*Biochemical Pharmacology*, Vol 35, No 13, pp 2233–2240, 1986
Printed in Great Britain

0006-2952/86 $3 00 + 0 00
Pergamon Journals Ltd

# DUAL LIGAND BINDING OF PYRIDYLALKANAMIDES TO MICROSOMAL CYTOCHROME P-450

CHRISTIAN REPOND,* ANITA BULGHERONI,† URS A MEYER,† JOACHIM M. MAYER* and BERNARD TESTA*‡

*School of Pharmacy, University of Lausanne, CH-1005 Lausanne, and †Department of Pharmacology, Biozentrum, University of Basle, CH-4056, Switzerland

(*Received* 30 *July* 1985; *accepted* 21 *November* 1985)

**Abstract**—Twelve homologous and regioisomeric pyridylalkanamides were examined spectrally for their binding affinity to cytochrome P-450 in phenobarbital- and 3-methylcholanthrene-induced rat liver microsomes The pKs values were calculated by the Lineweaver–Burk method and by non-linear analysis using both a one ligand–one acceptor and a one ligand–two acceptor model The latter model best fits most of the data, confirming that two pKs values exist for most derivatives in the 3-pyridyl and 4-pyridyl series Structure–binding relationships are discussed The two binding constants are hypothesized to arise from a dual mode of binding to the ferric ion. At low ligand concentrations, binding to hexacoordinated cytochrome P-450 occurs and involves displacement of an endogenous 6th ligand, at higher concentrations, the ligands bind to the pentacoordinated P-450, resulting in a high-to-low spin shift

Cytochrome P-450 monooxygenases are among the most important enzymes involved in the oxidation of xenobiotics as well as endogenous compounds The binding of substrates to cytochrome P-450 is a major step in their biotransformation. Additionally, binding to the enzyme may in some cases cause inhibition and possibly also be involved in its induction The binding process is thus a key event in determining the outcome of the interaction between a chemical compound and cytochrome P-450

Globally, two modes of binding to cytochrome P-450 are known, namely substrate and ligand binding [1]. The former involves binding to a hydrophobic region near the catalytic site and is the first step in the biotransformation process. Substrate binding is detected spectrally as type I binding spectra. In contrast, ligand binding involves the interaction between a heteroatom and the iron atom; it is detected spectrally as type II (nitrogenous ligands) or modified type II (oxygenated ligands) binding [1–3]. Ligand binding may produce inhibition of cytochrome P-450 function; it results in the conversion of a high spin form to a low spin form, or a low spin form to another low spin form. The spin state equilibrium was shown to modulate both the substrate binding and the redox potential of cytochrome P-450 in such a way that the reduction of the ferric high spin state is favoured as compared with the reduction of the ferric low spin state [4, 5]. The mechanisms of binding and the relationships influencing them are thus significant elements in a global understanding of cytochrome P-450 mediated biotransformation.

Pyridine and many pyridine derivatives are known to be strong ligands and direct-acting inhibitors of cytochrome P-450 [6, 7]. In the present study, various aspects of cytochrome P-450 binding and structure–binding relationships are examined using a series of regioisomeric and homologous pyridylalkanamides (Fig. 1) as model compounds of metyrapone

## MATERIALS AND METHODS

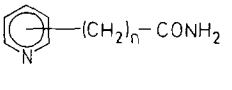
### *Pyridylalkanamides*

These compounds were synthesized as described [8] Their basicity ($pK_a$ values) and lipophilicity (log *P* values) have been reported [9]

### *Preparation of microsomes*

Sprague–Dawley male rats (150–200 g) received by i p. injection during 3 days phenobarbital (80 mg/kg/day) in physiological NaCl solution or 3-methylcholanthrene (20 mg/kg/day) in corn oil Control animals were injected only the vehicle The animals were killed by decapitation 24 hr after the last injection, they had received no solid food during the last 12 hr.

Subcellular fractionation was performed at 4° The livers were homogenised in a 0.25 M sucrose solution (1·3). The homogenisation was done in a 60 ml Elvejhem–glass homogeniser with a Teflon pestle (radial clearance 0.18–0.24 mm). The suspension was centrifuged for 15 min at 10,000 *g* to remove nuclei, mitochondria and debris. The supernatant was centrifuged for 60 min at 105,000 *g*. The microsomal pellets were resuspended in a sodium pyrophosphate 0 1 M/EDTA 0.1 mM solution (pH 7 4) with a 60 ml Dounce Homogeniser (small clearance 508 $\mu$m), then recentrifuged for 60 min at 105,000 *g*




n= 1 – 4

Fig 1 General formula for the 12 pyridylalkanamides investigated

‡ To whom correspondence should be addressed

The pellets were resuspended to concentrations of 10–20 mg protein/ml in a 0.1 M sodium phosphate solution (pH 7.4) and immediately frozen at −70°. The liver (*ca* 6 g) of each rat (100–150 g) yielded approximately 180 mg of microsomal protein. The protein concentration was measured colorimetrically using crystalline bovine serum albumin as standard [10]. The concentration of cytochrome P-450 was measured according to [11].

*Spectral studies*

The spectra were recorded at room temperature between 360 and 500 nm using an Aminco DW2 spectrophotometer. The two cuvettes each contained 750 μl of a standardized microsomal suspension (1.5 mg protein/ml) freshly prepared from the concentrated microsomal suspension. The amides were dissolved in a 0.1 M sodium phosphate buffer (pH 7.4) and added in portions of 0.5–10 μl into the sample cuvette. Corresponding volumes of the buffer solution were added to the reference cuvette. The dilution resulting from the addition of the ligand solution was always taken into account to calculate the ligand concentration in the cuvette and to correct the absorbance. The volume variation never exceeded 10%. The derivatives with one methylene group in the side chain present a detectable absorption ($\Delta A_{pyr}$). This absorption was taken into account too using the following formula

$$\Delta A_{corrected} = \Delta A_{measured} - \Delta A_{pyr}$$

*Calculation of Ks values*

*Assumptions.* For the calculation of Ks values a number of assumptions were made:

(a) The Lambert–Beer law is satisfied in the concentration range investigated.
(b) The ligand–P-450 complex has the same absorption coefficient ($\Delta\varepsilon_{max\text{-}min}$) for all used ligands with a defined type of microsomes.
(c) When a ligand displays two affinity constants, the corresponding complexes have the same $\Delta\varepsilon$ value.
(d) The ligands with the strongest affinity bind to the entire cytochrome P-450 population present when their concentration tends towards infinity.

Regarding assumption (b), a comment is necessary. Indeed, there is no theoretical proof that the $\Delta\varepsilon$ values are the same but this assumption is unavoidable since in this case there is no practical possibility to differentiate between both values. At low ligand concentrations the spectral maxima and minima are broad due to the small amplitude. The introduction of a second $\Delta\varepsilon$ value in the non-linear regression would lead to overparametrization of the model. Data analysis using the same $\Delta\varepsilon$ values yields consistent results thus favouring assumption (b).

*Mathematical model.* When the ligand (L) binds to the acceptor ($E_t$) following

$$E_t + L = E_tL \qquad (1)$$

its dissociation constant is

$$K_t = \frac{[E_t][L]}{[E_tL]} \qquad \text{for } t = 1, 2, \ldots n \qquad (2)$$

This dissociation constant is valid only when the metabolisation rate is nil or negligible. In our case this could be assumed because no cofactors were present in the cuvettes.

The mass balance for the acceptor gives

$$[E_t]_{tot} = [E_t] + [E_tL] \qquad \text{for } t = 1, 2, \ldots n \qquad (3)$$

Equations (2) and (3) give

$$[E_t - L] = \frac{[E_t]_{tot}}{1 + K_t/[L]} \qquad \text{for } t = 1, 2, \ldots n \qquad (4)$$

The mass balance for the ligand is

$$[L]_{tot} = [L] + \sum_{t=1}^{n}[E_tL] = [L] + \sum_{t=1}^{n}\frac{[E_t]_{tot}}{1 + K_t/[L]} \qquad (5)$$

The transformation of equation (5) with regard to (L) is a quadratic one for $n = 1$ and a third degree one for $n = 2$. In the two cases, the solution must respect the following condition

$$0 \leq [L] \leq [L]_{tot} \qquad (6)$$

The difference between maximum and minimum absorption $\Delta A$ is defined by

$$\Delta A = \Delta\varepsilon \sum_{t=1}^{n}[E_tL] = \Delta\varepsilon \sum_{t=1}^{n}\frac{[E_t]_{tot}}{1 + K_t/[L]} \qquad (7)$$

*Particular cases*

(a) *Representation according to* $\Delta A^{-1} = f([L]^{-1})$. When $n = 1$, equation (7) can be written

$$\frac{1}{\Delta A} = \frac{K_1}{\Delta\varepsilon[E_1]_{tot}}\,\frac{1}{[L]} + \frac{1}{\Delta\varepsilon[E_1]_{tot}} \qquad (8)$$

For this model we have to know [L]. When the ligand concentration is much higher than the cytochrome P-450, or when the ligands have little affinity for the acceptor, then $[L]_{tot} \approx [L]$, and equation (8) is the Lineweaver–Burk equation [12]

$$\frac{1}{\Delta A} = \frac{K_1}{\Delta\varepsilon[E_1]_{tot}}\,\frac{1}{[L]_{tot}} + \frac{1}{\Delta\varepsilon[E_1]_{tot}} \qquad (9)$$

For $n = 2$ this representation gives an hyperbola.

(b) *Simplification of the model to determine* $\Delta A_{max}$. When the following conditions are valid

$$[L]_{tot} > K_1 \text{ and } [L]_{tot} \gg K_t \qquad t = 2, 3 \ldots n$$

and

$$\sum_{1}^{n}[E_t]_{tot} \ll [L]_{tot} \qquad (10)$$

Then $[L]_{tot} \approx [L]$ and equation (7) simplifies to

$$\Delta A = \Delta\varepsilon \left(\sum_{t=2}^{n}[E_t]_{tot} + \frac{[E_1]_{tot}}{1 + K_1/[L]_{tot}}\right) \qquad (11)$$

We have analyzed the results according to

(1) Lineweaver–Burk (equation 9).
(2) non-linear model (equation 7) for $n = 1$ or 2, using the program "MODFIT" [13]. The iterative determination of five parameters ($\Delta\varepsilon$,

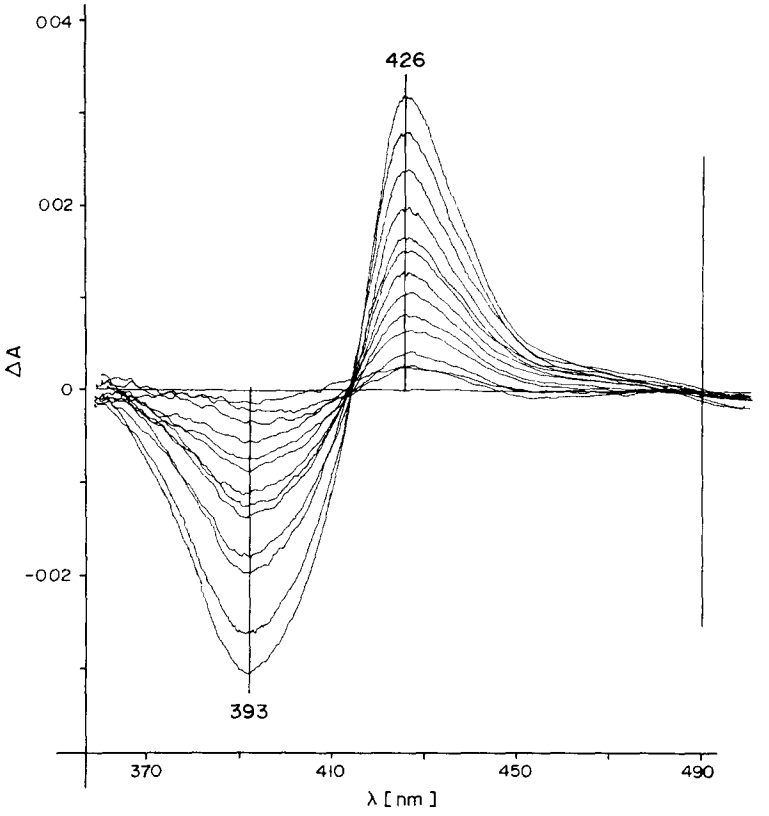



Fig 2 Type II binding spectra resulting from the interaction of 4-pyridylpentanamide (0 4–28 $\mu$M) with a microsomal suspension (initial P-450 concentration 3 11 $\mu$M) from PB-induced rats

$[E_1]_{tot}$, $[E_2]_{tot}$, $K_1$, $K_2$) cannot converge due to overparametrization For this reason $\Delta\varepsilon$ was determined independently from $\Delta A_{max}$ The latter was calculated from equation (11) using the ligand showing the highest affinity when [L] tends towards infinity (hypotheses d and c, see "Assumptions") The $\Delta\varepsilon_{PB}$ and $\Delta\varepsilon_{3MC}$ were calculated from the means of $\Delta A_{max}$ using

$$\Delta\varepsilon_{PB} \text{ or } \Delta\varepsilon_{3MC} = \frac{\Delta A_{max}}{\sum_{i=1}^{n} [E_i]_{tot}} = \frac{\Delta A_{max}}{[\text{P-450}]} \qquad (12)$$

The values of the four parameters for $n = 1$ or $n = 2$ were obtained when the difference between the last two iterations were less than 1% An F-test was performed to compare the results obtained for one acceptor ($n = 1$) and two acceptors ($n = 2$) The program also calculates the fraction $\beta_i$ of acceptor relative to the concentration of P-450

$$\beta_i = \frac{[E_i]_{tot}}{[\text{P-450}]} \qquad (13)$$

## RESULTS AND ANALYSIS OF DATA

The content of cytochrome P-450 by CO-binding spectra was found to be 0 792, 2 073 and 1 656 nmol P-450/mg protein in non-induced, PB-induced, and 3MC-induced rat liver microsomes, respectively The interaction of the pyridylalkanamides with the PB- and 3MC-induced microsomes resulted in each case in typical type II spectra Figure 2 exemplifies the spectra obtained with 4-pyridylpentanamide

The spectral characteristics are the same for the 3- and 4-pyridyl series ($\lambda_{max} = 425 \pm 2$ nm, $\lambda_{min} = 393 \pm 2$ nm, isobestic point = $415 \pm 2$ nm) We observed a little shift toward the low wavelength for the 2-pyridyl series ($\lambda_{max} = 422 \pm 2$ nm, $\lambda_{min} = 391 \pm 2$ nm, isobestic point = $409 \pm 3$ nm)

### *Lineweaver–Burk analysis of data*

Figure 3 shows a double reciprocal plot typical of several investigated pyridylalkanamides The 3- and 4-pyridyl series yield two straight lines Slope changes were determined by selecting the pair of straight lines which minimizes the residuals The 2-pyridylalkanamides present only one line Tables 1–3 report the results obtained with the three series of amides

The Lineweaver–Burk plot is characterized by its simplicity However, and like other methods based on the transformation of experimental data, it exhibits a number of shortcomings stressed by Klotz [14] and Burgisser [15] Thus, the distorted distribution of points can lead to inaccuracies and oversimplified plots two straight lines are apparent in Fig 3 when the real function should be a hyperbola (interaction of a ligand with two acceptors) Non-linear methods overcome these limitations [14]

### *Non-linear analysis of data*

The $\Delta A_{max}$ values were calculated with equation

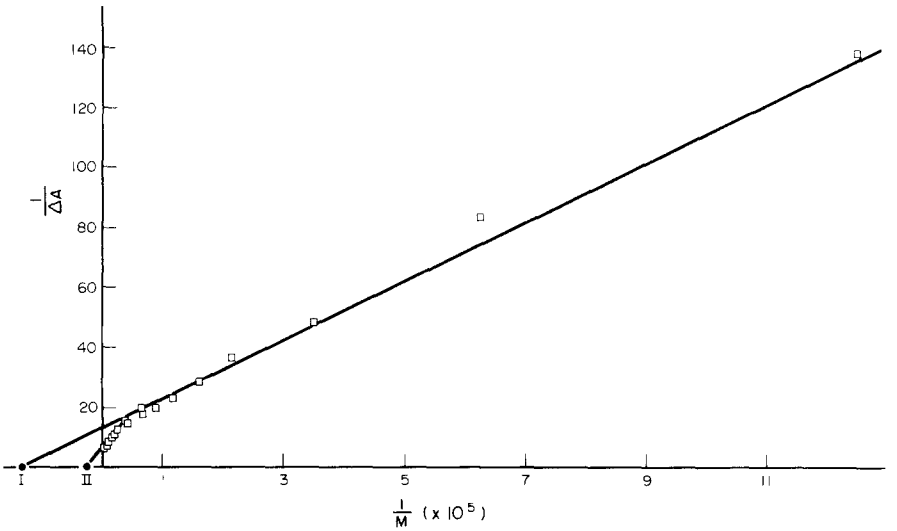



Fig 3 Double reciprocal plot for the binding of 4-pyridylbutanamide to PB-induced rat liver microsomes $\Delta A = A_{393}-A_{426}$, M = ligand concentration in M, I = $-1/Ks_1$, II = $-1/Ks_2$

(11) respecting conditions (10) The $\Delta\varepsilon$ values determined from equation (12) are 50 $mM^{-1}$ $cm^{-1}$ for PB-induced microsomes and 65 08 $mM^{-1}$ $cm^{-1}$ for the 3MC-induced ones.

Figure 4 represents the individual binding data of 4-pyridylpentanamide plotted according to the one- and two-acceptor models. Visual inspection clearly reveals that the two-acceptor model yields a better fit This is confirmed statistically: the residual variance is 1 12 × $10^{-3}$ and 2 24 × $10^{-4}$, respectively, for the one-acceptor and two-acceptor model ($P < 0.001$). Tables 4–6 present the pKs values calculated for the three isomeric series of pyridylalkanamides.

## DISCUSSION

### *Comparison of the pKs values calculated by the Lineweaver–Burk and non-linear methods*

Globally, the pKs values calculated by the two methods (symbolized by "LB" and "nonlin", respectively) are remarkably similar (Fig. 5) The regression equation is

$$pKs(LB) = 0.79(\pm 0\ 04)\,pKs(nonlin) + 0\ 78(\pm 0\ 15)$$
$$n = 40 \qquad r^2 = 0\ 900 \qquad (16)$$

This similarity must be due to the fact that the ligands were tested in the broadest possible concentration range, between the detection limit and the limit of spectral saturation A more narrow concentration range could have yielded pKs(LB) values intermediate between $pKs_1$ and $pKs_2$(LB) values

A closer examination of the tables and Fig 5 shows that $pKs_2$(LB) values are smaller than $pKs_1$(nonlin), whereas the reverse holds for $pKs_2$ values These systematic biases may originate from the simplifications of the Lineweaver–Burk model, which identifies a hyperbola with two straight lines

The above comparison shows that the Line-

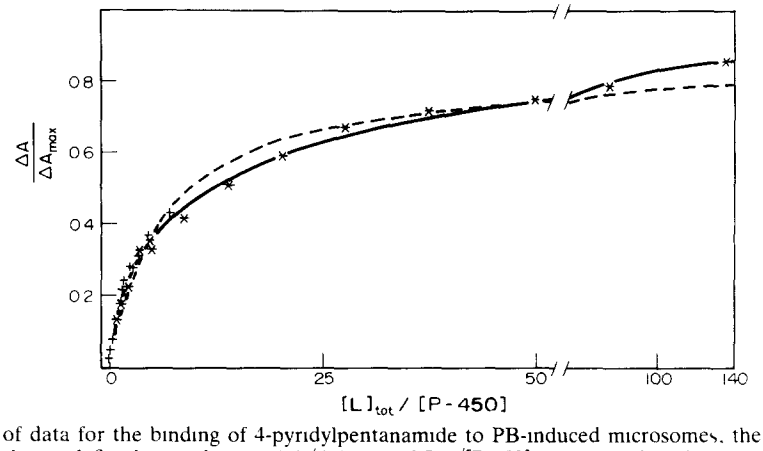



Fig 4 Plot of data for the binding of 4-pyridylpentanamide to PB-induced microsomes, the ordinate and abscissa being defined according to $\Delta A/\Delta A_{max}$ and $L_{tot}/[P\text{-}450]$, respectively The curves for the one acceptor (- - -) and two acceptor models (——) were calculated according to equation (11) Note the different scale of the abscissa above 50

Table 1 pKs values of 2-pyridylalkanamides (Lineweaver–Burk analysis)

| | PB-induced microsomes | | | | 3MC-induced microsomes | | | |
|---|---|---|---|---|---|---|---|---|
| nC* | conc [M]† | r‡ | N§ | pKs[M]‖ | conc [M]† | r‡ | N§ | pKs[M]‖ |
| 1 | $2\,0\ 10^{-3}$–$20\ 10^{-3}$ | 0 992 | 25 | 1 71 ± 0 09 | $2\,0\ 10^{-3}$–$43\ 10^{-3}$ | 0 804 | 17 | 2.23 ± 0 38 |
| 2 | $2\,7\ 10^{-3}$–$24\ 10^{-3}$ | 0 993 | 15 | 1 76 ± 0 10 | $0\,7\ 10^{-3}$–$58\ 10^{-3}$ | 0 994 | 19 | 2.20 ± 0 06 |
| 3 | $4\,0\ 10^{-3}$–$50\ 10^{-3}$ | 0 980 | 26 | 1 97 ± 0 07 | $1\,0\ 10^{-3}$–$50\ 10^{-3}$ | 0 985 | 21 | 2 37 ± 0 07 |
| 4 | $0\,4\ 10^{-3}$–$11\ 10^{-3}$ | 0 987 | 18 | 2 69 ± 0 09 | $1\,0\ 10^{-3}$–$17\ 10^{-3}$ | 0 993 | 12 | 2 48 ± 0 08 |

* nC = number of —$CH_2$— group in side chain
† conc = concentration range investigated
‡ r = correlation coefficient
§ N = number of points in the regression
‖ The errors were calculated from the 95% confidence limits of the slope

Table 2 pKs values of 3-pyridylalkanamides (Lineweaver–Burk analysis)

| nC | conc [M] | r | N | $pKs_1$ [M] | conc [M] | r | N | $pKs_2$ [M] |
|---|---|---|---|---|---|---|---|---|
| | | | | PB-induced microsomes | | | | |
| 1 | $0\,4\ 10^{-4}$–$40\ 10^{-4}$ | 0 985 | 28 | 3 32 ± 0 09 | $0\,6\ 10^{-2}$–$2\,2\ 10^{-2}$ | 0 921 | 8 | 2 40 ± 0 24 |
| 2 | $0\,4\ 10^{-4}$–$60\ 10^{-4}$ | 0 997 | 11 | 2 98 ± 0 16 | $0\,6\ 10^{-2}$–$3\,0\ 10^{-2}$ | 0 971 | 12 | 2 34 ± 0 10 |
| 3 | $0\,4\ 10^{-5}$–$24\ 10^{-5}$ | 0 979 | 22 | 4 52 ± 0 10 | $3\,0\ 10^{-4}$–$97\ 10^{-4}$ | 0 975 | 19 | 3 55 ± 0 07 |
| 4 | $0\,2\ 10^{-5}$–$37\ 10^{-5}$ | 0 991 | 17 | 4 58 ± 0 08 | $3\,7\ 10^{-4}$–$18\ 10^{-4}$ | 0 888 | 5 | 3 80 ± 0 39 |
| | | | | 3MC-induced microsomes | | | | |
| 1 | $1\,2\ 10^{-4}$–$20\ 10^{-4}$ | 0 977 | 13 | 3 34 ± 0 14 | $0\,2\ 10^{-2}$–$2\,4\ 10^{-2}$ | 0 990 | 16 | 2 65 ± 0 05 |
| 2 | $0\,4\ 10^{-4}$–$16\ 10^{-4}$ | 0 999 | 12 | 3 36 ± 0 03 | $1\,6\ 10^{-3}$–$29\ 10^{-3}$ | 0 991 | 18 | 2 72 ± 0 04 |
| 3 | $1\,0\ 10^{-5}$–$49\ 10^{-5}$ | 0 987 | 14 | 4 20 ± 0 09 | $4\,9\ 10^{-4}$–$97\ 10^{-4}$ | 0 991 | 8 | 3 43 ± 0 18 |
| 4 | $2\,0\ 10^{-6}$–$58\ 10^{-6}$ | 0 971 | 20 | 4 86 ± 0 13 | $0\,6\ 10^{-4}$–$18\ 10^{-4}$ | 0 995 | 22 | 3 87 ± 0 03 |

See Table 1 for column headings

Table 3 pKs values of 4-pyridylalkanamides (Lineweaver–Burk analysis)

| nC | conc [M] | r | N | $pKs_1$ [M] | conc [M] | r | N | $pKs_2$ [M] |
|---|---|---|---|---|---|---|---|---|
| | | | | PB-induced microsomes | | | | |
| 1 | $0\,4\ 10^{-4}$–$19\ 10^{-4}$ | 0 976 | 31 | 3 69 ± 0.09 | $2\,0\ 10^{-3}$–$11\ 10^{-3}$ | 0 994 | 8 | 2 79 ± 0 07 |
| 2 | $0\,2\ 10^{-4}$–$21\ 10^{-4}$ | 0 910 | 34 | 4 02 ± 0 14 | $2\,0\ 10^{-3}$–$17\ 10^{-3}$ | 0 988 | 7 | 2 89 ± 0 10 |
| 3 | $0\,4\ 10^{-5}$–$68\ 10^{-5}$ | 0 972 | 30 | 4 50 ± 0 09 | $0\,7\ 10^{-3}$–$10\ 10^{-3}$ | 0 973 | 12 | 3 38 ± 0 08 |
| 4 | $0\,4\ 10^{-6}$–$28\ 10^{-6}$ | 0 993 | 22 | 5 15 ± 0 12 | $2\,8\ 10^{-5}$–$42\ 10^{-5}$ | 0 999 | 8 | 4 46 ± 0 03 |
| | | | | 3MC-induced microsomes | | | | |
| 1 | $0\,4\ 10^{-4}$–$10\ 10^{-4}$ | 0 980 | 20 | 3 70 ± 0 10 | $1\,0\ 10^{-3}$–$3\,0\ 10^{-3}$ | 0 994 | 6 | 3 11 ± 0 10 |
| 2 | $1\,0\ 10^{-5}$–$60\ 10^{-5}$ | 0 996 | 13 | 3 85 ± 0 09 | $0\,6\ 10^{-3}$–$19\ 10^{-3}$ | 0 996 | 10 | 3 13 ± 0 04 |
| 3 | $0\,8\ 10^{-5}$–$72\ 10^{-5}$ | 0 998 | 18 | 4 22 ± 0 03 | $7\,2\ 10^{-4}$–$92\ 10^{-4}$ | 0 989 | 12 | 3 43 ± 0 06 |
| 4 | $1\,6\ 10^{-6}$–$16\ 10^{-6}$ | 0 991 | 8 | 5 28 ± 0 12 | $0\,2\ 10^{-4}$–$15\ 10^{-4}$ | 0 973 | 19 | 4 02 ± 0 12 |

See Table 1 for column headings

Table 4 pKs values of 2-pyridylalkanamides (non-linear analysis)

| nC | pKs* [M] | $\sigma_1$† | df‡ | $pKs_1$* [M] | $pKs_2$* [M] | $\sigma_2$† | df‡ | P§ |
|---|---|---|---|---|---|---|---|---|
| | | | | PB-induced microsomes (total P-450 concentration in cuvettes 3 11 $\mu$M) | | | | |
| 1 | 1 58 ± 0 04 | $1\ 77\ 10^{-4}$ | 45 | 1 51 ± 0 27 | 0 36 ± 1 19 | $3\ 22\ 10^{-4}$ | 43 | NS |
| 2 | 1 86 ± 0 05 | $1\ 05\ 10^{-4}$ | 20 | 1 87 ± 0 05 | 0 66 ± 2 67 | $1\ 17\ 10^{-4}$ | 18 | NS |
| 3 | 1 95 ± 0 03 | $2\ 02\ 10^{-4}$ | 30 | 1 78 ± 0 28 | 2 68 ± 1 12 | $1\ 96\ 10^{-4}$ | 28 | NS |
| 4 | 2 57 ± 0 05 | $1\ 53\ 10^{-4}$ | 20 | 1 28 ± 0.36 | 2 76 ± 0 09 | $1\ 48\ 10^{-4}$ | 18 | NS |
| | | | | 3MC-induced microsomes (total P-450 concentration in cuvettes 2 49 $\mu$M) | | | | |
| 1 | 1 82 ± 0 10 | $1\ 57\ 10^{-4}$ | 20 | 1 55 ± 0 32 | 0 0001‖ | $4\ 07\ 10^{-4}$ | 18 | NS |
| 2 | 1 84 ± 0 04 | $8\ 37\ 10^{-5}$ | 20 | 1 71 ± 0 04 | 4 24 ± 0 59 | $2\ 72\ 10^{-5}$ | 18 | <0 01 |
| 3 | 2 11 ± 0 05 | $3\ 80\ 10^{-4}$ | 23 | 1 70 ± 0 07 | 3 75 ± 0 26 | $8\ 22\ 10^{-5}$ | 21 | <0 001 |
| 4 | 2 40 ± 0 05 | $1\ 48\ 10^{-4}$ | 14 | 1 92 ± 0 17 | 3.27 ± 0 26 | $4\ 40\ 10^{-5}$ | 12 | <0 05 |

* pKs resp $pKs_1$ and $pKs_2$ = pKs values obtained with one resp two binding sites model
† $\sigma_1$ resp $\sigma_2$ = residual variance obtained with one resp two binding sites model
‡ df = degrees of freedom
§ P assessed by F-test (NS = non-significant)
‖ Starting value in iteration, otherwise non-calculable

Table 5 pKs values of 3-pyridylalkanamides (non-linear analysis)

| nC | pKs [M] | $\sigma_1$ | df | $pKs_1$ [M] | $pKs_2$ [M] | $\sigma$ | df | P |
|---|---|---|---|---|---|---|---|---|
| PB-induced microsomes (total P-450 concentration in cuvettes 3.11 $\mu$M) | | | | | | | | |
| 1 | 2.70 ± 0.04 | $0.90\ 10^{-3}$ | 34 | 2.10 ± 0.14 | 3.46 ± 0.16 | $2.57\ 10^{-1}$ | 32 | 0.001 |
| 2 | 2.78 ± 0.08 | $3.60\ 10^{-3}$ | 19 | 1.39 ± 0.28 | 3.36 ± 0.10 | $5.00\ 10^{-1}$ | 17 | 0.001 |
| 3 | 3.78 ± 0.05 | $2.95\ 10^{-3}$ | 39 | 2.96 ± 0.08 | 4.53 ± 0.08 | $3.42\ 10^{-1}$ | 37 | 0.001 |
| 4 | 4.19 ± 0.06 | $2.05\ 10^{-3}$ | 19 | 2.98 ± 0.33 | 4.62 ± 0.12 | $6.28\ 10^{-1}$ | 17 | 0.01 |
| 3MC-induced microsomes (total P-450 concentration in cuvettes 2.49 $\mu$M) | | | | | | | | |
| 1 | 2.77 ± 0.03 | $0.88\ 10^{-3}$ | 27 | 2.42 ± 0.07 | 3.71 ± 0.19 | $1.86\ 10^{-1}$ | 25 | 0.001 |
| 2 | 2.79 ± 0.03 | $1.13\ 10^{-3}$ | 27 | 2.35 ± 0.06 | 3.53 ± 0.10 | $1.25\ 10^{-1}$ | 25 | 0.001 |
| 3 | 3.64 ± 0.04 | $1.78\ 10^{-3}$ | 20 | 2.88 ± 0.11 | 4.17 ± 0.08 | $2.01\ 10^{-1}$ | 18 | 0.001 |
| 4 | 3.87 ± 0.02 | $0.48\ 10^{-3}$ | 40 | 3.65 ± 0.05 | 4.79 ± 0.24 | $1.95\ 10^{-1}$ | 38 | 0.01 |

See Table 4 for column headings

weaver–Burk analysis is only approximate for a one ligand–two acceptor model The approximation is satisfactory if sufficient points have been measured over a large concentration range, as evidenced by the 3- and 4-pyridylalkanamides The approximation is not as good if the concentration range is relatively narrow, or if the number of acceptors is uncertain as is the case for the 2-pyridylalkanamides Non-linear methods, while less straightforward to employ, are globally more reliable

*Hypothesis regarding the existence of two pKs values*

The non-linear analysis of data has unambiguously established the existence of two dissociation constants for most of the ligands investigated here A few similar cases have already been reported in the literature [17], but no explanations have been offered

A number of hypotheses can be postulated to explain dual affinity of a ligand to cytochrome P-450

(a) The binding occurs to two distinct isozyme populations However, we note that the pKs values are practically the same for PB- and 3MC-induced microsomes This could indicate that the difference in the relative proportion of isocytochromes P-450 has no influence on the ligand-binding of pyridylalkanamides

(b) Hajek *et al* [18], using other ligands and purified isocytochromes P-450, also found two affinities This would suggest that dual affinity results from dual binding to a given isozyme either as 5th and 6th ligand, an improbable hypothesis in the light of studies published by White and Coon [19] showing that the 5th ligand (—S ) does not change during the metabolic activity of cytochrome P-450 or as 6th ligand and as substrate on an adjacent protein region resulting in a modification of the type II spectrum [20], which however was never detected or may also be due to an additional non-specific binding to a protein region removed from the heme moiety a hypotheses which must be excluded since such a binding would not be detectable with the spectral technique used

Thus no acceptable explanation can be offered which would satisfy a dual binding to a single isozyme in a single state

(c) The most likely possibility involves changes in the spin state of the ferric ion We postulate that at low concentrations, the pyridines bind as the 6th ligand to the population of cytochrome P-450 existing in the low-spin state due to the presence of an endogenous 6th ligand the latter is thus displaced by the exogenous ligand and this produces a type IIb spectrum At higher concentrations the ligand binds to the high spin (pentacoordinated) iron ion producing a shift to the low-spin state (hexacoordinated) and type II spectrum which represents the sum of type IIb and IIa spectra [17]

In the context of this hypothesis one could have expected different $pKs_1$ values depending on the type of inducer being used Indeed cytochrome P-450 in hepatic microsomes from PB-pretreated rats exists preferentially in the low spin form whereas after 3MC pretreatment the high-spin form predominates

Table 6 pKs values of 4-pyridylalkanamides (non-linear analysis)

| nC | pKs [M] | $\sigma_1$ | df | $pKs_1$ [M] | $pKs_2$ [M] | $\sigma$ | df | P |
|---|---|---|---|---|---|---|---|---|
| PB-induced microsomes (total P-450 concentration in cuvettes 3.11 $\mu$M) | | | | | | | | |
| 1 | 3.08 ± 0.04 | $1.65\ 10^{-3}$ | 39 | 2.55 ± 0.15 | 3.83 ± 0.19 | $0.76\ 10^{-1}$ | 37 | 0.01 |
| 2 | 3.68 ± 0.04 | $4.05\ 10^{-3}$ | 57 | 2.55 ± 0.18 | 4.09 ± 0.08 | $1.65\ 10^{-1}$ | 55 | 0.001 |
| 3 | 3.94 ± 0.05 | $6.54\ 10^{-3}$ | 46 | 2.69 ± 0.17 | 4.50 ± 0.10 | $2.11\ 10^{-1}$ | 44 | 0.001 |
| 4 | 4.73 ± 0.03 | $1.12\ 10^{-3}$ | 27 | 4.14 ± 0.10 | 5.34 ± 0.11 | $2.24\ 10^{-1}$ | 25 | 0.001 |
| 3MC-induced microsomes (total P-450 concentration in cuvettes 2.49 $\mu$M) | | | | | | | | |
| 1 | 3.44 ± 0.04 | $0.64\ 10^{-3}$ | 24 | 2.33 ± 0.64 | 3.79 ± 0.15 | $3.43\ 10^{-1}$ | 22 | NS |
| 2 | 3.32 ± 0.04 | $2.40\ 10^{-3}$ | 20 | 2.87 ± 0.07 | 4.28 ± 0.15 | $3.52\ 10^{-1}$ | 18 | 0.001 |
| 3 | 3.83 ± 0.04 | $2.48\ 10^{-3}$ | 27 | 2.74 ± 0.13 | 4.25 ± 0.06 | $3.14\ 10^{-1}$ | 25 | 0.001 |
| 4 | 3.99 ± 0.05 | $3.57\ 10^{-3}$ | 26 | 3.89 ± 0.09 | 6.15 ± 1.59 | $3.37\ 10^{-1}$ | 24 | NS |

See Table 4 for column headings

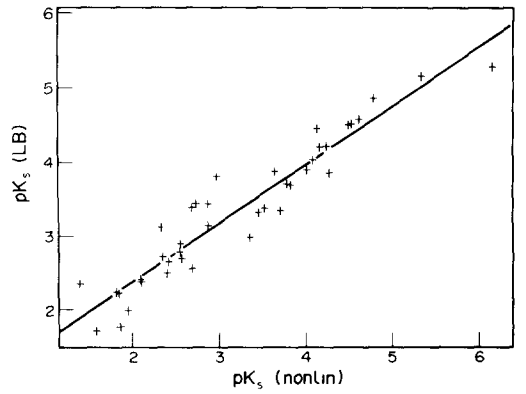



Fig 5 Comparison of the pKs values obtained by Lineweaver–Burk (LB) and non-linear (nonlin) analysis The calculated line is described by equation (16)

[21, 22]. Our results show a great similarity in the pKs$_1$ values of both types of microsomes, as also found by Jefcoate *et al.* [17] when studying aliphatic amines. These observations make sense since there are indications [21] that the preparation of microsomes may easily alter the spin state of cytochrome P-450, and since measurements of spin state by EPR yield only relative and not very accurate data.

Hypothesis (c) is supported by data from other studies White and Coon [19] showed that imidazole presents more affinity for low spin cytochrome P-450 ($LM_2$ induced by PB) than for the high spin form ($LM_4$ induced by 5,6-benzoflavone). Moreover,

Table 7 $\beta_1$ values obtained from equation 13 for a one acceptor- (n = 1) and a two acceptor-model (n = 2)

| Substances tested | n = 1 | n = 2 | |
|---|---|---|---|
| | $\beta_1$ [%] | $\beta_1$ [%] | $\beta_2$ [%] |
| PB* 2†–1‡ | 42 ± 2 | 49 ± 29 | 0 1 ± 282 |
| 2–2 | 43 ± 2 | | |
| 2–3 | 44 ± 1 | 41 ± 12 | 6 ± 16 |
| 2–4 | 34 ± 2 | | |
| 3†–1‡ | 58 ± 2 | 53 ± 3 | 19 ± 4 |
| 3–2 | 78 ± 3 | 95 ± 30 | 44 ± 5 |
| 3–3 | 70 ± 2 | 50 ± 2 | 33 ± 3 |
| 3–4 | 68 ± 2 | 49 ± 9 | 44 ± 6 |
| 4†–1‡ | 78 ± 2 | 69 ± 5 | 25 ± 7 |
| 4–2 | 87 ± 2 | 49 ± 4 | 57 ± 5 |
| 4–3 | 83 ± 2 | 56 ± 5 | 51 ± 4 |
| 4–4 | 83 ± 2 | 63 ± 4 | 33 ± 6 |
| 3MC* 2†–1‡ | 20 ± 2 | | |
| 2–2 | 33 ± 1 | 34 ± 1 | 2 ± 0 4 |
| 2–3 | 37 ± 2 | 39 ± 2 | 6 ± 1 |
| 2–4 | 37 ± 2 | 40 ± 3 | 8 ± 3 |
| 3*–1‡ | 76 ± 2 | 64 ± 4 | 18 ± 4 |
| 3–2 | 86 ± 1 | 65 ± 4 | 29 ± 5 |
| 3–3 | 78 ± 2 | 50 ± 4 | 40 ± 5 |
| 3–4 | 81 ± 1 | 74 ± 4 | 12 ± 5 |
| 4*–1‡ | 57 ± 2 | 63 ± 42 | 33 ± 9 |
| 4–2 | 90 ± 2 | 71 ± 4 | 26 ± 5 |
| 4–3 | 85 ± 2 | 44 ± 3 | 56 ± 4 |
| 4–4 | 102 ± 4 | 99 ± 5 | 5 ± 4 |

* PB and 3MC = microsomes induced by PB and 3MC, respectively

† 2, 3 and 4 = 2-, 3- and 4-pyridylalkanamides, respectively

‡ 1 to 4 = number of —$CH_2$— groups in side chain

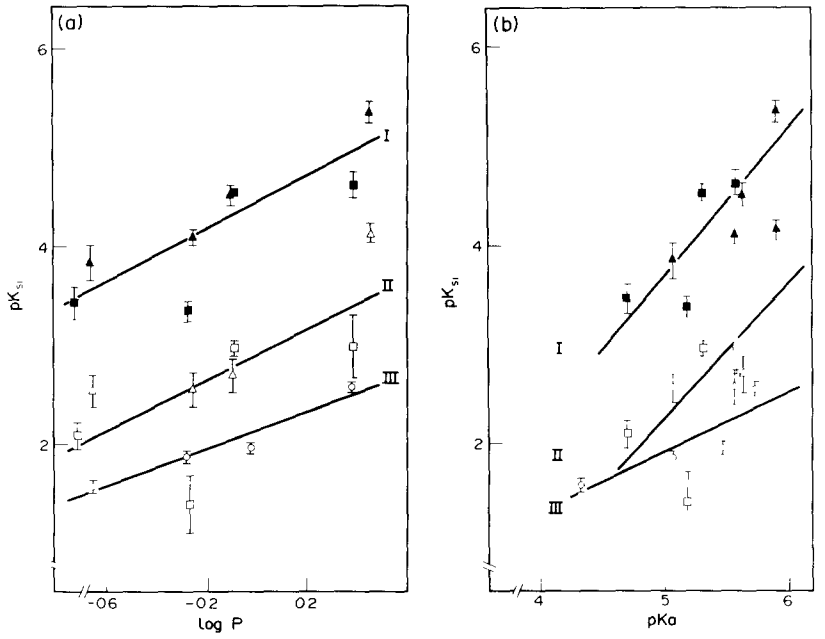



Fig 6 Plots of pKs values (PB-induced microsomes, non-linear analysis) versus (a) log P (I r = 0 844, n = 8, II r = 0 691, n = 8, III r = 0 962, n = 4) and (b) p$K_a$ (I r = 0 846, n = 8, II r = 0 700, n = 8, III r = 0 872, n = 4) values of pyridylalkanamides Full symbols pKs$_2$, open symbols pKs$_1$. ●, ○ = 2-pyridyl series, ■, □ = 3-pyridyl series, ▲, △ = 4-pyridyl series

Kumaki *et al* [21] observed that a type IIb ligand has a higher affinity for the cytochrome P-450 than a type IIa ligand.

The particular results of the 2-pyridylalkanamides are of interest The single pKs with these compounds implies that the only detectable binding is to the high spin form (low affinity site, Table 7) resulting in a type IIa spectrum The little shift toward the lower wavelength as compared to the regioisomers suggests a decreased contribution of a type IIb component in the spectrum The fact that the sum $\beta_1 + \beta_2$ (expressing % enzyme molecules occupied) is smaller than 50% (see Table 7) is compatible with binding to the high spin form only

*Structure-binding relationships*

Tables 4–6 show that the affinity of pyridylalkanamides for cytochrome P-450 is dependent upon their chemical structure However, clear-cut structure-binding relationships are not apparent Qualitatively, two effects can be seen the affinity decreases in the series 4- > 3- > 2-pyridylalkanamides, and the affinity increases with increasing length of the side chain

The first effect is postulated to be steric in nature, steric shielding of the pyridyl nitrogen being unfavorable for its ligand binding to the ferric ion The particular mode of binding of the 2-pyridyl series indicates the importance of steric effects permitting binding only to the pentacoordinated iron which is sterically more accessible

As regards the increase in affinity with the length of side chain, its origin cannot be assessed with certainty. Indeed, we have shown [8, 9], that the lipophilicity (log P) and the basicity ($pK_a$) of these compounds increase with increasing length of the side chain Typical plots of pKs versus log P and $pK_a$ values are shown in Fig 6 They indicate a global increase in affinity for both increasing lipophilicity and basicity But because these two physicochemical properties are intercorrelated in this series ($r = 0.85$), it is not possible to conclude whether the binding of the pyridylalkanamides to cytochrome P-450 is controlled by lipophilicity and/or basicity Plots of pKs values (measured with 3MC-microsomes) versus log P and $pK_a$ are similar to those shown in Fig 6 This work and others [17, 19, 23] indicate that the dissociation constant is influenced by lipophilicity and basicity Indeed the ligand must present a sufficient solubility to approach the heme and nucleophilicity to bind to the $Fe^{3+}$

The correlations between pKs and log P or $pK_a$ values are only partial (Fig 6), perhaps suggesting other structural factors to be influential The thermodynamics of distribution of pyridylalkanamides between organic solvents and water ([24], Repond *et al* , in preparation) has shown that in media of low polarity the 2-pyridyl homologs having 1 to 3 methylene groups in the side chain can form an intramolecular H-bond between the amide group and the heterocyclic nitrogen (Fig 7) This conformation amplifies the steric effect of the 2-substitution and contributes to the masking of the nitrogen for the binding to the iron atom

In conclusion, the present work brings evidence for a dual mode of binding of a number of ligands

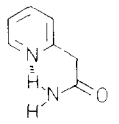


Fig 7 Representation of the intramolecular H-bond for 2-pyridylethanamide

to cytochrome P-450 High affinity binding is postulated to involve the low spin, hexacoordinated ferric cation, whereas low affinity binding is believed to occur to the high spin, pentacoordinated ferric cation, and to induce a high spin to low spin conversion This hypothesis, however, needs experimental confirmation. Furthermore, it may be of interest to investigate whether the pyridylalkanamides are inducers of cytochrome P-450, and whether there is a relationship between their affinity and inducing properties Such studies have been performed and a publication is in preparation

*Acknowledgements*—The authors gratefully acknowledge the financial support of the Swiss National Science Foundation (grants 3 013–0 81 and 3 539–0 83 to B T , grant 3 806–0 84 to U A M )

## REFERENCES

1 G J Mannering, in *Concepts in Drug Metabolism* (Eds P Jenner and B Testa), Part B, p 53 Dekker New York (1981)
2 K Kumaki and D W Nebert, *Pharmacology* **17** 262 (1978)
3 B Testa and P Jenner, *Drug Metab Rev* **12**, 1 (1981)
4 S G Sligar, *Biochemistry* **15**, 5399 (1976)
5 O Ristau, H Rein, G R Janig and K Ruckpaul *Biochim biophys Acta* **536** 226 (1978)
6 K C Leibman and E Ortiz, *Drug Metab Dispos* **1**, 184 (1973)
7 U Giger and U A Meyer *Biochem Pharmac* **31** 1735 (1982)
8 J M Mayer and B Testa, *Helv chim Acta* **65** 1868 (1982)
9 J M Mayer, B Testa, H van de Waterbeemd and A Bornand-Crausaz, *Eur J med Chem* **17**, 453 (1982)
10 O H Lowry, N J Rosebrough, A L Farr and R J Randall *J biol Chem* **193**, 265 (1951)
11 T Omura and R Sato *J biol Chem* **239** 2370 (1964)
12 H Lineweaver and D Burk *J Am chem Soc* **56** 658 (1934)
13 J E A McIntosch and R P McIntosch, *Mathematical Modelling and Computer in Endocrinology* p 250 Springer, Berlin (1980)
14 I M Klotz, *TIPS* **4**, 253 (1983)
15 E Burgisser, *TIPS* **5**, 142 (1984)
16 H A Feldman, *Analyt Biochem* **48**, 317 (1972)
17 C R E Jefcoate, J L Gaylor and R L Calabrese *Biochemistry* **8**, 3455 (1969)
18 K K Hajek, N I Cook and R F Novak, *J Pharmac exp Ther* **223**, 97 (1982)
19 R E White and M J Coon, *J biol Chem* **257** 3073 (1982)
20 J B Schenkman *Biochemistry* **9** 2081 (1970)
21 K Kumaki, M Sato H Kon and D W Nebert *J biol Chem* **253**, 1048 (1978)
22 I O Stern J Peisach, W E Blumberg A Y H Lu and W Levin *Archs Biochem Biophys* **156** 404 (1973)
23 J L Born and S Early, *J pharm Sci* **69**, 850 (1980)
24 C Repond, Propriétés biologiques et physico-chimiques de pyridylalkylamines et de pyridylalkanamides Doctorate Thesis, University of Lausanne (1985)