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INTERACTIONS OF PERHEXILINE MALEATE AND ARYLALKYLAMINE DERIVATIVES WITH CYTOCHROME P-450 AND *IN-VITRO* HYDROXYLATION

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Abstract—In order to find a substitute for perhexiline maleate, an antiangor drug, for which several side effects due to poor hydroxylation have been reported, an arylalkylamine series with antianginal properties has been synthesized. The aim of the present work was to select a more rapidly hydroxylated compound than perhexiline maleate in this series. Two criteria have been retained. The binding of the molecules to liver microsomal cytochrome P-450 and their rate of hydroxylation were both studied *in vitro* using phenobarbital induced rat liver microsomes. Incubation with cofactors and extraction procedures have been tested on one of the molecules of the series taken as example: *N*-2-dicyclohexyl-2-phenethylamine (3). All of the molecules tested in the series substrates were type I substrates; nevertheless, no correlation was found between binding on cytochrome P-450 and oxydative metabolism of the drugs. Two of the studied molecules were more easily hydroxylated than the others and than perhexiline maleate: (3) (*N*-2-dicyclohexyl-2-phenethylamine) and (II) (*N*-cyclohexyl-2-diphenylethylamine) with the following respective kinetics: Apparent V_{max}: 0.073 and 0.32 units, apparent K_m: 6.9×10^{-5} M and 22.2×10^{-5} M.

Effective antianginal drugs continue to be needed. Perhexiline maleate [Pexid®; 1,1 dicyclohexyl-2-(2-piperidyl) ethane] has proven to be very effective in the treatment of angina pectoris [1, 2]. However, it is of somewhat limited interest due to serious side effects, notably peripheral neuropathy [3], severe weight loss [4] and effects on hepatic functions, including cirrhosis [5]. Singlas *et al.* [6] reported that patients who develop peripheral neuropathy hydroxylate the drug more slowly than those patients who do not show this side effect. A hydroxylation polymorphism has been associated with perhexiline in humans and rats [7].

In the hope of reducing the adverse effects of perhexiline, Leclerc *et al.* [8] synthesized 24 arylalkylamine derivatives related to perhexiline and examined their antianginal activities.

In the light of Singlas' work, the fate of these drugs in the organism and their rate of metabolism were also of importance in choosing a potential drug within the series. As *in-vivo* metabolic studies are tedious and expensive in a series, an *in-vitro* approach was proposed to achieve this metabolic aim.

The *in-vitro* hydroxylation of the arylalkylamine derivatives by liver microsomes from phenobarbital pre-treated rats and the interaction of these structures with the heme iron of cytochrome P-450 were examined.

This paper deals with the analytical approach and with the spectral interaction. We also discuss the correlation between both methods. The most rapidly hydroxylated arylalkylamine derivatives had to be selected, in the working hypothesis of detecting a rapidly metabolized compound.

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MATERIALS AND METHODS

Animals and preparation of microsomal liver fractions. The microsomal liver fractions from phenobarbital pre-treated male Sprague–Dawley rats (180–200 g) [phenobarbital was administered intraperitoneally (80 mg/kg) for 5 days] were prepared by differential centrifugation according to the method of Beaufay *et al.* [9]. The buffer consisted of phosphate 100 mM, DTT 1 mM, EDTA 1 mM, glycerol 20% (v/v).

Chemicals. Perhexiline maleate was donated by Merrell Toraude (2, Place de la Sorbonne, 75005 Paris) and arylalkylamine derivatives were synthesized by Leclerc (Institut de Pharmacologie, Strasbourg). The structures are indicated in Fig. 1. Other chemicals were purchased as follows: methanol, cyclohexane, ethylacetate from Merck (Darmstadt, F.R.G.), diethyl ether from Prolabo; trifluoroacetic anhydride from Fluka (Buchs, Switzerland); K_2HPO_4, KH_2PO_4 from Merck; DTT, EDTA, glycerol from Sigma (St. Louis, MO); NAD, NADP, Glucose-6-phosphate dehydrogenase, Glucose-6-phosphate from Boehringer (Mannheim, F.R.G.).

Apparent K_m and V_m for hydroxylation. The incubations (2.2 ml) contained substrates at concentrations varying from 2.3×10^{-6} M to 4.4×10^{-4} M, microsomal suspension equivalent to 5 nmol of cytochrome P-450 in buffer and a NADPH generating system (NAD: 0.5 mM, NADP: 0.42 mM, glucose-6-phosphate: 3.2 mM, glucose-6-phosphate dehydrogenase: 97 units, $MgCl_2$: 5.5 mM and Tris–HCl buffer: 5.5 mM pH 7.4). The incubations were run in duplicate, started by the introduction of microsomes and incubated by shaking at 37° for 6–15 min, depending on the molecule being examined.

100 μl 1.5×10^{-3} M of internal standard (III) were

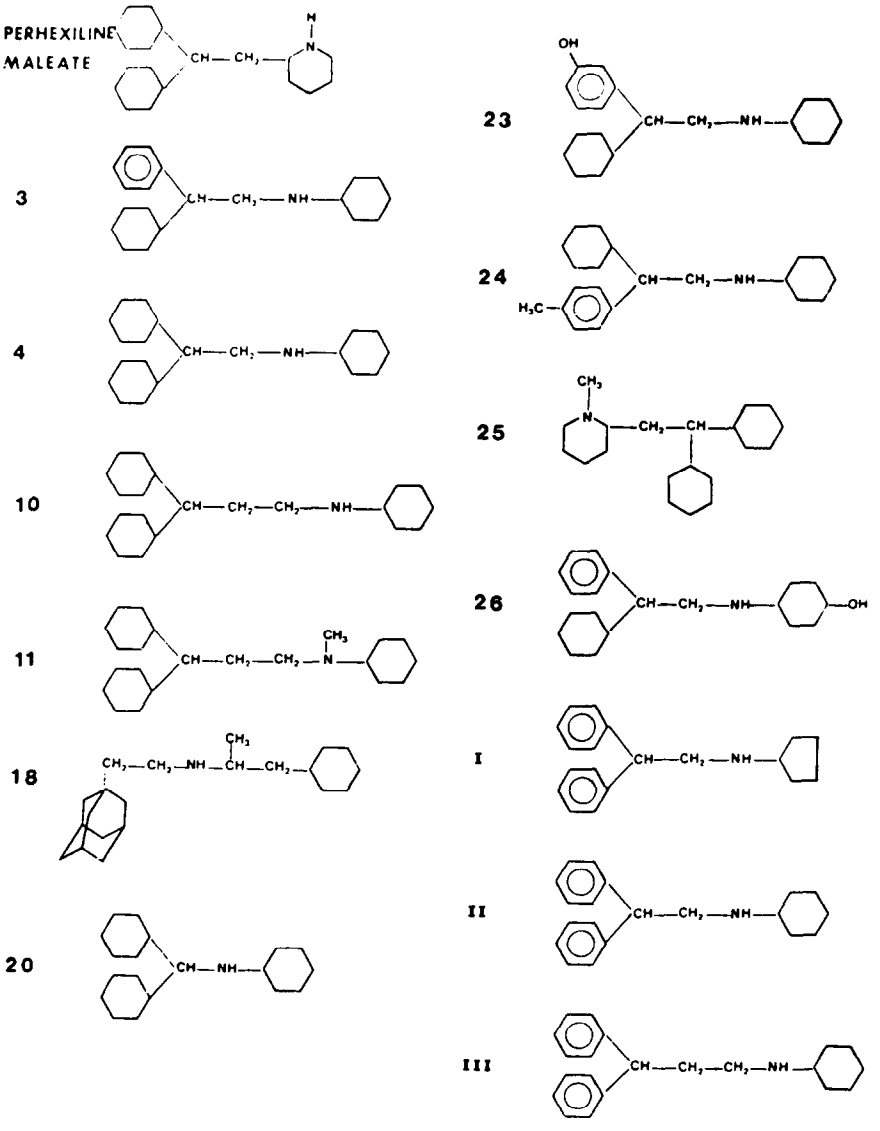


Fig. 1. Arylalkylamine series.

added. The unmetabolized substrate, the metabolites and the internal standard were extracted in two steps. In the first step, 5 ml cyclohexane was added to the incubation mixture. The tubes were shaken for 10 min and centrifuged at 3000 rpm for 10 min. The organic layer was discarded. The aqueous layer was then alkalinized by adding 1 N NaOH (pH > 11) and extracted twice with 5 ml diethylether. After 10 min of shaking and centrifuging, the diethylether was collected and evaporated to dryness in a Rotavapor (Büchi) at 40°. The dried residue was extracted twice with 0.6 ml methanol and transferred into derivatization vials (1.5 ml). Methanol was evaporated under nitrogen in a sand bath (50°). 100 μl trifluoroacetic anhydride and 100 μl ethyl acetate were added and the tubes were heated at 60° for 20 min. Excess derivatization reagents were evaporated and the dried residue was dissolved in 400 μl ethyl acetate of which 2 μl were injected into the GLC.

A Packard model 429 gas chromatograph was used. It was equipped with a thermoionic detector, specific for N and P, and fitted with a 1.8 × 2 mm glass column packed with 3% OV 17 on 100–120 mesh chromosorb W-AW DMCS. The operating conditions were as follows: oven temperature 225°; injector temperature 250°; detector temperature 260°; carrier gas flow (N_2) 30 ml/min. Chromatograms were recorded on an LTTI CAP 10 integrator with a LTT 510 printing system. The mass spectrometry, for the identification of the types of metabolites produced from (3), was carried out on a Riebermag R 10-10/Sidar connected to a Carlo Erba 4160 GLC, fitted with a 25 m capillary column (ID = 0.32 mm) coated with SE 30 (Dr J. M. Ziegler, Mass Spectrometry Laboratory, University of Nancy I). The samples were injected according to the split procedure (ratio 1/10). Carrier gas flow (He) was 2 ml/min, injector and oven temperatures 220 and 200°, respectively. Electron impact mass spectra

were recorded at 70 eV and processed by a PDP 11/23 computer.

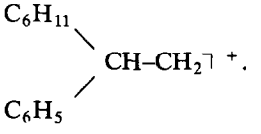
The substrate concentrations were determined *in vitro* from the previous incubations and GLC analysis. Apparent K_m and V_{max} were obtained from double reciprocal plots, calculated by linear regression.

Interaction with microsomal cytochrome P-450. Cytochrome P-450 was measured by the method of Omura and Sato [10]. Protein concentrations were determined by the method of Lowry *et al.* [11]. Microsomal suspensions were diluted to 1 nmol cytochrome P-450/ml in phosphate buffer 100 mM, DTT 1 mM, EDTA 1 mM, glycerol 20% (v/v), pH 7.4. Difference spectra were recorded on a Beckman UV 5230 spectrophotometer, from 360 to 460 nm, using tandem cells thermostated at 25° [12]. Spectra were recorded after the addition of 5 µl aliquots of the substrate dissolved in methanol. The final concentrations varied from 0.4×10^{-5} M to 5×10^{-5} M. The same amount of solvent was added to the reference cell. The spectral dissociation constants (K_s) or binding affinities were obtained as follows: the reciprocal of the difference in absorption between the peak and valley in each difference spectrum was plotted against the reciprocal of the concentration of each compound. The intercept on the abscissal axis, equivalent to $-1/K_s$, was determined by linear regression. The maximal difference in absorption (ΔA_{max}) was calculated from the intercept on the ordinate ($\frac{1}{2}\Delta A_{max}$).

RESULTS

Identification of the metabolites of N,2-*dicyclohexyl*-2-*phenethylamine* (3)

The GLC profile obtained after incubation of (3) with the phenobarbital induced rat liver microsomes and derivatization of the extracts is shown in Fig. 2. Respective retention time of (3), and internal standard were 4.85 min and 10.15 min. After a 30-min incubation (in order to obtain a sufficient quantity of metabolites) with microsomes and NADPH generating system, (3) was almost totally metabolized. Two metabolites appeared with retention times 6 min (M_1) and 6.73 min (M_2), respectively. The mass spectra of the TFAA derivatives of (3), M_1 and M_2, are shown in Fig. 3. The molecular ion at m/z 381 in the spectrum of (3) and at m/z 493 in the M_1 and M_2 spectra were consistent with the monohydroxylation of the TFAA derivative of the metabolites: m/z 493 including a $COO–CF_3$ species. In the M_1 and M_2 spectra the ion m/z 186, corresponding to



was absent. The m/z 298 peak that appeared corresponded with the previous species that had been hydroxylated and TFAA-derivatized (185 + 16 + 97). This was consistent with a hydroxylation on the ion m/z 186. The m/z 208 peak corresponding to the species

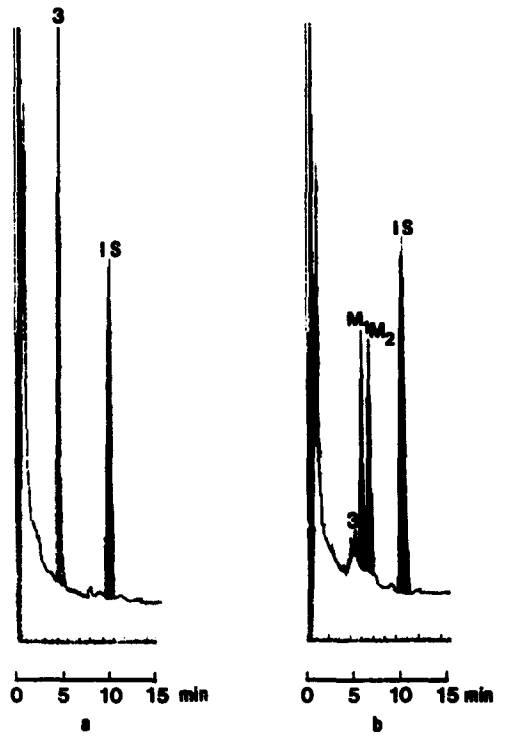
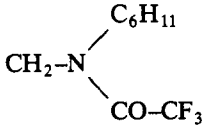


Fig. 2. Gas chromatographic profile of [3] before (a) and after (b) incubation (30 min) of 1.5×10^{-3} M with 5 nmoles of cytochrome P-450 from phenobarbital pretreated rat liver microsomes.



which was still present in the mass spectra of M_1 and M_2, as well as in the spectrum of (3), also indicated that this part of the molecule (3) was not hydroxylated. The m/z 91 ($C_6H_5–CH_2^+$) and m/z 104 ($C_6H_5–CH–CH_2^+$) peaks, in the mass spectra of M_1 and M_2 show that the aromatic ring is not substituted, and indicate likely hydroxylation on the cyclohexyl moiety close to the aromatic ring. The position of the metabolically introduced hydroxyl group in M_1 and M_2 was not determined.

GLC quantitative analysis of N,2-*dicyclohexyl*-2-*phenethylamine* (3)

The GLC response to incubation, extraction and derivatization of (3) was linear in function of the concentration range of the substrate (1.7×10^{-5} M–2.1×10^{-4} M). Variation coefficient for 20 samples was estimated to determine the repeatability of extraction (20 samples were extracted only, without previous incubation procedure) and of incubation (20 other samples were incubated 30 min at 37° and then extracted). C.V. were 1.8 and 5.8%, respectively.

Enzyme kinetics of hydroxylation and interaction with cytochrome P-450

Table 1 shows apparent K_m and V_{max} values for the substrates. The data have been arranged in

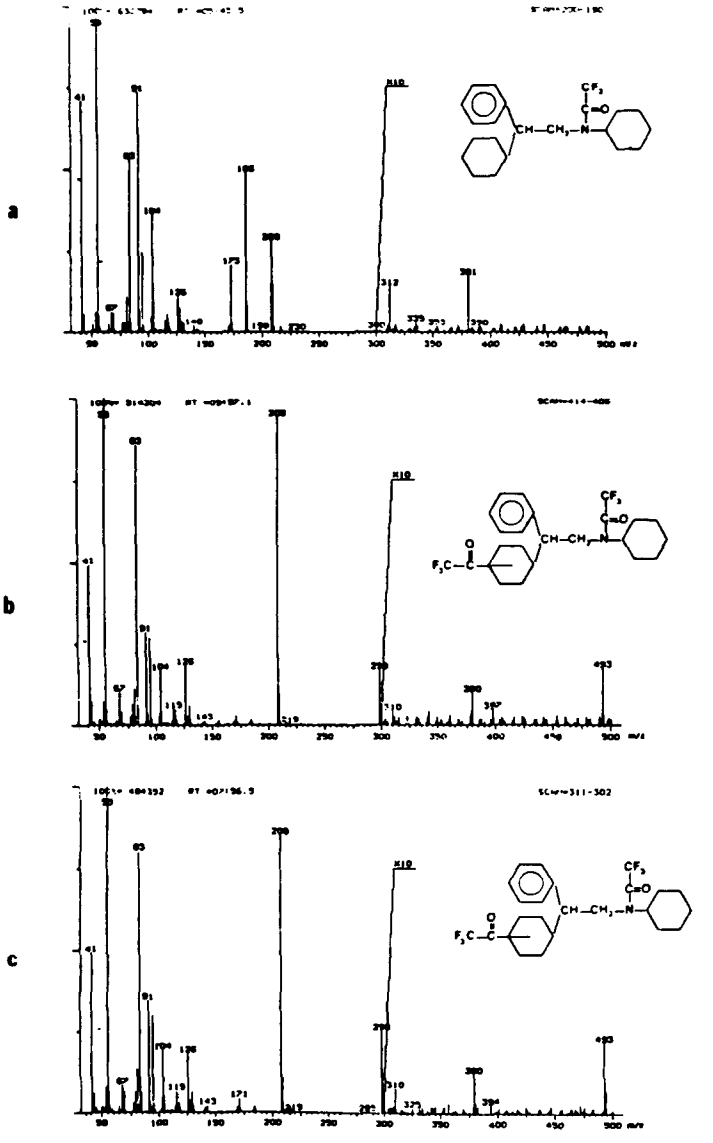


Fig. 3. EI mass spectra of (3) (a) and M_1 (b) and M_2 (c) metabolites obtained after incubation of (3) with phenobarbital pretreated rat liver microsomes.

decreasing order of V_{max}. The highest values were obtained with [II] and (3).

The highest affinity for metabolism (K_m 2.2×10^{-5} M) was observed with (18), with the lowest apparent V_{max}. (Figure 4 gives an example of the double reciprocal plots used to compute K_m, V_{max} and K_s.)

When added to the microsomes the arylalkylamine derivatives studied produced type I spectra (max ~ 390 nm) (min ~ 420 nm) with cytochrome P-450, indicating substrate-type interaction (see Table 1).

The maximal amplitude of the difference between peak and valley (ΔA_{max}) was the highest with (24), a compound substituted by a p-toluyl ring (Fig. 5).

Binding affinities (K_s) were in the same order of magnitude, from 1 to 3×10^{-5} M, for most compounds examined, except for (25) which exhibited a binding constant $K_s = 0.5 \times 10^{-5}$ M in this molecule. The piperidyl ring of perhexiline was substituted by a methyl-piperidyl ring. The presence of a CH_3 substituent seems to facilitate the interaction with cytochrome P-450, possibly followed by dealkylation.

No metabolites were detectable with (23) and (26), which were themselves possible metabolites of (3) (hydroxylated on the benzyl and *N*-cyclohexyl rings, respectively), although they were not identified as *in-vitro* metabolites by mass spectrometry. Nevertheless they still interacted with cytochrome P-450. The binding affinity of (23) was similar to that of the parent compound. A similar lack of *in-vitro* metabolites was observed with perhexiline itself and with (20) which differed from the metabolized (4) by one methane in the chain.

Binding affinities in the series were correlated neither with apparent K_m values ($r = 0.14$) nor with apparent V_{max} ($r = -0.32$). Binding amplitudes were correlated with neither V_{max} ($\simeq 0.42$) nor K_m ($\simeq 0.35$).

Table 1. *In-vitro* interaction and enzyme kinetics in the aralkylamine series (the apparent V_{max} values correspond to the $\frac{\text{Metabolite surface}}{\text{I.S. surface}}$ ratio)

Molecules	App. V_{max} (Units)	App. K_m (10^{-5} M)	Absorbance max (nm)	Absorbance min (nm)	ΔA_{max}	K_s ($M.10^{-5}$)
II	0.32	22.2	386	418	0.022	1.09
3	0.073	6.9	381	418	0.029	1.04
10	0.034	79.5	376	422	0.020	2.18
25	0.023	21.7	386	422	0.068	0.55
I	0.018	3.1	387	418	0.033	1.29
24	0.016	8.7	384	422	0.098	1.79
11	0.014	11.4	384	420	0.051	2.92
4	0.013	8.4	385	421	0.042	1.33
III	0.0051	4.2	386	420	0.046	2.51
18	0.0032	2.2	384	420	0.046	1.55
23	ND	ND	389	422	0.013	1.10
26	ND	ND	385	418	0.043	2.52
20	ND	ND	386	421	0.059	2.45
Perhexiline	ND	ND	380	422	0.020	1.51

N.D., not detectable.

DISCUSSION

Analysis of metabolites

In the presence of phenobarbital treated rat liver microsomes *N*,2-dicyclohexyl-2-phenethylamine (3) was hydroxylated on the cyclohexyl ring close to the benzyl substituent. GLC indicated the formation of two metabolites that were both hydroxylated. No metabolism was observed on the aromatic ring or on the *N*-cyclohexyl moiety.

The GLC analytical method was applied to the series of arylalkylamine derivatives. As more than one metabolite might appear, the appearance of these metabolites was measured to determine the kinetics of metabolism. The method could be reproduced on a routine basis.

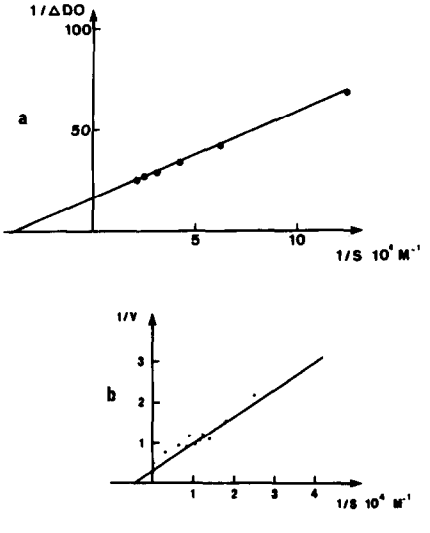


Fig. 4. Example of the double reciprocal plots used to compute K_s (a: example of [20], $r = 0.999$) and K_m and V_{max} (b: example of (II), $r = 0.994$).

Choice of the most actively metabolized compound

Interaction with cytochrome P-450. All of the compounds interacted with type I spectra when added to the microsomal preparation from phenobarbital treated rats, indicating a substrate-type interaction. The highest binding affinity or amplitude were observed with the compounds which can be demethylated. With the exception of these methylated compounds, K_s varied threefold. This difference did not seem sufficiently discriminative to select a compound in the series. ΔA_{max}, indicating the maximal binding amplitude, varied fivefold. The binding affinity and amplitude depended on the spin state of the heme iron in cytochrome P-450. Type I binding represents the shift from low spin to high spin iron. Phenobarbital pretreatment of rats increased the amount of low spin iron to about 58% [13], allowing only this part of cytochrome P-450 to bind the substrate. The spin state also depends on the pH [14], temperature [15], animal species and pre-treatment [16]. For example, phenobarbital pretreated pig liver microsomes corresponded to 70% low spin iron. Moreover, different forms of cytochrome P-450 are present in each animal species which might bind at a different affinity.

Kinetics

Apparent K_m varied 35-fold from one compound to another and apparent V_{max} varied 100-fold. The extreme cases were represented by (III) and (18) in which K_m and V_{max} were low, indicating that the enzymatic system was rapidly saturated and the metabolites produced at low velocity, leading to a final low yield of metabolites.

In decreasing order of velocity, the second compound was (3), exhibiting a low K_m, and relatively high V_{max}; it was also selected for its pharmacological properties [8]. This compound could thus represent a good compromise. Moreover, in a previous work

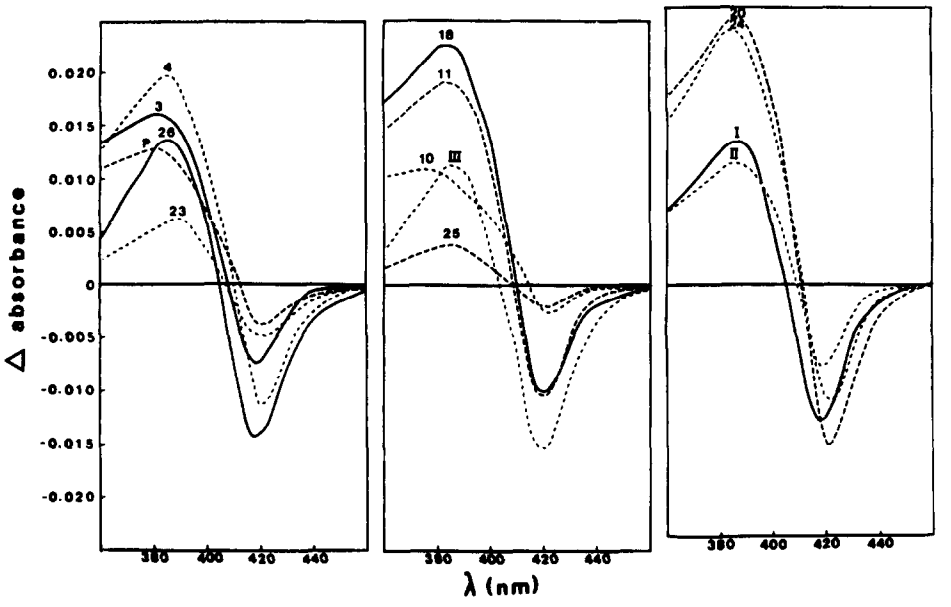


Fig. 5. Difference spectra of liver microsomes from phenobarbital pretreated rats in the presence of aralkylamine molecules.

conducted with partially purified liver microsomal cytochrome P-450 from phenobarbital pretreated pig, (3) was also one of the two compounds exhibiting the highest substrate-binding affinity.

As a control of the system used, perhexiline, which was known to be slowly metabolized *in vivo*, produced no detectable metabolites *in vitro*.

Lack of correlation between spectral dissociation constants (K_s) and oxidation kinetic constants (K_m and V_{max}). Spectral dissociation constants and binding amplitude had no correlation with apparent K_m and V_{max}, although the binding was a substrate-type interaction and the compounds were metabolized *in vitro*. Correlations between these parameters have been reported [12, 17, 18] by several authors, but a similar lack of correlation was also reported by Davies *et al.* [19] for ethylmorphine demethylation. This implies that a correlation between the extent of hydroxylation and type I binding to cytochrome P-450 is not a rule. Rein *et al.* [13] reported a correlation between the V_{max} for the hydroxylation of benzphetamine derivatives and the spectral dissociation constants for the same molecules, all of them being metabolized by *N*-demethylation. In the present series, metabolism can take place at different positions in the compounds. In microsomal preparations, different forms of cytochrome P-450 do co-exist. Moreover, non-specific protein binding might occur, possibly decreasing the substrate concentration. The system might be improved by using purified forms of cytochrome P-450 and determining which among the isolated forms was responsible for the metabolism. Another hypothesis regarding a certain lack of correlation was discussed by Yih and Van Rossum [20] who observed two compounds being metabolized quite slowly in a series of barbiturates, despite a high binding affinity. They concluded that even if a high affinity for cytochrome P-450 was a necessity, other factors such as the reactivity of the C–H bond, play an important role. According to this hypothesis, it was observed here that all compounds studied were bound, but some were not metabolized *in vitro*.

The present work leads to the conclusions that (i) in the present situation of discordance between metabolism kinetics and binding constants, the data for kinetics were the most determinative, (ii) the binding studies are improved by using purified forms of cytochrome P-450, which is now in progress in the laboratory and (iii) a rapid rate of metabolism does not indicate an absence of toxicity, as the metabolism might itself create toxic intermediates.

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