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A STUDY OF MONOOXYGENASE ACTIVITY IN HUMAN PLACENTAL HOMOGENATES: *IN VITRO* BEHAVIOUR TOWARDS A NUMBER OF SUBSTRATES AND INHIBITORS

ALFRED C. KAELIN* and ALAN J. CUMMINGS
Division of Perinatal Medicine, Clinical Research Centre, Watford Road, Harrow, Middlesex HA1 3UJ, U.K.

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Abstract—The *in vitro* effects of cimetidine, metyrapone, SKF-525A and α-naphthoflavone on the monooxygenase activity in human placental tissue have been determined by indirect fluorimetric assay methods in placental homogenates from five maternal smokers. The inhibitor concentrations producing half-maximum inhibition (I_{50} values) were calculated for the *O*-deethylation of 7-ethoxycoumarin and 7-ethoxyphenoxazone, and the hydroxylation of 2,5-diphenyloxazole. The results indicate that cimetidine is a weak inhibitor of the placental monooxygenase system, resembling metyrapone and SKF-525A in its effects rather than α-naphthoflavone. Characterization of the behaviour of the three substrates towards placental monooxygenase activity indicates a much greater enzymic affinity for 7-ethoxyphenoxazone than for 7-ethoxycoumarin or 2,5-diphenyloxazole.

The human placenta has the ability to metabolize a number of xenobiotics by means of an inducible cytochrome P-448 associated monooxygenase enzyme system. This enzyme system is induced in placentas as a result of maternal cigarette smoking during pregnancy [1]. The hydroxylation of zoxazolamine, benzo[*a*]pyrene and 2,5-diphenyloxazole, the *O*-deethylation of 7-ethoxyphenoxazone and 7-ethoxycoumarin and the *N*-demethylation of 3-methyl-4-monomethylaminoazobenzene by preparations of placental microsomes or homogenates have been demonstrated *in vitro* [2–9].

The co-administration of the histamine H_2-receptor antagonist cimetidine, extensively used to treat gastric hyperacidity [10], has been reported to decrease the clearance of several drugs including diazepam [11], theophylline [12], warfarin [13] and caffeine [14]. Recent evidence has indicated that these effects result from direct action of cimetidine on cytochrome P-450 rather than from an indirect effect of histamine H_2-receptor blockade [15].

Preliminary studies of the effect of cimetidine on human placental monooxygenase activity carried out by us [16] indicated the need for a more fundamental approach to the characterization of this enzyme system, in particular its behaviour towards a number of different substrates and inhibitors. In this study we present the results of an investigation of the metabolism of 2,5-diphenyloxazole, 7-ethoxyphenoxazone and 7-ethoxycoumarin substrates by human placental homogenates and compare and contrast the effects of cimetidine with the known monooxygenase inhibitors α-naphthoflavone, metyrapone and SKF-525A.

* To whom correspondence should be addressed.

MATERIALS AND METHODS

Chemicals. 7-Ethoxycoumarin, umbelliferone (7-hydroxycoumarin), cimetidine, α-naphthoflavone (7,8-benzoflavone), metyrapone and NADPH were purchased from Sigma (London) Chemical Co. (Poole, Dorset, U.K.). SKF-525A was kindly provided by Dr. M. D. Burke, Department of Pharmacology, Marischal College, University of Aberdeen.

Sample preparation. Placentas were obtained from women who smoked cigarettes during their pregnancy and who had delivered at term after a normal pregnancy and labour. Placentas were stored frozen (−20°) until required for assay when they were allowed to thaw out overnight at 4° before being minced using a Bauchnecht tissue mincer after removal of the membranes and umbilical cord. A portion of the mince was homogenized (14,500 rpm, 1 min) in ice-cold 50 mM Tris–HCl buffer (pH 7.5) using a Silverson homogenizer to yield a 20% (w/v) homogenate.

Assay methods for determination of placental monooxygenase activity. The three methods of assay used in this work were, in most part, major modifications of previously described techniques [17–19]. The modified assay methods using the substrates 2,5-diphenyloxazole and 7-ethoxyphenoxazone (7-ethoxyresorufin), which have already been reported [3, 5], were very similar to the method using 7-ethoxycoumarin as substrate, which is described here.

For the determination of monooxygenase activity using 7-ethoxycoumarin as substrate the assay medium contained NADPH (2.4 mM) and 7-ethoxycoumarin (0.67 mM) in 50 mM Tris–HCl buffer (pH 7.5). Placental homogenate (0.5 ml) was equilibrated in duplicate (5 min, 37°) before the

enzyme reaction was initiated with assay medium (1.0 ml). After incubation (10 min, 37°) the reaction was stopped by the addition of 4.5 ml of a solution of acetone (10% v/v) in chloroform. Blank solutions were prepared by adding the placental homogenates after stopping the reaction. The tubes were vortex-mixed (15 sec) and after centrifugation (1500 *g*, 5 min) the metabolic product, 7-hydroxycoumarin, was back-extracted from an aliquot (3.0 ml) of the lower, organic phase into 1.0 ml of 50 mM Tris buffer (pH 9.8) by vortex-mixing (15 sec). After centrifugation (1500 *g*, 5 min) the fluorescence of the upper, aqueous layer from this mixture was determined in an Aminco–Bowman spectrophotofluorometer (American Instrument Co. Inc., MD) at uncorrected excitation and emission wavelengths of 368 and 456 nm, respectively. Quinine sulphate solution (10 μg/ml in 0.1 M H_2SO_4) was used for calibration of the spectrophotofluorometer. The sensitivity of the instrument was set to an arbitrary value of 34% relative to the fluorescence of this standard (100%) at the excitation and emission wavelengths quoted above.

The recovery of metabolite was determined by adding a known quantity of 7-hydroxycoumarin to the complete reaction mixture after addition of acetone and chloroform. Addition of homogenate to the assay tubes resulted in a fall in metabolite recovery from 81.1% ± 6.3% (mean ± S.D., $n = 16$) to 50.0% ± 1.9% ($n = 12$). In the absence of homogenate almost quantitative transfer of solvent (4.4 ml) was possible whereas with homogenate present formation of a homogenate 'disc' occurred during centrifugation. The removal of this disc resulted in a reduced amount of solvent transfer (3.0 ml) together with a fall in metabolite recovery.

The effect of inhibitors upon placental monooxygenase activity *in vitro*, as measured using 2,5-diphenyloxazole, 7-ethoxyphenoxazone and 7-ethoxycoumarin substrates, was assessed by adding appropriate amounts of each of the inhibitors, dissolved in dimethyl sulphoxide, to the corresponding assay media. The range of inhibitor concentrations used in the assays were: cimetidine (3.3–67 mM), α-naphthoflavone (0.067–3.3 μM), metyrapone (0.17–5.0 mM) and SKF-525A (0.067–3.3 mM).

Assay characteristics. Whilst the fluorescence of the metabolite(s) formed as a result of 2,5-diphenyloxazole hydroxylation appeared to be quite stable in 1 M NaOH for several hours, both 7-hydroxyphenoxazone (resorufin) and 7-hydroxycoumarin fluorescence decayed rapidly under these conditions. Optimum metabolite stability and fluorescence were obtained in the standard assays by final extraction into 50 mM Tris buffer at pH 9.8. Using this extracting medium resorufin was found to be light-insensitive and the loss of fluorescence intensity of 2 μM solutions of resorufin and 7-hydroxycoumarin was found to be minimal (9 and 2%, respectively, after 1 hr).

Calculation of enzyme activity. Because the metabolite(s) resulting from hydroxylation of 2,5-diphenyloxazole have not been characterized, activities were expressed as net percentage fluorescence intensity (sample minus blank) per g of wet-weight placental tissue per min of incubation time. In the case of the two other assay systems, quantities of metabolite formed as a result of deethylation were calculated by comparing the net percentage fluorescence of the final alkaline extract with a standard plot of metabolite concentration vs percentage fluorescence. Activities were corrected for percentage recovery of product, and are expressed as nmole of metabolite formed per g of wet-weight tissue per min of incubation time.

RESULTS

Reaction characteristics

Assessment of the effect of incubation time upon placental *O*-deethylase and hydroxylase activity using the three substances showed that both 7-ethoxyphenoxazone and 7-ethoxycoumarin deethylase activities and 2,5-diphenyloxazole hydroxylase activity varied linearly with time up to 5, 10 and 20 min, respectively. With all three substrates, placental monooxygenase activity was found to be proportional to homogenate concentration up to 30% (w/v).

The rate of metabolite formation was substrate concentration dependent in the case of 7-ethoxyphenoxazone up to a substrate concentration of 1.2 μM. Further increases in substrate concentration resulted in a sharp decrease in the rate of metabolite formation indicating the possibility of substrate inhibition. All assays of placental monooxygenase activity in homogenates involving this substrate contained a standard concentration of 7-ethoxyphenoxazone in the incubation mixtures (1.0 μM). A similar, though much less pronounced, decrease in rate of metabolite formation with increasing substrate concentration was found to occur using 7-ethoxycoumarin when substrate concentrations exceeded 0.67 mM. A standard substrate concentration in the incubation mixtures of 0.45 mM was adopted for all determinations of placental 7-ethoxycoumarin deethylase activity.

Substrate concentrations giving half the maximum observed reaction rate were found to be 0.3, 4 and 30 μM for 7-ethoxyphenoxazone, 2,5-diphenyloxazole and 7-ethoxycoumarin substrates, respectively.

A double-reciprocal plot of reaction velocity against substrate concentration for the *O*-deethylation of 7-ethoxyphenoxazone produces a non-linear curve of the type shown in Fig. 1a. A double-reciprocal plot for the *O*-deethylation of 7-ethoxycoumarin results in a similar form of curve although experimental data at high substrate concentrations are restricted in the latter case due to the limited solubility of the substrate in the assay medium. It is interesting to note that the double-reciprocal plot obtained for the hydroxylation of 2,5-diphenyloxazole and shown in Fig. 1b, although also non-linear, has a markedly different curve shape to that shown in Fig. 1a.

Under normal assay conditions maximum rates of metabolite formation were observed when the NADPH concentration in the incubation mixture was in excess of 1.2 mM (Fig. 2). The concentration of NADPH used in the assays was standardized at 1.6 mM.

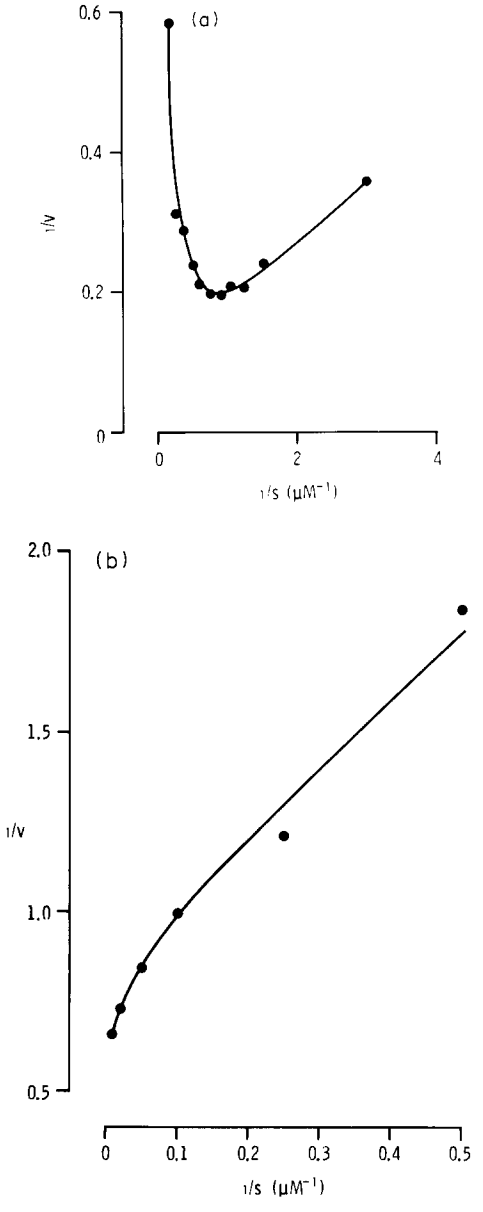

Fig. 1. Double-reciprocal plots for: (a) *O*-deethylation of 7-ethoxyphenoxazone and (b) hydroxylation of 2,5-diphenyloxazole. Reaction velocities are given as nmole resorufin formed/g per min and % fluorescence/g per min for 7-ethoxyphenoxazone and 2,5-diphenyloxazole substrates, respectively. Substrate concentrations are expressed as μmole/l.

Maximum monooxygenase activity towards the three substrates was found to occur in placental homogenates incubated at pH 7.5, and changing this pH by 0.5 unit either way resulted in a sharp decrease in enzyme activity (Fig. 3).

Inhibitor studies

The effect of cimetidine, metyrapone, SKF-525A and α-naphthoflavone on monooxygenase activity in placentas from maternal smokers is shown in Fig. 4(a–c). The placental system was markedly inhibited by α-naphthoflavone but only weakly inhibited by metyrapone, SKF-525A and cimetidine.

Inhibitor concentrations producing 50% inhibition of maximum monooxygenase activity (I_{50} values) were calculated in order to quantitate the effect of the four inhibitors upon placental enzyme activity as measured by the three substrates, and are shown in Table 1. The I_{50} values for inhibition of placental *O*-deethylase activity are generally lower than those obtained for inhibition of 2,5-diphenyloxazole hydroxylation. The I_{50} values for cimetidine ($4.5–27.2 \times 10^{-3}$ M) resemble more closely those for metyrapone ($3.4–15.0 \times 10^{-4}$ M) and SKF-525A ($6.1–7.7 \times 10^{-4}$ M) than those found for α-naphthoflavone ($1.3–12.1 \times 10^{-7}$ M), thus the inhibitory effects of cimetidine towards placental monooxygenase activity that has been induced by smoking are quite small in comparison with those produced by α-naphthoflavone.

Care has to be taken when interpreting both the substrate and inhibitor constants, especially in the case of 7-ethoxyphenoxazone. The standard assay concentration of both 7-ethoxycoumarin and 2,5-diphenyloxazole is some 15-fold greater than the substrate concentration that gives half-maximum observed velocity. Because of the problem of substrate inhibition encountered with 7-ethoxyphenoxazone the assay concentration used for this compound is only about three times greater than that required to give half-maximum velocity. Should any of the inhibitors tested act in a competitive manner then this difference in substrate concentration could affect differences in I_{50} values seen between this substrate and the others.

DISCUSSION

The assay methods presented in this report are based on measuring placental monooxygenase activity in homogenates whereas many published methods of determining such activity have used microsomal preparations [17, 18, 20]. Preparation of such samples by techniques such as differential centrifugation undoubtedly leave a substantial amount of monooxygenase activity residing in other fractions [21, 22]. The use of homogenates increases the likelihood of the total monooxygenase activity in the sample being assayed and, because the assays are based on a simple two-stage extraction procedure, problems caused by sample turbidity and other fluorescence quenching effects, such as protein binding of resorufin in the case of the 7-ethoxyphenoxazone assay [23], are avoided.

We found appreciable differences between the substrate concentrations required to give half the maximum observed rate for the three substrates used in this study, which suggest that there are marked differences between placental enzyme affinity for these substrates. The values that we obtained for the substrate concentrations giving half the maximum observed rate for the *O*-deethylation of 7-ethoxycoumarin (30 μM) and 7-ethoxyphenoxazone (0.3 μM) agree well with the results of similar studies of human placental monooxygenase activity reported by Pelkonen and Moilanen (25–70 μM, [6]) and Manchester (0.08–0.38 μM, [7]), respectively.

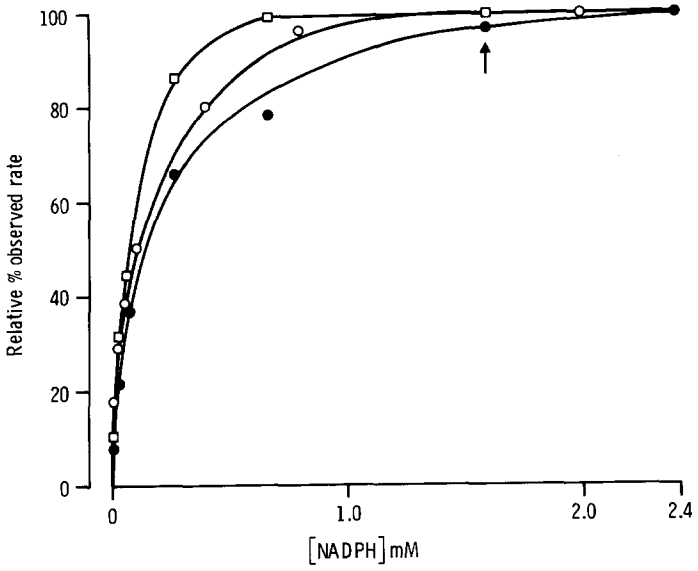

Fig. 2. Relationship between human placental monooxygenase activity and NADPH concentration for assays using 2,5-diphenyloxazole (○), 7-ethoxycoumarin (●) and 7-ethoxyphenoxazone (□) as substrates. Enzyme activities are expressed as relative % observed rate where 100% rate values are 1.64 (% fluorescence/g per min), 0.45 and 2.82 (nmole product formed/g per min) for 2,5-diphenyloxazole, 7-ethoxycoumarin and 7-ethoxyphenoxazone substrates, respectively. The arrow denotes the NADPH concentration used in the standard assay (1.6 mM).

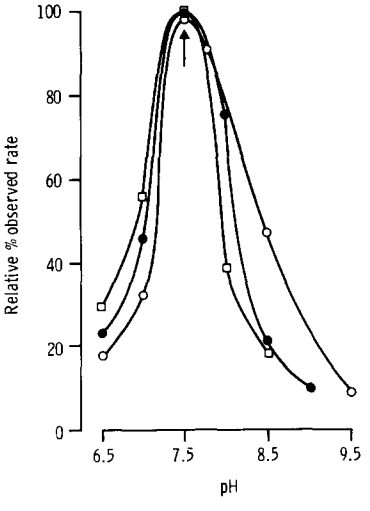

Fig. 3. pH dependence of placental monooxygenase activity for 2,5-diphenyloxazole (○), 7-ethoxycoumarin (●) and 7-ethoxyphenoxazone (□) substrates. 100% rate values are 0.34 (% fluorescence/g per min), 0.70 and 5.38 (nmole product formed/g per min) for 2,5-diphenyloxazole, 7-ethoxycoumarin and 7-ethoxyphenoxazone substrates, respectively. The arrow denotes the pH used in the standard assay (pH 7.5).

It has been reported that resorufin, in certain concentrations, can have an inhibitory effect on the deethylation reaction [24]. Whilst we found no evidence of product inhibition we did find a strong indication of substrate inhibition. Plotting 7-ethoxyphenoxazone deethylase activity against substrate concentration produces a curve whose shape is consistent with that produced by an enzymic reaction inhibited by its own substrate [25]. Although there is a suggestion of substrate inhibition also occurring in the case of the *O*-deethylation of 7-ethoxycoumarin, this is more readily apparent from a double-reciprocal plot than it is from a plot of reaction velocity against substrate concentration. The double-reciprocal plot obtained for the hydroxylation of 2,5-diphenyloxazole, although also non-linear, has a curve shape which is characteristic of a reaction catalysed by two or more distinct enzymes. Similar findings have been reported by other groups of workers for the demethylation of aminopyrine [26] and the hydroxylation of aniline [27]; however, non-linear double-reciprocal plots may also arise for a number of other reasons, namely, the presence of endogenous substrates, negative co-operativity or complex steady-state kinetics. All the substrates used in the present study are metabolized by the cytochrome P-448-dependent monooxygenase system but there are indications, both from the data presented here and elsewhere [6], that the hydroxylation of 2,5-diphenyloxazole and the *O*-deethylation of 7-ethoxycoumarin in the human placenta may be dependent upon more than one cytochrome P-450 system.

The inhibitor data presented in this study confirm and extend previous reports [20, 28] that cimetidine is capable of inhibiting monooxygenase activity. The present work shows that the inhibitory effects of cimetidine upon the human placental metabolism of 2,5-diphenyloxazole, 7-ethoxyphenoxazone and 7-ethoxycoumarin appear to resemble more closely those exhibited by metyrapone and SKF-525A than they do those shown by α-naphthoflavone.

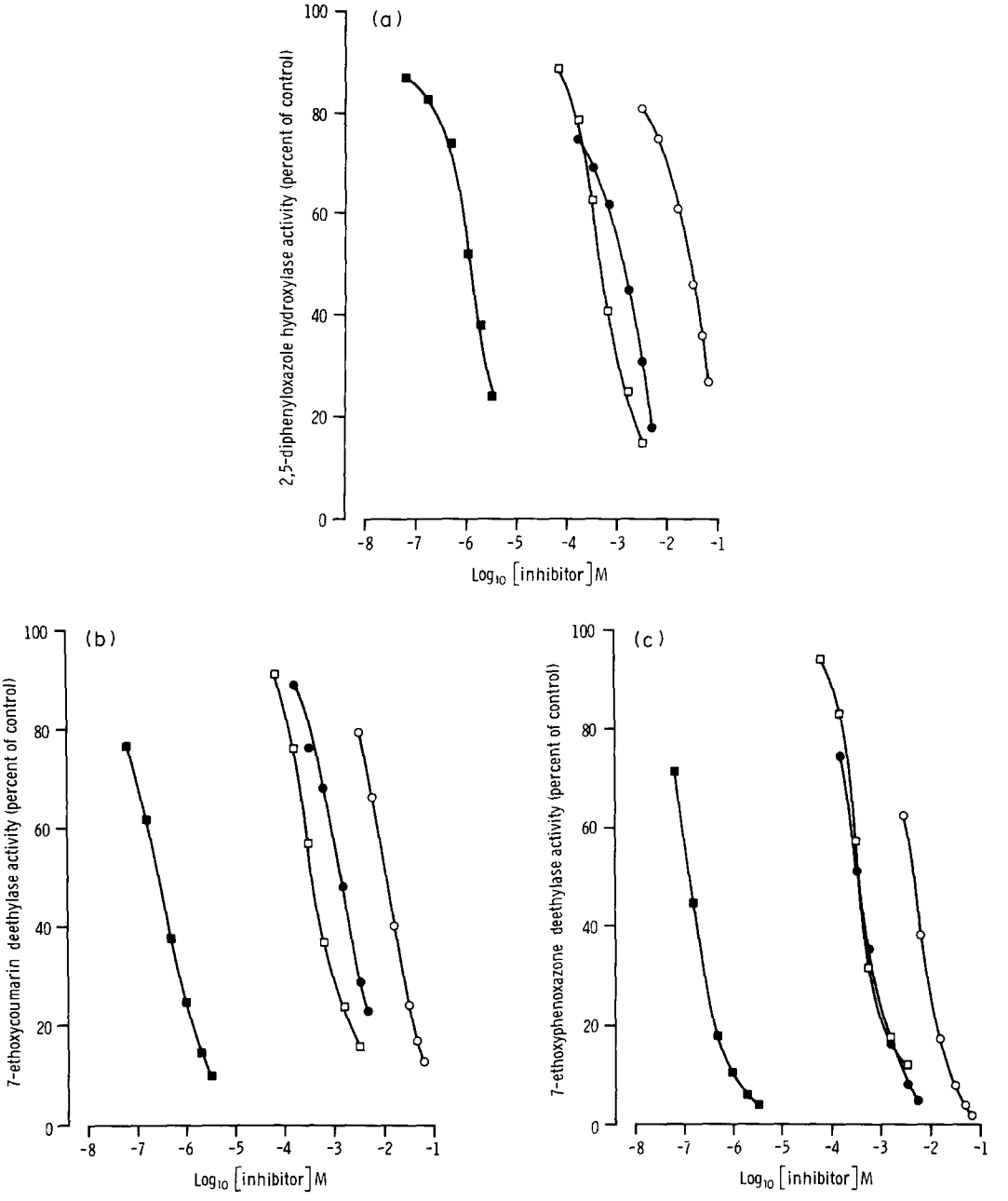

Fig. 4. Inhibition of human placental monooxygenase activity by cimetidine (○), metyrapone (●), SKF-525A (□) and α-naphthoflavone (■) using: (a) 2,5-diphenyloxazole; (b) 7-ethoxycoumarin; and (c) 7-ethoxyphenoxazone as substrates. Experimental details are indicated in the text. The values given are the means of duplicate results from assays of five different placental samples.

The finding that cimetidine does not appear to be a potent inhibitor of monooxygenase activity [28, 29] unlike other imidazole compounds [30] is probably largely dependent upon the type of cytochrome P-450-dependent system it is modifying. It has been shown [31] that a number of compounds including metyrapone, SKF-525A and phenylimidazole derivatives markedly inhibit one form of cytochrome P-450, α-naphthoflavone having only a minimal effect, whilst the converse was found to apply with a different form of cytochrome P-450.

Inhibition by low concentrations of α-naphthoflavone and high concentrations of metyrapone and SKF-525A has been generally regarded as evidence for the involvement of cytochrome P-448-dependent hydroxylase and dealkylase activity while the converse finding is thought to signify involvement of the constitutive (uninduced) cytochrome P-450-dependent monooxygenase system [32, 33].

It can be concluded from the results of these and previous investigations [34] that the placental monooxygenase most obviously associated with the

Table 1. Comparison of the inhibitory effects of cimetidine, metyrapone, SKF-525A and α-naphthoflavone on 2,5-diphenyloxazole hydroxylase and 7-ethoxyphenoxazone and 7-ethoxycoumarin deethylase activities in human placental homogenates from maternal smokers

Inhibitor	Inhibition of monooxygenase activity (I_{50})		
	2,5-Diphenyloxazole hydroxylase	7-Ethoxyphenoxazone deethylase	7-Ethoxycoumarin deethylase
Cimetidine	$27.2 \pm 5.6 \times 10^{-3}$	$4.5 \pm 0.9 \times 10^{-3}$	$12.1 \pm 1.9 \times 10^{-3}$
Metyrapone	$15.0 \pm 6.1 \times 10^{-4}$	$3.4 \pm 0.6 \times 10^{-4}$	$14.9 \pm 4.0 \times 10^{-4}$
SKF-525A	$7.7 \pm 1.8 \times 10^{-4}$	$6.1 \pm 1.1 \times 10^{-4}$	$6.5 \pm 1.0 \times 10^{-4}$
α-Naphthoflavone	$12.1 \pm 2.2 \times 10^{-7}$	$1.3 \pm 0.2 \times 10^{-7}$	$3.0 \pm 0.7 \times 10^{-7}$

I_{50} values (means ± S.D.) were determined from inhibition curves derived from six inhibitor concentrations and are expressed as molar concentrations for each inhibitor. Each value represents the mean of duplicate results from five different placental homogenates.

metabolism of xenobiotics is dependent upon cytochrome P-448 rather than upon cytochrome P-450. All the substrates used in the present studies are known to be metabolized by cytochrome P-448-dependent monooxygenases in laboratory animal tissue preparations; ethoxycoumarin, however, appears to be metabolized to a significant extent also by cytochrome P-450-dependent monooxygenases. Low levels of activity towards these substrates have been demonstrated in the placentas of non-smoking women, but their rate of metabolism is markedly increased in the placentas of women who smoked cigarettes while pregnant. Whether the activities in placentas from maternal smokers and non-smokers are due to the same monooxygenase is not known. The present data are insufficient to establish the presence of more than one monooxygenase system in the placenta although certain of our results, particularly those obtained with 2,5-diphenyloxazole, may be interpreted as evidence pointing to the existence of multiple monooxygenase activities in the human placenta.

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