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THE MICROSOMAL DEALKYLATION OF *N,N*-DIALKYLBENZAMIDES

JIM ILEY* and LUIS CONSTANTINO†

Physical Organic Chemistry Research Group, Chemistry Department, The Open University, Milton Keynes MK7 6AA, U.K. and †CECF, Faculdade de Farmacia de Lisboa, Avenida das Forças Armadas, 1600 Lisboa, Portugal

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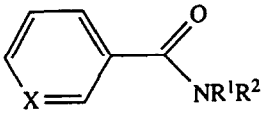
Abstract—The *in vitro* metabolism of *N,N*-dialkylamides by phenobarbital-induced rat liver microsomes yields an *N*-alkylamide and the corresponding aldehyde. Although, *N*-hydroxymethyl-*N*-alkylamide intermediates can be detected from *N*-methyl-*N*-alkylamides, no *N*-hydroxyalkyl-*N*-alkylamide intermediates are detected from the *N,N*-dialkylamide substrates. V_{max} values were independent of amide structure, whereas V_{max}/K_m values were dependent on the lipophilicity of the *N,N*-dialkylbenazamide studied. These results suggest that diffusion of substrate into the membrane-bound enzyme active site limits the rate of the microsomal oxidation of the amides. Metabolism of *N*-alkyl-*N*-methylamides reveals identical values of V_{max} for demethylation and dealkylation. Values of V_{max}/K_m for demethylation depend upon the lipophilicity of the *N*-alkyl group, whereas V_{max}/K_m values for dealkylation appear to be dependent upon the steric bulk of the alkyl group, particularly around the α-carbon. Moreover, V_{max}/K_m values for demethylation are larger than for dealkylation, implying the reactions are under kinetic control. Comparison of the kinetic data with theoretical AM1 semi-empirical molecular orbital calculations suggests a mechanism involving formation of a carbon-centred radical. Use of an *N*-cyclopropylmethylbenzamide substrate to trap such a radical failed, presumably because oxygen rebound is faster than radical rearrangement. An *N*-cyclopropylamide substrate did not undergo metabolism of the cyclopropyl ring, consistent with carbon-centred radical, but not nitrogen radical cation, formation.

Cytochrome P450 enzymes are membrane bound, iron porphyrin-containing monooxygenases that catalyse several reactions having a common unifying feature, namely the incorporation of one oxygen atom into the substrate molecule. As part of an ongoing interest in the molecular mechanisms of such processes, we have studied the oxidative dealkylation of *N,N*-dimethylbenzamides using both the biomimetic system tetraphenylporphyrinatoiron(III) chloride/*t*-butylhydroperoxide [1], as well as rat liver microsomes [2]. Our choice of substrates was prompted in part by the fact that several *N,N*-dialkylamides which have important biological activity—for example, the insect repellent *N,N*-diethyl-3-toluamide [3], the respiratory stimulant *N,N*-diethylnicotinamide [4], and the industrial solvent *N,N*-dimethylformamide [5]—are all known to undergo either oxidative N-dealkylation or oxidation of an *N*-alkyl group by liver metabolism *in vivo*. The substituent and kinetic deuterium isotope effects (KDIEs) obtained suggested a mechanism involving direct hydrogen atom abstraction from the substrate, though the data do not preclude a mechanism involving H^+ abstraction from an amide radical cation. We have continued our studies to gain further insight into the mechanism of amide dealkylation, and have studied the demethylation and dealkylation of a series of *N,N*-dialkylamides and *N*-alkyl-*N*-methylamides. The compounds used in the present study are **1a-h** (Table 1).

* Corresponding author. Tel. (0908) 652513; FAX (0908) 653744.

Table 1



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	X	R^1	R^2
(a)	CH	Me	Me
(b)	CH	Et	Et
(c)	N	Et	Et
(d)	CH	Me	Et
(e)	CH	Me	Bu^n
(f)	CH	Me	Pr^i
(g)	CH	Me	CH_2—$CHCH_2CH_2$
(h)	CH	Me	$CHCH_2CH_2$

MATERIALS AND METHODS

Substrates. The *N,N*-dialkylamides **1a-f** and the product *N*-alkylamides were synthesized by the Schotten–Baumann method [6] from the appropriate amine and acid chloride. The *N,N*-dialkylamide substrates were purified by column chromatography using ethyl acetate/hexane (5:2) as eluent, the *N*-alkylamides were recrystallized from ethanol.

N-Methyl-*N*-cyclopropylmethylbenzamide, **1g**, and *N*-methyl-*N*-cyclopropylbenzamide, **1h**, were synthesized as follows: the appropriate monoalkyl-

benzamide (0.012 mol) was dissolved in dry tetrahydrofuran (THF) (30 cm^3) under N_2 at room temperature, *n*-butyllithium (1.1 mol equiv.) was injected slowly into the solution and, after 15 min, a solution of iodomethane (2 mol equiv.) in dry THF (10 cm^3) was added and the solution stirred for a further 45 min. Ethanol (5 cm^3) was added, the solvents evaporated, and the residue extracted with ethyl acetate (30 cm^3). The organic extract was washed with water (2 × 10 cm^3), dried ($MgSO_4$), concentrated and subjected to chromatography on silica using ethyl acetate/hexane (5:2) as eluent.

Substrate lipophilicity. Partition coefficients of the *N,N*-dialkylamide substrates between octanol and 0.068 mol/dm^3 pH 7.4 phosphate buffer containing 0.074 mol/dm^3 NaCl were determined by adding each substrate (2 mg) to a biphasic system comprising 0.5 cm^3 of *n*-octanol (previously saturated with the phosphate buffer) and 0.5 cm^3 of phosphate buffer (previously saturated with octanol). The mixture was agitated for 2 min then centrifuged. Aliquots (0.02 cm^3) of each phase were diluted with ethanol (10 cm^3) then analysed for amide content by HPLC.

Microsomes. These were prepared as described previously [2]. Phenobarbital induces the cytochrome P450 IIB1 and IIB2 isoenzymes. From comparative studies using microsomes induced by phenobarbital, 3-methylcholanthrene (P450 IA1 and IA2) or pregnenolen-16α-carbonitrile (P450 IIIA1), Hall and Hanzlik [7] have shown that the major tertiary amide metabolizing isoenzyme is P450 IIB1. We did not assay the individual isoenzyme content of our microsomal preparation, but the work described herein was conducted using the same batch of pooled microsomal protein. Protein concentration was determined by the method of Lowry *et al.* [8] and cytochrome P450 analysis was performed according to Omura and Sato [9]. Cytochrome P450 levels were *ca.* 1.50 nmol/mg protein.

Product analysis. Reactions were quenched by the sequential addition of 0.20 mol/dm^3 $ZnSO_4$ and 0.16 mol/dm^3 $BaCl_2$. After centrifugation, the supernatant was analysed by HPLC before both and after treatment with NaOH for amide content. The aldehydes produced were also analysed by HPLC after derivatization with 2,4-dinitrophenylhydrazine [10]. Separation of the amides was performed using a 5 μm C-18 25 cm Jones chromatography column, with an eluent consisting of 20% acetonitrile in water for **1a**, 14% acetonitrile in 0.05 mol/dm^3 acetate buffer pH for **1c**, or a gradient from 15% acetonitrile in 0.05 M pH 2.6 phosphate buffer at the time of injection to 42% of acetonitrile in 0.05 M pH 2.6 phosphate buffer at 22 min for the other amides. Detection was carried out at 230 or 254 nm; the limit of detection was *ca.* 5×10^{-7} mol/dm^3. Aldehyde analysis involved the use of the same Jones column, together with a solvent comprising of 54% acetonitrile in 0.05 mol/dm^3 phosphate buffer at pH 2.6. Detection of the aldehydes was performed at 350 nm; the limit of detection was 2.5×10^{-7} mol/dm^3.

Reaction kinetics. Incubations were performed as described previously [2]. Values of V_i, which were obtained from four separate determinations ranged from 3.8×10^{-4} to 2.5×10^{-3} mol/dm^3/hr and were measurable to 6×10^{-5} mol/dm^3/hr.

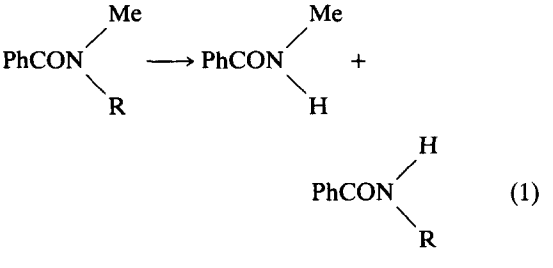
Mass spectral assay for reaction products. Substrates (usually 10 mmol/dm^3) were subjected to microsomal oxidation as described above. The reactions were followed by HPLC and every 2 hr more glucose-6-phosphate, NADPH and glucose-6-phosphate dehydrogenase were added until *ca.* 50% of the substrate was metabolized. The reactions were quenched as usual, and after centrifugation, the supernatant was extracted three times with ethyl acetate, the organic phase was dried with sodium sulphate, concentrated and analysed by GC–MS (BP-5 column 25 m, start temperature 8° then 10°/min up to 200°, Hewlett Packard 5890-A/VGT Masslab 20–250).

Molecular orbital calculations. Structure determinations, ΔH_f values and ionization potentials were calculated using the semi-empirical AM1 self-consistent field molecular orbital program within the MOPAC 4 package [11]. Radical structures were determined using unrestricted Hartree–Fock calculations. All structures were geometry optimized using the Broyden–Fletcher–Goldfarb–Shanno procedure and were performed on a VAX cluster.

RESULTS

Incubation of *N,N*-diethylbenzamide **1b** and *N,N*-diethylnicotinamide **1c** with rat liver microsomes produces only one metabolite detectable by HPLC, the retention time and UV spectrum of which is identical to that of the corresponding *N*-ethylamide. In contrast to the metabolism of *N,N*-dimethylbenzamide **1a**, the formation of an intermediate *N*-hydroxyethyl-*N*-ethylbenzamide is not observed. This is unsurprising since it is known that *N*-hydroxyalkyl groups are less stable than the corresponding *N*-hydroxymethyl group and, moreover, the stability of an *N*-hydroxymethyl group decreases with the increasing size of the other *N*-alkyl group [12, 13]. Independent experiments demonstrated that neither *N*-ethylbenzamide nor *N*-ethylnicotinamide is metabolized to any significant extent over the timescale of the reactions. Plots of V_i versus [substrate] for **1a–c** produce the usual rectangular hyperbolae of Michaelis—Menten behaviour (data not shown). Interpolation of the data, using a non-linear least squares method, gives rise to the V_{max} and V_{max}/K_m values contained in Table 2.

For the oxidative dealkylation of the amides **1d–h**, both N-dealkylation to form an *N*-methylbenzamide and N-demethylation to form an *N*-alkylbenzamide are observed (Eqn 1).



(1)

As might be anticipated from the observations of the metabolism of individual *N,N*-dimethyl- and

Table 2. Kinetic parameters for demethylation and dealkylation of *N,N*-dialkylbenzamides by rat liver microsomes at 37° and pH 7.4*

	Total dealkylation		Demethylation		Dealkylation		
Substrate	$10^3 V_{max}$ (mol/dm³/hr)	V_{max}/K_m (/hr)	$10^3 V_{max}$ (mol/dm³/hr)	V_{max}/K_m (/hr)	$10^3 V_{max}$ (mol/dm³/hr)	V_{max}/K_m (hr)	log *P*
1a	3.31 (± 0.10) *0.55*	1.32 (± 0.14) *0.22*	3.31 (± 0.01) *0.55*	1.32 (± 0.14) *0.22*			0.76
1b	2.33 (± 0.06) *0.58*	3.22 (± 0.45) *0.83*			2.33 (±0.06) *0.58*	3.22 (± 0.45) *0.81*	1.56
1c	1.0 (± 0.07) *0.25*	0.35 (± 0.08) *0.09*			1.0 (± 0.07) *0.25*	0.35 (± 0.08) *0.09*	0.25
1d	3.26 (± 0.13) *0.65*	2.02 (± 0.32) *0.40*	1.98 (± 0.08) *0.66*	1.61 (± 0.29) *0.57*	1.34 (± 0.09) *0.65*	0.51 (± 0.13) *0.25*	1.19
1e	3.02 (± 0.19) *0.60*	6.09 (± 1.71) *1.22*	1.82 (± 0.08) *0.61*	4.04 (± 0.89) *1.34*	1.11 (± 0.33) *0.55*	2.19 (± 0.108) *1.1*	2.25
1f	2.45 (± 0.06) *0.61*	4.46 (± 0.57) *1.12*	2.38 (± 0.06) *0.79*	3.97 (± 0.49) *1.32*	0.061 (± 0.01) *0.061*	0.058 (± 0.01) *0.058*	1.81
1g	1.65 (± 0.04) *0.33*	4.96 (± 0.82) *0.99*	1.11 (± 0.03) *0.37*	6.38 (± 0.153) *2.13*	0.57 (± 0.02) *0.28*	0.54 (± 0.06) *0.27*	1.92
1h	1.83 (± 0.04) *0.61*	2.17 (± 0.20) *0.72*	1.83 (± 0.04) *0.61*	2.18 (± 0.20) *0.73*	0	0	1.53

* Italicized values are corrected for number of metabolizable α-hydrogen atoms.

N,N-diethylbenzamides, no intermediate can be detected for the N-dealkylation process, whereas N-demethylation always produces an HPLC-detectable intermediate that behaves like an *N*-hydroxymethyl compound (e.g it decomposes in NaOH to form the *N*-alkylamide and has an HPLC retention time shorter than the *N*-alkylamide) [2]. Plots of V_i versus [substrate] for total dealkylation yield the values for V_{max} and V_{max}/K_m shown in Table 2. Since both dealkylation and demethylation can be quantified independently by means of the *N*-alkyl- and *N*-methylamides so produced, V_{max} and V_{max}/K_m values can be obtained for both processes, and these data are also contained in Table 2.

As well as *N*-methyl- and *N*-cyclopropylmethylbenzamides, upon metabolism compound **1g** gives rise to two aldehydes: formaldehyde, from loss of the methyl group; and cyclopropanecarboxaldehyde, from loss of the cyclopropylmethyl group. More significantly, within the errors of the analytical methods employed, the amounts of cyclopropanecarboxyaldehyde formed correspond to the amounts of *N*-methylbenzamide formed (Table 3). Thus, ring opening of the cyclopropyl group in **1g** is not observed.

Metabolism of compound **1h** gives rise to *N*-cyclopropylbenzamide (via metabolism of the methyl group) as the *only* monoalkylamide product. No *N*-methylbenzamide can be detected. However, concurrent hydrolysis of the amide bond is apparent by the formation of benzoic acid. Metabolism of **1h** by microsomes treated with CO (to inhibit cytochrome P450) yields benzoic acid with rates similar to those of the non-CO-treated microsomes, but formation of *N*-cyclopropylbenzamide is completely inhibited. Thus, amide hydrolysis is not associated with a cytochrome P450-catalysed process but probably arises from the presence of a microsomal amidase. However, this amidase must have a high substrate specificity, since we have not observed benzoic acid formation from any other substrate studied so far.

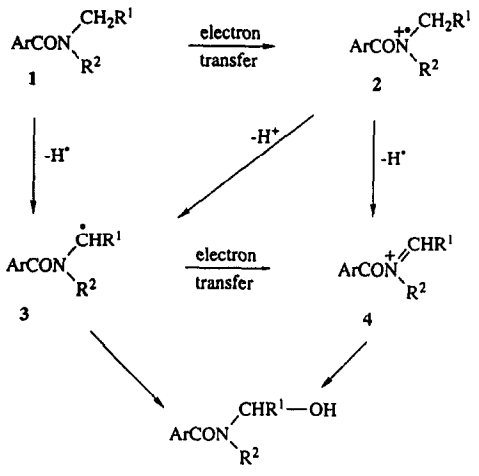
Semi-empirical AM1 self consistent field molecular orbital calculations were performed to further aid our understanding of the potential metabolic processes (Scheme). Values for the heats of formation, ΔH_f, for *E*- and *Z*-rotamers of the amides **1a, d–f**, together with $\Delta\Delta H_f$ values for the corresponding radical cations **2**, radicals **3** and iminium ions **4**, where $\Delta\Delta H_f = \Delta H_f$ (**2**, **3** or **4**)$-\Delta H_f$ (**1**), are contained in Table 4. Since the data relate to the gas phase, values of ΔH_f for charged species are much larger than for neutral species. Therefore absolute comparisons should not be made, rather the trends in ΔH_f for similar species should be inspected.

DISCUSSION

Both V_{max} and V_{max}/K_m for the dealkylation process appear to vary from compound to compound (Table 1). However, on correction for the number

Table 3. V_i values determined from amide and aldehyde analyses for the metabolism of compound **1g**

	$10^3 V_i$/mol/dm³/hr			
[Substrate]/mmol/dm³	PhCONHCH₂CHCH₂CH₂	HCHO	PhCONHMe	CH₂CH₂CH—CHO
1	1.13	0.86	0.15	0.16
5	0.95	0.90	0.21	0.18



Scheme. Pathways for the oxidative dealkylation of *N,N*-dialkylamides.

of α-H atoms capable of being metabolized (e.g. six in **1a**, four in **1b** and **1c**) it appears that there is less variation in V_{max} than there is in V_{max}/K_m. Indeed, V_{max} is largely substrate independent, with a value of *ca.* 0.6×10^{-3} mol/dm^3/hr/hydrogen atom. In contrast, V_{max}/K_m is dependent upon the lipophilicity of the substrate and there is a direct correlation between log V_{max}/K_m and log P, where P is the partition coefficient of the substrate between octanol and aqueous phosphate buffer (Fig. 1). Such a correlation is entirely consistent with the substrates having to enter a lipid membrane to reach the enzyme active site.

Several further observations arise from the data in Table 1 which are worthy of note. First, for compounds **1d** and **1e** with linear alkyl substituents, the maximum rates for the metabolism of the methyl and alkyl groups *per metabolizable hydrogen atom*, V^H_{max}, are essentially identical and, moreover, the same as those obtained for compounds **1a** and **1b**. Thus, there is no observed preference, other than statistical, for the loss of a methyl rather than an alkyl group. Second, for the compounds studied the

Table 4. Heats of formation, ΔH_f, and differences in heats of formation, $\Delta\Delta H_f$, calculated by the AM1 method for the amides **1a**, **d–f** and the corresponding species **2–4**

Amide	ΔH_f (kJ/mol)	IP (eV)	Radical cation **2** $\Delta\Delta H_f$ (kJ/mol)	Radical **3** $\Delta\Delta H_f$ (kJ/mol)		Iminium ion **4** $\Delta\Delta H_f$ (kJ/mol)	
				Methyl loss	Alkyl loss	Methyl loss	Alkyl loss
PhC(=O)N(Me)Me	−21.7	9.55	806.7	102.8 (*Z*)* 98.2 (*E*)		776.4 (*Z*)* 776.0 (*E*)	
PhC(=O)N(Me)Et	−45.6	9.57	797.3	102.0	81.1	766.0	741.3 (*E*)† 742.2 (*Z*)
PhC(=O)N(Et)Et	−46.4	9.56	801.3	97.8	92.0	769.5	739.8 (*E*)† 743.0 (*Z*)
PhC(=O)N(Me)Bu	−103.7	9.55	795.9	102.8	83.6	765.0	737.7
PhC(=O)N(Bu)Bu	−100.7	9.50	795.4	97.8	87.4	764.3	732.0 (*E*)† 737.1 (*Z*)
PhC(=O)N(Me)Pr^i	−58.9	9.54	792.1	102.4	79.4	750.3	714.1
PhC(=O)N(Pr^i)Pr^i	−55.2	9.54	792.1	97.4	73.6	755.8	710.3

* Stereochemical relationship between the iminium moiety or carbon-centred radical and the carbonyl oxygen.
† Streochemistry of the double bond of the iminium moiety.

maximum rate for demethylation, $^{Me}V^{H}_{max}$, is largely constant and independent of the structure of the other *N*-alkyl group. In contrast, the maximum rate of dealkylation, $^{R}V^{H}_{max}$, is very sensitive to the nature of the alkyl groups, the branched chain substrates all exhibiting reduced rates of metabolism especially when the branching occurs at the α-carbon. There is clearly some steric inhibition of metabolism in these cases. Third, the sub-maximal rates of dimethylation, $^{Me}V^{H}_{max}/K_m$, appear to depend upon the lipophilic nature of the other *N*-alkyl group, as defined by either log *P* or π (Fig. 2), whereas the sub-maximal rates of dealkylation, $^{R}V^{H}_{max}/K_m$, have no clear dependence upon lipophilicity or steric factors. Fourth, the sub-maximal rates for demethylation are larger than the corresponding rates for dealkylation.

Semi-empirical MO calculations were undertaken in an attempt to explain these trends. The data in Table 4 predict that (i) the ionization potential of the parent amide is independent of the alkyl group, but that formation of the cation radical **2** is more favoured for alkyl groups with the greater electron donating power, (ii) no matter whether reaction proceeds via the carbon-centred radical **3** or the iminium ion **4**, metabolism of the alkyl group is favoured (lower $\Delta\Delta H_f$ values) over metabolism of the methyl group, (iii) iminium ion formation from the methyl group is favoured by the electron-donating effect of the alkyl group (there being no preference between the *E*- and *Z*- forms, or, at most, a slight preference for the methylene group *Z*- to the carbonyl oxygen atom), and iminium ion formation from the alkyl group also is favoured by the electron-donating ability of its component substituents, and (iv) carbon-centred radical formation from either the *E*- or *Z*- methyl group is independent of the nature of the alkyl group (but radical formation from the *E*- methyl group is preferred), whereas radical formation from the alkyl group does depend upon the structure of the alkyl group.

These theoretical observations, in particular the predicted preference for loss of the alkyl, rather than the methyl, group, demonstrate that the microsomal dealkylation reaction must be under kinetic, rather than thermodynamic, control. Indeed, on thermodynamic grounds the *N*-isopropyl group of **1f** should be metabolized most readily, yet it has one of the lowest rates of dealkylation. Metabolism of the methyl group in compound **1f** proceeds normally, indicating that the substrate is reaching the active site. In fact, using the biomimetic system employed previously [1], we observe that the ratio for oxidative dealkylation of compound **1f** is Pr^i/Me = 1.25 (± 0.2). The conclusion must be either that the substrate binds to the active site in such a way that there is steric inhibition to loss of the α-hydrogen, or that the alkyl group sits in a hydrophobic pocket of the enzyme that protects it from dealkylation. This latter seems unlikely since other groups do undergo metabolism at much higher rates.

The data in Tables 2 and 4 show that the independence of $^{Me}V^{H}_{max}$ upon the alkyl group parallels the independence upon the alkyl group of $\Delta\Delta H_f$ for the formation of the radical **4** from the corresponding methyl group. We can infer from this that the reaction most likely proceeds via formation of a carbon-centred radical **3** rather than the iminium ion **4** or radical cation **2**. We have previously arrived at this conclusion from kinetic deuterium isotope effects and the lack of an effect of the substituents in the aryl ring on the rate of dealkylation [2]. In an

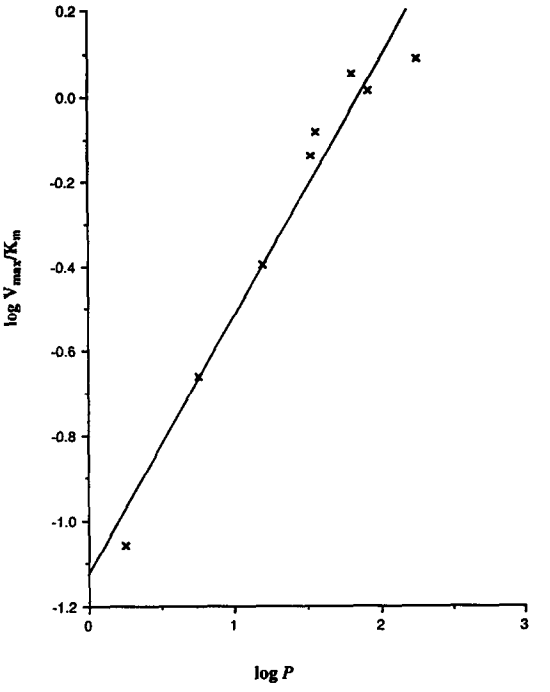


Fig. 1. Plot of log V_{max}/K_m versus log *P* for the metabolism of compounds **1a–h**.

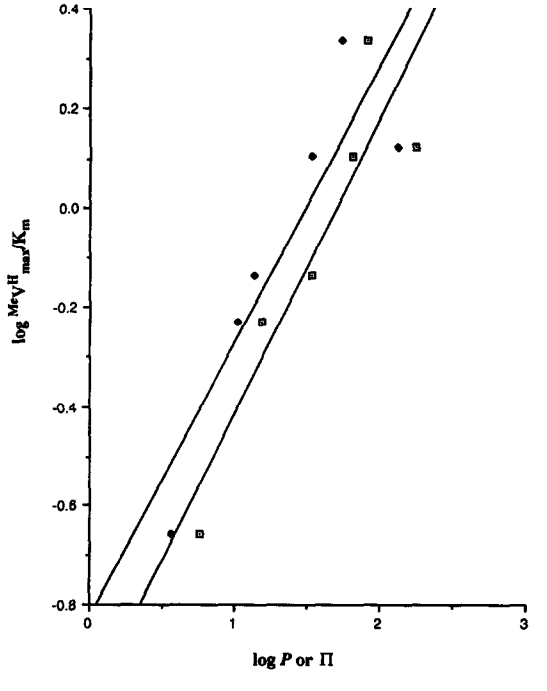


Fig. 2. Plot of log $^{Me}V_{max}/K_m$ versus log *P* (⊡) or π (◆).

attempt to more clearly identify the possible intermediacy of the radical cation **2** and carbon-centred radical **3** we investigated the metabolism of compounds **1g** and **1h**. Compound **1g** was studied because the cyclopropyl ring adjacent to a methylene group can potentially intercept the radical **3** [14], by opening of the cyclopropyl ring and diverting the reaction into other products. Compound **1h**, which bears a cyclopropyl ring adjacent to the amide *N*-atom, can potentially intercept the cation radical **2**, again by cyclopropyl ring opening. This is a strategy that has been adopted to intercept radical cations in the oxidative dealkylation of tertiary amines by cytochrome P450 [15, 16]. Moreover, formation of a carbon-centred radical at the α-carbon of the cyclopropyl ring is expected to be an unlikely process [17]. In the event, compound **1g** undergoes metabolism of the cyclopropylmethyl group without any detectable ring opening (Table 3). However, this observation does not rule out the radical **3** as an intermediate, because it may be that such a radical is trapped by the haem-hydroxyl moiety of the enzyme faster than it can rearrange. This situation has certainly been observed for saturated hydrocarbon substrates [18]. Moreover, the data in Table 3 reveal compound **1g** to have a V_{max} value much smaller than the other compounds studied. One explanation for this might be that, since cytochrome P450 levels are much smaller than substrate concentrations, a small amount of ring opening, which would escape detection by the analytical method employed, results in an inhibition of cytochrome P450. Further work is clearly required to clarify this point.

The cytochrome P450-dependent metabolism of compound **1h** suggests that the carbon-centred radical **3** rather than the radical cation **2** is involved as an intermediate in the metabolism of amides. If the radical cation were involved it is unlikely that *intermolecular* processes such as proton or hydrogen-atom loss, which have second-order rate constants of *ca.* 10^5–10^9 dm^3/mol/sec, [19, 20] would compete with *intramolecular* ring opening of the cyclopropyl group, which has a first-order rate constant of 10^8/sec [14], unless the effective molarity of the haem–hydroxyl complex is greater than 10^8. However, we see no metabolism other than loss of the methyl group, and this proceeds at rates that differ little from other *N*-methylamides. In the biomimetic system, the ratio of oxidative dealkylation for cyclopropyl versus methyl loss for compound **1h** is 0.65. The exclusive metabolism of the methyl group can be ascribed both to steric inhibition, as observed for the Pr^i group, and to the greater relative ease of formation of an α-carbon radical from a methyl group than from a cyclopropyl group [16]. Work is currently in hand to probe more fully the involvement of intermediates **2**, **3** and **4** in the oxidative dealkylation of amides.

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