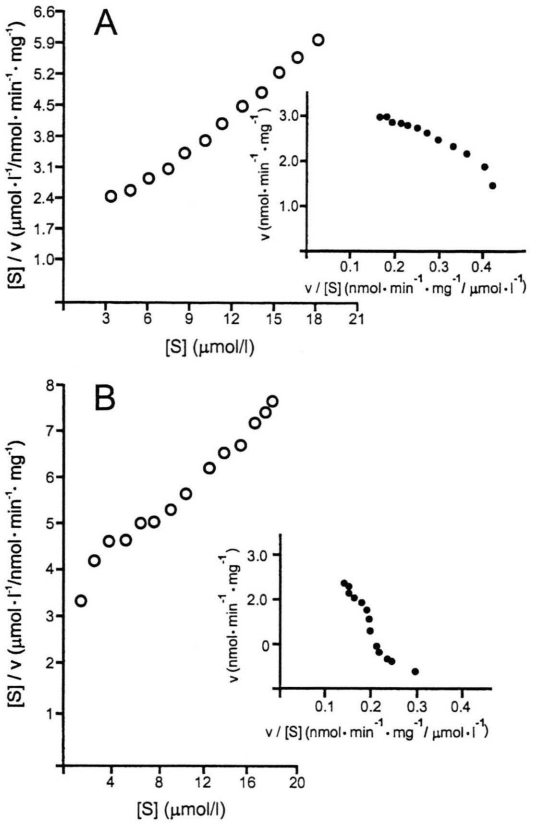

Fig. 4. Representative examples of Hanes-Wilkinson and Eadie-Hofstee (inserts) transformations of 3-chloro-7-methoxy-4-methylcoumarin demethylation in microsomes of untreated rats (A) and rats pretreated with phenobarbital (B). [*S*] is given in µmol/l, *v* is given in nmol · min · mg)$^{-1}$. Each point represents the average of two determinations obtained in a representative experiment.
The regression equations were $Y = 0.265X + 1.13$ (A) and $Y = 0.227X + 3.39$ (B).

380 nm and 480 nm respectively. In rat liver microsomes the O-demethylation proceeds linearly with protein concentration and time. We found a very low back ground drift (Fig. 3) and a high level of activity with uninduced samples.

So, we described here a direct microsomal test, in which the microsomal activity can be measured without sample clean up, which has an advantage over the procedure, described by De Luca *et al.* (1986). Contrary to EFC, CMMC can be dissolved in buffer without requiring the addition of DMSO, which inhibits the reaction. We obtained evidence that more than one isoenzyme metabolized 3-chloro-7-methoxy-4-methylcoumarin. The demethylation of 3-chloro-7-methoxy-4-methylcoumarin was moderately induced by PB but not by MC. The isoenzymes induced by PB had low affinity relative to control. DeLuca *et al.* (1986) examined the induction of EFC O-deethylase with PB and MC. While both reagents clearly induced EFC O-deethylase activity the isoenzymes induced by MC appeared to have high affinity for EFC whereas those induced by PB had – comparable with our results – low affinity relative to control.

Metyrapone, one of the most frequently employed P450 inhibitors, which is known as an inhibitor of phenobarbital induced monooxygenases, inhibited the CMMC O-demethylase activity in microsomes of rats pretreated with PB and untreated rats by 62% and 28%, respectively. Chlorzoxazone, which is considerd to be a specific substrate of P450 2E1 (Peter *et al.*, 1990) inhibited CMMC O-demethylation in PB- induced microsomes by 61% and in uninduced microsomes by 28%. Reactions catalysed by cytochrome P450 2E1 often show cross reactivity with P450 1A2 (Raucy *et al.*, 1989; Buters *et al.*, 1993; Yamazaki *et al.*, 1996). But, α-naphthoflavone, an inhibitor of the cytochrome 1A family (P450 1A1 and 1A2) (Murray and Reidy, 1990; Boobis *et al.*, 1990; Guengerich, 1991) did not inhibit the metabolic generation of CHMC in untreated rats and rats pretreated with phenobarbital. No inhibition by α-naphthoflavone in uninduced rat and human livers was also reported with regard to the O-deethylation of 7-EC (Kremers *et al.*, 1981; Buters *et al.*, 1993), which has shown to be catalysed by cytochromes P450 2E1 and 1A2 in microsomes of rat liver (Ryan and Levin, 1990) and human liver (Yamazaki *et al.*, 1996).

Due to the inhibition by chlorzoxazone we suppose that cytochrome P450 2E1 is one of the enzymes involved in the demethylation of CMMC. A participation of P450 1A2 in this reaction cannot be excluded.

There are several similar direct fluorometric assays for the determination of cytochrome P450 enzyme activity available, utilising in addition to 7-ethoxycoumarin (Ullrich and Weber, 1972), alkoxy resorufins (Mayer *et al.*, 1990), 3-cyano-7-ethoxycoumarin (White, 1988) and scoparone (Müller-Enoch *et al.*, 1981) as substrates. The assay described by DeLuca *et al.* (1986), using the substrate

7-ethoxy-4-trifluoromethylcoumarin offers as confirmed by Buters *et al.* (1993) some distinct advantages over these assays. The assay described here, which exhibits the advantages of the EFC-test, has the further advantage that the substrate – in the concentration required – does not need the addition of a solubiliser due to a higher solubility in buffer. CMMC is metabolised mainly to CHMC. The fluorescence of a minor metabolite, a hydroxylated derivative of the substrate, under the conditions of the test, accounted for only about 1% of the fluorescence of the main product. Compared to the EFC-test, the amount of microsomal protein for an assay with CMMC as substrate is very low. In conclusion, CMMC is a useful substrate for monitoring cytochrome P450 activity in rat liver microsomes. It represents a valuable addition to the tools available for investigation of this enzyme system.

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