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PRODUCT INHIBITION DURING THE HEPATIC MICROSOMAL *N*-DEMETHYLATION OF AMINOPYRINE IN THE RAT*

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Abstract—The main metabolites formed during the aminopyrine (DMAP) *N*-demethylation reaction, mediated by cytochrome P-450, are 4-monomethylaminoantipyrine (MMAP) and 4-aminoantipyrine (AAP). MMAP and AAP may bind to cytochrome P-450. Using hexobarbital (3 mM), a relatively pure type I compound, as a modifier, we were able to distinguish between the type I and the type II binding components of DMAP and its metabolites with cytochrome P-450. The affinity for type I (substrate) and type II (ligand) binding correlates positively with the lipophilicity and the sterical accessibility of the nitrogen atom containing non bonded electrons of the compounds, respectively.

The DMAP type I binding sites can only partially be occupied by MMAP or AAP. Moreover, our spectral results indicate that the interaction between DMAP and MMAP for their common binding sites is competitive. The *in vitro* *N*-demethylation of [dimethyl-^{14}C]aminoantipyrine, assessed by measuring $H^{14}CHO$ (K_m = 0.38 mM) is also competitively inhibited by MMAP (K_i = 0.85 mM) and AAP (K_i = 2.43 mM). MMAP will accumulate, relative to AAP, in the incubate. From *in vivo* data in the literature, it is evident both in the human and the rat that MMAP may reach concentrations equal to, or higher than, the DMAP plasma concentrations. The data in this report suggest that product inhibition of DMAP *N*-demethylation by MMAP is likely to occur—both *in vitro* and *in vivo*.

Using rat hepatic microsomes, aminopyrine (DMAP) is mainly metabolized by two successive oxidative *N*-demethylation reactions [1] to produce 4-monomethylaminoantipyrine (MMAP) and 4-aminoantipyrine (AAP) respectively.

In addition, formaldehyde is formed (Fig. 1). The rapidity of the reaction and the ease with which formaldehyde can be measured [2, 3] has led to the extensive use of aminopyrine as a model substrate to determine cytochrome P-450 dependent *N*-demethylase activity in the liver, and also other tissues, e.g. the brain [4] or the lung [5]. Although estimation of aminopyrine *N*-demethylation is apparently easy, several analytical difficulties [6] as well as biochemical problems [7] may arise. This paper will deal with some of these biochemical complications.

If aminopyrine and its metabolites bind at the same sites on cytochrome P-450, they will mutually inhibit the metabolism each of the other. In this way the overall velocity of the *N*-demethylation reaction of DMAP may be a complex of forward and inhibitory reactions.

MATERIALS AND METHODS

Chemicals. Aminopyrine was purchased from Brocacef, Rotterdam; 4-aminoantipyrine from E. Merck, Amsterdam; *dl*-sodium-hexobarbital from Bayer (Federal Republic Germany); dimethyl^{14}C-aminoantipyrine (specific activity 9.6 mCi/mmole, radiochemical purity 97%) and [^{14}C]formaldehyde (specific activity 4.4 mCi/mmole, radiochemical purity 99%) from the Radiochemical Centre, Amersham (Great Britain); $NADP^+$, glucose-6-phosphate (disodium salt) and glucose-6-phosphate dehydrogenase were obtained from Boehringer (Mannheim GmbH, Mannheim, Federal Republic Germany). Bovine serum albumin from Poviet Producten N.V., Amsterdam (The Netherlands). Monomethyl-aminoantipyrine was a gift from Hoechst Pharma, Amsterdam (The Netherlands). All other chemicals and solvents used were of analytical grade purity.

Preparation of microsomes. Liver microsomes from at least six rats were prepared as described previously [8], pooled and stored at −70° after quick-freezing in liquid nitrogen.

Spectral measurements. Difference spectra were recorded at 37°, using an Aminco DW 2 UV-Vis spectrophotometer in the split beam mode. Substrates, dissolved in water, were added as indicated in the text. The concentration of cytochrome P-450 was estimated according to Estabrook *et al.* [9], by means of a sodium dithionite difference spectrum. Mean changes in absorbance were expressed per nmole cytochrome P-450 per ml from at least three experiments, unless otherwise stated in the text. The microsomal preparations contained approximately 3 nmoles cytochrome P-450/ml.

Enzyme assays. Microsomes (2 mg protein/ml incubate) were incubated at 37°, with shaking, air being freely admitted. The incubate contained 0.5 mM $NADP^+$, 4.2 mM $MgCl_2$, 4.2 mM glucose-6-phosphate, 0.3 I.U./ml incubate glucose-6-phosphate dehydrogenase and substrate.

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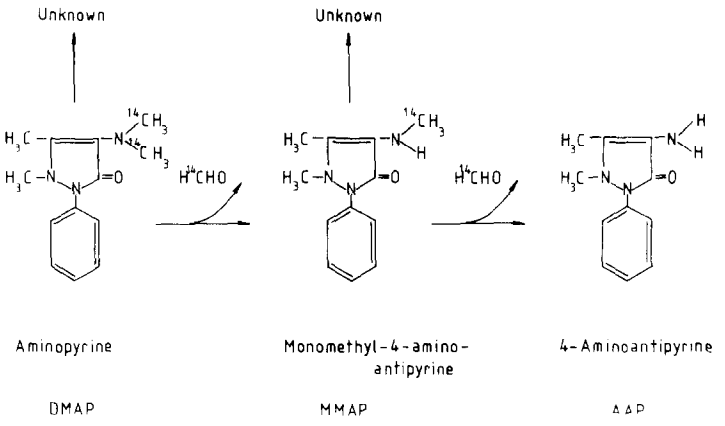

Fig. 1. The microsomal *N*-demethylation reaction of [dimethyl-^{14}C]aminoantipyrine (DMAP). During two successive oxidative *N*-demethylation reactions, 4-monomethylaminoantipyrine (MMAP) and 4-aminoantipyrine (AAP) are formed and formaldehyde is liberated. Other reactions with DMAP or MMAP as substrate may play a role as shown in [6] and are indicated by "unknown".

Radiometric assays. The assay was carried out in a total volume of 0.5 ml, as described by Poland *et al.* [3], with some minor modifications. The reaction was terminated after 5 min incubation by adding 0.5 ml semicarbazide (20 mM), 0.5 ml 0.2 N NaOH and 4 ml cold $CHCl_3$. After mixing and centrifugation, 1.2 ml and subsequently 0.8 ml of the aqueous layer were again extracted with 4 ml $CHCl_3$.

A 0.5 ml sample of the aqueous phase of the third extraction was then added to 10 ml Dioxane scintillation fluid (Packard-Becker B.V., Groningen; The Netherlands) and counted in a Packard TriCarb Scintillation counter model 3255. Far quenching was corrected by means of an external standard method.

High performance liquid chromatographic assay. The incubation was performed in a total volume of 3 ml and stopped with 0.1 N HCl—bringing the mixture to pH 6.0 [10]—and 15 ml $CHCl_3$. After mixing and centrifugation, 12 ml of the $CHCl_3$ layer were evaporated under reduced pressure at 35°. The residue was redissolved in 1 ml methanol. An aliquot (25 μl) was chromatographed on a Lichrosorp 5 RP 8 (150 × 4.6 mm) column (Chrompack, The Netherlands) using a Hewlett–Packard 1084 B liquid chromatograph equipped with a fixed wavelength U.V. (254 nm) detector. The elution system consisted of methanol (35% v/v) and 0.05 M phosphate buffer pH 6.5 (65% v/v). The flow rate was 1 ml/min and the oven temperature was set at 40°.

Protein assay. Microsomal protein was assayed according to the method of Lowry *et al.* [11], using crystalline bovine serum as a standard.

RESULTS

It is known that substrates may interact with cytochrome P-450 via a mixed type of binding [12]. Using the relatively pure type I compound hexobarbital as a modifier (3 mM), we were able to block the type I binding sites more or less selectively. In this way it is possible to separately determine type I and type II binding [8, 12, 13] of DMAP and MMAP. This is illustrated for MMAP in Fig. 2. The MMAP difference spectrum is shown to contain both a type I and a type II component.

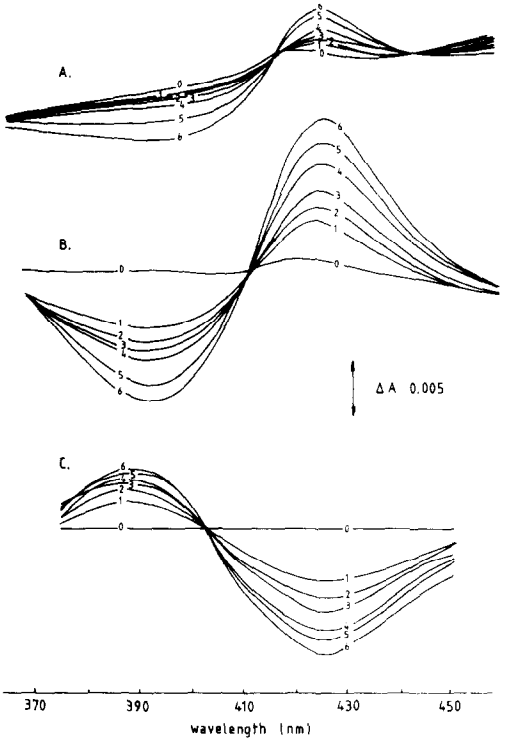

Fig. 2. (A) The observed difference spectrum of 4-monomethylaminoantipyrine (MMAP). After establishing the base line (0), the contents of the sample cuvette were titrated with MMAP (1–6 are respectively 0.33, 0.67, 1.0, 1.33, 2.0 and 3.0 mM) and the spectra recorded. (B) The type II component of the MMAP binding to cytochrome P-450. Both the sample and the reference cuvettes contained 3 mM hexobarbital and the contents of the sample cuvette alone were titrated with MMAP. Concentrations of MMAP as indicated in Fig. 2A. (C) The type I component of the MMAP binding to cytochrome P-450, obtained by subtracting the type II component (Fig. 2B) from the observed difference spectrum (Fig. 2A). Concentrations of MMAP as indicated in Fig. 2A.

Table 1. Binding of aminopyrine and its metabolites to cytochrome P-450

	Type I		Type II	
	K_s (mM)	ΔA_{max}/nmole P-450/ml*	K_s (mM)	ΔA_{max}/nmole P-450/ml†
Aminopyrine	0.17	0.0084	7.79‡	0.0120
	0.92	0.0167		
Monomethyl-4-aminoantipyrine	1.26	0.0064	1.49	0.0096
4-Aminoantipyrine	—	—	0.48	0.0060

Results were obtained by the method of Lineweaver–Burk in the concentration range 0.3 mM–3.0 mM (‡in the range 0.3 mM–7.0 mM).

* Difference in absorbance was determined between 385 nm and 420 nm (Type I) and † between 392 nm and 427 nm (Type II).

By plotting the reciprocal of the peak-to-trough absorbance versus the substrate concentration for each binding site, the maximal absorbance (ΔA_{max}/nmole P-450/ml) and the spectral dissociation constant (K_s) are obtained [14]. From the non-linear Lineweaver–Burk plot for DMAP type I binding to cytochrome P-450, two binding affinities were apparent (Table 1). In the concentration range studied (0.3 mM–3.0 mM), no type I binding component for AAP could be detected (Table 1).

The observed absorbance ($\Delta A_{385-420\,nm}$/nmole P-450/ml) originating from the DMAP–cytochrome P-450 interaction cannot be reduced to zero by increasing concentrations of MMAP or AAP (Fig. 3). This indicates that only a fraction of the DMAP binding loci can be occupied by its metabolites. The initial increase in type I absorption of DMAP by AAP (Fig. 4b) most likely originates from inhibition of type II binding of DMAP by AAP. It is only visible at high concentrations of DMAP, which is in accord with the K_s value for DMAP type II binding as mentioned in Table 1.

In order to further examine the mutual effects of DMAP and MMAP on their binding to cytochrome P-450, the contents of the sample cuvette were titrated with DMAP while MMAP was present at a fixed concentration in both sample and reference cuvettes. The reciprocal plots obtained, indicative of a spectral interaction between DMAP and MMAP with cytochrome P-450, cannot be interpreted in the classical way. This is because the difference spectrum which is actually measured is not the spectral change elicited by DMAP. The spectral contribution of MMAP in the reference cuvette is greater than the magnitude of the spectral change elicited by MMAP in the sample cuvette owing to the simultaneous presence of DMAP in the latter. As a consequence, the spectral change of MMAP in the reference cuvette does not simply cancel the spectral perturbation by MMAP in the sample cuvette.

A K_i can still be calculated according to the method of van den Berg *et al.* [13], if the same number of binding sites are occupied by DMAP and MMAP. But as shown in Fig. 3, this condition is not fulfilled.

Recently, however, we described a method as an expansion of the work of van den Berg *et al.* [13] in which a partial overlap in binding of two substrates to cytochrome P-450 is allowed [8]. Using this method, it is necessary to characterize the pool of cytochrome P-450 subforms binding only DMAP and not MMAP. This is possible with the data from Fig. 3. By subtraction of the spectral magnitude of this pool from the observed data, according to the formulae described [8], Fig. 4 can be transformed to the graphical result shown in Fig. 5. The data in Fig. 5 refer to the pool of cytochrome P-450 subforms

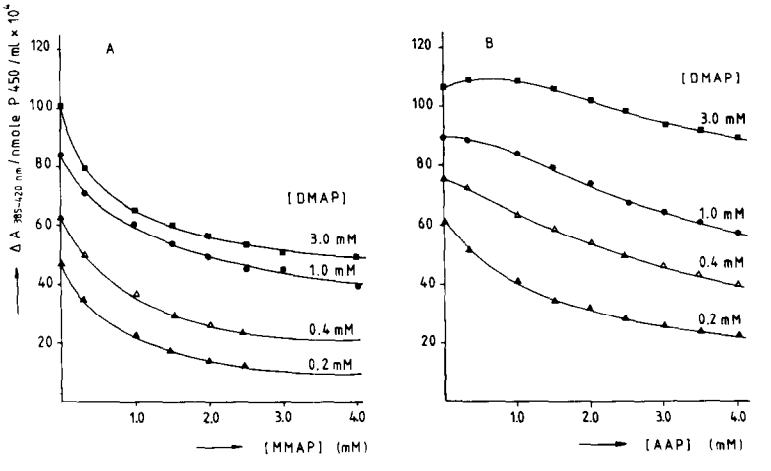

Fig. 3. (A) Influence of MMAP on DMAP binding to cytochrome P-450. The sample cuvette contained a fixed concentration of DMAP and the contents of both sample and reference cuvettes were titrated with MMAP. The magnitude of the difference spectrum ($\Delta A_{385-420\,nm}$/nmole P-450/ml) is plotted against the MMAP concentration. (B) Data as in Fig. 3(A) except that instead of MMAP, AAP was used.

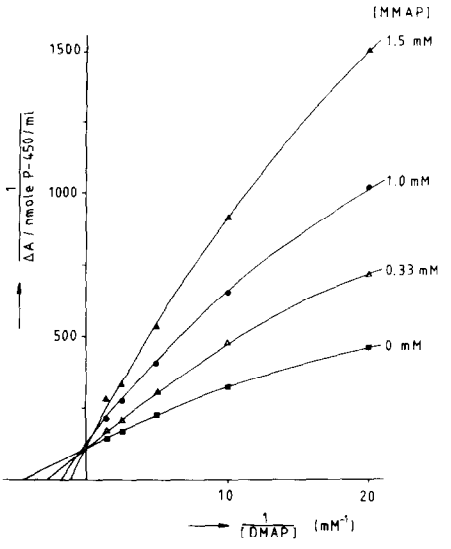

Fig. 4. Effect of MMAP upon the observed difference spectrum ($\Delta A_{385-420\,nm}$/nmole P-450/ml) elicited by DMAP. The sample and the reference cuvettes contained a fixed concentration of MMAP and the contents of the sample cuvette alone were titrated with DMAP. The inverse of $\Delta A_{385-420\,nm}$/nmole P-450/ml was plotted against the inverse of the DMAP concentration.

which DMAP and MMAP have in common. A K_s of 0.07 mM for DMAP can be determined. For MMAP a K_i of 1.57 mM (inset Fig. 5) can be calculated by plotting the inverse of the apparent ΔA_{max}/nmole P-450/ml versus the MMAP concentration [8, 13]. This is permissible because the same number of binding sites for DMAP and MMAP are now involved.

The *in vitro* *N*-demethylation of [^{14}C]-DMAP can be estimated by measuring the H^{14}CHO formation. It appears that the *N*-demethylation (K_m = 0.38 mM) is competitively inhibited by MMAP (K_i = 0.85 mM) and AAP (K_i = 2.43 mM) (Fig. 6). The K_i values were obtained by plotting the slope of the Lineweaver–Burk plot versus the inhibitor concentration.

Figure 7 shows the time–course of the formation of MMAP and AAP during the DMAP *N*-demethylation in hepatic rat microsomes, using two DMAP concentrations. It is obvious that MMAP reaches a much higher level than AAP. The MMAP:AAP ratio becomes greater with an increase in DMAP concentration.

DISCUSSION

The first step in oxidative drug metabolism mediated by cytochrome P-450 is binding of the substrate to the ferric form of cytochrome P-450 [14]. Interference with this binding may inhibit the metabolism of a substrate [15]. Cytochrome P-450 exists in multiple forms and may bind substrates in a rather broad and overlapping manner [16]. In addition to this, hydroxylated or demethylated substrates (products) resemble, as far as their molecular structure is concerned, their precursors. Consequently, binding of substrates and metabolites, on similar binding loci is very likely to occur. This would imply that metabolites are able to produce product inhibition.

If DMAP and MMAP are metabolized via the same cytochrome P-450 subforms they will interfere with the metabolism of each other. Even the formation of the endproduct AAP may have an influence on the DMAP *N*-demethylation. To check this interesting possibility—from both a theoretical and a practical point of view—the binding of aminopyrine and its main metabolites on cytochrome P-450 was characterized (Table 1). The lipophilicity of the compounds shown in Table 1, as determined by Noda *et al.* [17], is positively correlated with the affinity ($1/K_s$) for type I binding. Such a correlation has also been shown for other series of substrates [18–20]. The affinity for type II binding follows the order

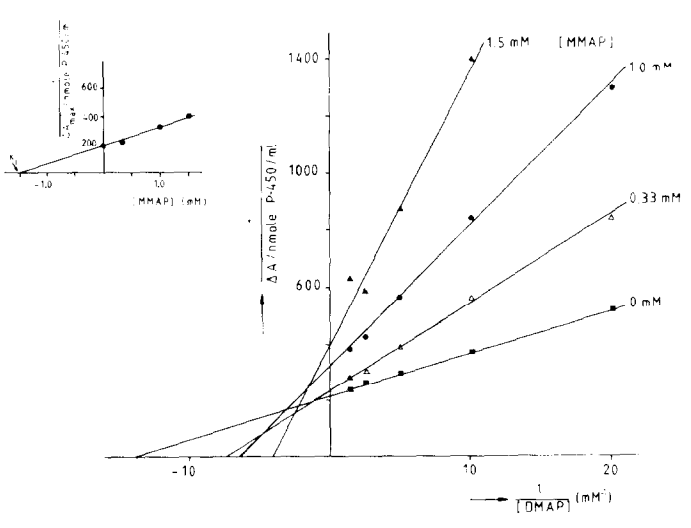

Fig. 5. Effect of MMAP upon the difference spectrum ($\Delta A_{385-420\,nm}$/nmole P-450/ml) elicited by DMAP, by competition for common type I binding sites of DMAP and MMAP. The calculation to obtain these plots is described in the text and the plot is the same as in Fig. 4. *Inset:* Plot of the inverse of the apparent ΔA_{max}/nmole P-450/ml versus the MMAP (inhibitor) concentration in order to obtain a K_i concerning the common binding sites on cytochrome P-450 of DMAP and MMAP.

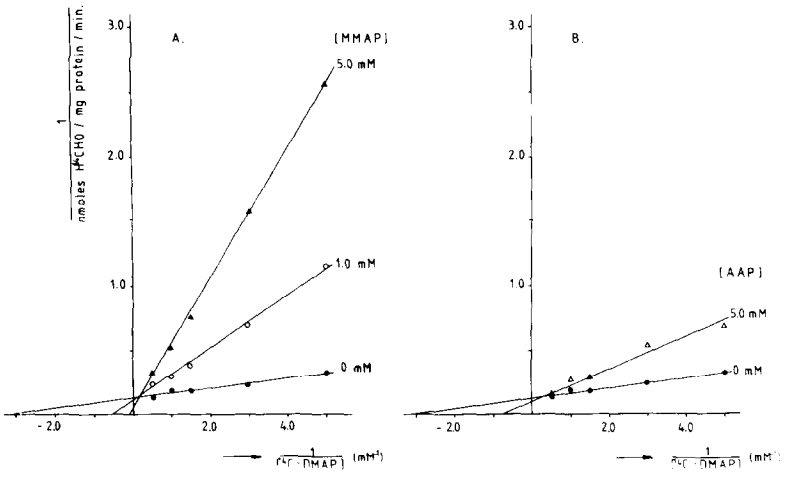

Fig. 6. (A) Lineweaver–Burk plots showing competitive inhibition of DMAP-*N*-demethylase activity ($H^{14}CHO$ formation/mg protein/min) by MMAP. (B) Data obtained as in 6(A) except AAP was used instead of MMAP.

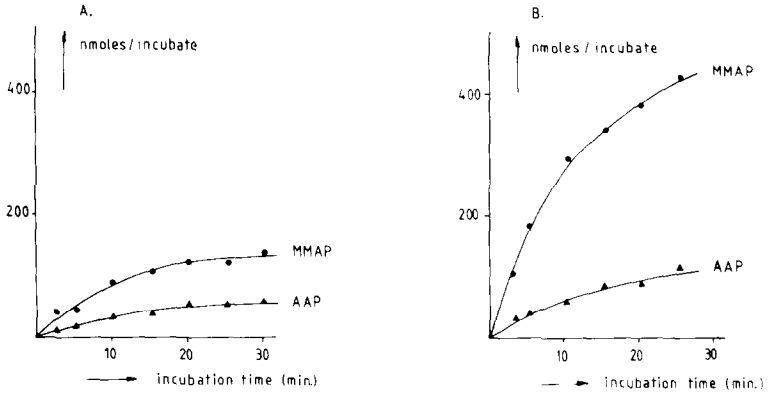

Fig. 7. (A) Time–course of the formation of MMAP and AAP during the metabolism of 0.2 mM DMAP incubated with rat liver microsomes (6 mg protein/3 ml). The data points are the means of two experiments. (B) As in Fig. 7(A) except 3.33 mM DMAP was used.

AAP > MMAP > DMAP and is probably dependent upon the accessibility of the nitrogen atom, which has a pair of non-bonded electrons [21]. The biphasic properties of the interaction of DMAP with type I binding sites on cytochrome P-450 has been attributed to cytochrome P-450 which may form two types of enzyme substrate complexes, containing one (P-450–S) or two (P-450–S_2) substrate molecules [22]. In these studies, however, the type II binding component was not taken into account.

Alternatively, binding to different cytochrome P-450 forms may be responsible for this phenomenon.

Furthermore, it is noticeable from Table 1 that ΔA_{max}/nmole P-450/ml for DMAP is the greatest for both type I and type II binding and decreases in the order DMAP > MMAP > AAP. The ΔA_{max} is probably proportional to the maximal number of binding sites occupied by the substrate. Consequently, it follows from Table 1 that neither MMAP nor AAP can displace DMAP completely from all of its binding sites.

This conclusion is supported by the finding that the spectral change caused by DMAP cannot be fully reduced to zero by MMAP or AAP (Fig. 3). To determine whether the interaction between DMAP and MMAP for the same cytochrome P-450 binding sites is a competitive one, the formulae as described previously [8] were applied. The result is shown in Fig. 5, in which it can be seen that the apparent K_s as well as the apparent ΔA_{max} depend on the concentration of the inhibitor (MMAP). This is different from normal competitive inhibition kinetics but is indeed the theoretical pattern for competitive spectral interaction with cytochrome P-450 [8, 13] if the same binding sites for the substrate and the inhibitor are involved.

The *in vitro* microsomal *N*-demethylation of [^{14}C]-DMAP, measured by $H^{14}CHO$ formation, is also competitively inhibited by MMAP and by AAP (Fig. 6). The K_i values for MMAP and AAP are comparable with the affinity constants these compounds exhibit for type I binding (Table 1).

From progress curves of two concentrations of DMAP it is evident that MMAP may accumulate relative to AAP in the incubate (Fig. 7).

The extent of the accumulation of MMAP, and thereby the concentration of MMAP versus DMAP, depends upon the separate velocities of the two *N*-demethylation reactions. In the literature there is confusion about this. Gram *et al.* [1] stated that the demethylation of DMAP is much faster than that of MMAP, while La Du *et al.* [23] stated that MMAP was demethylated four times faster than DMAP. These contradictory results may be caused firstly by analytical pitfalls in the formaldehyde determination [6] and secondly by the complex mutual regulation of the overall velocity by inhibitory and forward reactions of DMAP and MMAP, also with respect to unknown reactions. Variables such as substrate concentration and incubation time may play a crucial role.

AAP probably has little effect on demethylation, based on both spectral data (Table 1), the high K_i (Fig. 6) and the relatively low concentration in the incubate (Fig. 7). The third reason for the paradoxical data in the literature may be that the DMAP *N*-demethylation may have a bi- or even triphasic character [3, 24–29]. The finding that at least two cytochrome P-450 forms are involved in the binding of DMAP supports this idea.

However, non-linear Lineweaver–Burk plots have to be interpreted with care because of the above mentioned analytical [6] and biochemical problems.

Despite these difficulties, we have cogently shown that product inhibition during DMAP *N*-demethylation *in vitro* is possible. *In vivo*, MMAP may reach concentrations equal to or higher than that of the DMAP plasma concentration, both in rats and humans [10, 30]. Therefore, *in vivo* product inhibition may also take place.

This phenomenon may be a general one. Indeed, product inhibition of diazepam metabolism by desmethyldiazepam has been suggested [31]. Inhibition of the metabolism of their precursor by hydroxylated metabolites of desmethylimipramine, diphenylhydantoin, or phenylbutazone has also been observed or suggested by circumstantial evidence [32–34]. As lipophilicity is an important determinant for the affinity of substrates for cytochrome P-450, this type of inhibition is indeed likely to occur for strongly lipophilic compounds, as mentioned above.

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