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THE METABOLIC FATE OF PARGYLINE IN RAT LIVER MICROSOMES

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Abstract—The availability of a sensitive analytical assay for the simultaneous quantitation of pargyline (PARG) and four of its major metabolites have made possible a detailed study on the metabolism of the drug in rat liver microsomes with emphasis put on comparisons between optional *N*-dealkylation reactions and *N*-oxide formation. Pargyline is a lipophilic amine with a low pK_a-value of 6.6 and undergoes extensive metabolism. The conversion of the substrate is rapid and comprizes three *N*-dealkylation and one *N*-oxidation reactions, yielding *N*-benzylpropargylamine (BPA), *N*-methylpropargylamine (MPA), *N*-benzylmethylamine (BMA) and pargyline *N*-oxide (PNO), respectively. Phenobarbital (PB) pretreatment of the rats causes a pronounced increase in the metabolism with about 90% of the substrate being consumed within the first minute of incubation at 100 μM substrate concentration. At this substrate concentration the most pronounced induction is seen in the formation of BPA and also in its further metabolism, while levels of BMA and MPA remain fairly constant. Pargyline *N*-oxide is the most abundant metabolite in microsomes from untreated rats and its formation is not increased by PB induction. Moreover, the inhibition of PNO formation by typical cytochrome P-450 inhibitors is marginal, while that of BPA, BMA and MPA formation is not. *N*-Debenzylation, yielding MPA, is the least important of the *N*-dealkylation reactions and the effect of PB induction on this reaction becomes noticeable only at high substrate concentrations. The studies suggest that various cytochrome P-450 enzymes are involved in the *N*-dealkylation reactions of PARG while *N*-oxidation appears to occur mainly by a cytochrome P-450-independent pathway. As propiolaldehyde, a potential hepatotoxin, is formed concomitant to BMA, and as PNO, under certain conditions, can decompose to acrolein, another well-known hepatotoxin, both these quantitatively important metabolic routes have to be considered in evaluating the toxicity of pargyline.

The irreversible monoamine oxidase inhibitor pargyline although, due to undesirable side effects [1], no longer used clinically, is a useful tool in experimental pharmacology [2]. The compound also proves a suitable model substrate for investigations of metabolic functionalization reactions occurring at or around a nitrogen atom. Providing the analytical methodology is available, with this substrate one would be able to investigate simultaneously the relative importance of three competing *N*-dealkylation reactions as well as *N*-oxide formation (Fig. 1).

In the course of studies on metabolic functionalization of α-acetylenic amines [3–7], the biotransformation of pargyline has been investigated and we have previously reported [8] *N*-demethylation and *N*-oxidation to be important metabolic routes in liver microsomes. These studies, however, gave limited information due mainly to analytical limitations. The development of a sensitive analytical assay [9] for simultaneous quantation of pargyline (PARG) and its four primary metabolites [*N*-benzylpropargylamine (BPA), *N*-methylpropargylamine (MPA), *N*-benzylmethylamine (BMA) and pargyline *N*-oxide (PNO)] has now made possible more detailed studies on the metabolism of pargyline. The assay [9] is based on a two-phase derivatization of the various amines with isobutyl chloroformate and subsequent gas chromatographic (GC) analysis. Using this technique we now report results from a more comprehensive study on the metabolism of PARG in which emphasis is put on comparisons between the various *N*-dealkylation reactions and *N*-oxide formation (cf. Fig. 1). The effects of some enzyme inducers as well as enzyme inhibitors on the reactions are also reported. The inducers used were phenobarbital (PB) and β-naphthoflavone; the inhibitors, carbon monoxide, sodium azide, metyrapone, 2,4-dichloro-6-phenylphenoxyethylamine (DPEA) and *N*(1-benzyl-2-methylpropyl)hydroxylamine.

MATERIALS AND METHODS

Instrumentation. A Bergman and Beving PHM62 standard pH meter was used for pH measurements and a Beckman model 25 spectrophotometer was used in the protein and cytochrome P-450 assays.

Gas chromatographic analysis was performed on a Hewlett–Packard 5730A gas chromatograph with thermoionic detector and equipped with a 1.2 m × 2 mm i.d. glass column with a packing of 10% SP-1000 on supelcoport 80/100 mesh. The column was operated at 130 and 190° with a carrier gas flow of 30 ml/min (8% H_2 in He).

Linear regression analysis was done with a Hewlett–Packard model HP-9810A calculator.

Chemicals. *N*-Benzylmethylamine, *N*-methylbutylamine, isobutylchloroformate and sodium phenobarbital were purchased from Fluka AG

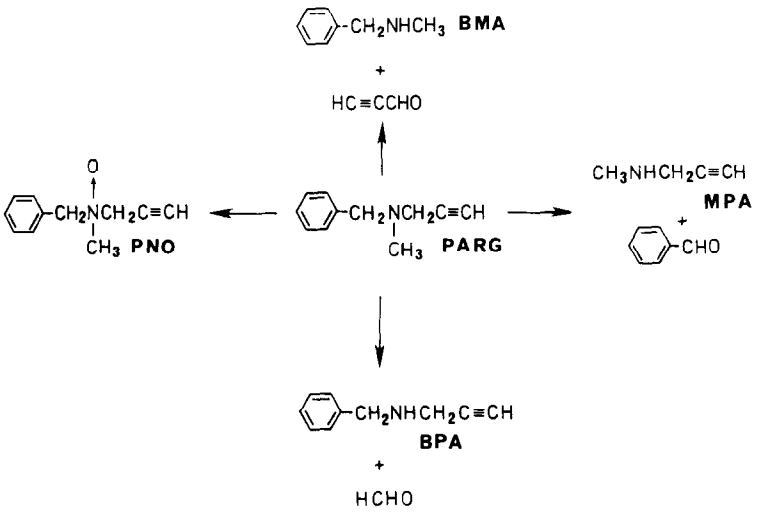

Fig. 1. Primary metabolic pathways of pargyline (PARG) in rat liver microsomes, yielding *N*-benzylpropargylamine (BPA), *N*-benzylmethylamine (BMA), *N*-methylpropargylamine (MPA) and pargyline *N*-oxide (PNO).

Buchs, Switzerland, and metyrapone from Ciba AG, Basel, Switzerland. *N*-Methylamphetamine was supplied by ACO Läkemedel, Solna, Sweden. 2,4-Dichloro-6-phenylphenoxyethylamine was a gift of the late Dr. R. McMahon, Eli Lilly, Indianapolis, IN, U.S.A.

Pargyline [10], BPA [10], MPA [11], PNO [8] and *N*(1-benzyl-2-methylpropyl)hydroxylamine [12] were synthesized according to the cited published methods.

NADP, glucose-6-phosphate, glucose-6-phosphate dehydrogenase, serum albumin and β-naphthoflavone were obtained from Sigma Chemical Co., St. Louis, MO, U.S.A.

The O_2–N_2 (4:96) and CO–O_2–N_2 (40:4:56) gas mixtures were supplied by the AGA Corporation, Lidingö, Sweden.

Other chemicals were obtained from local commercial sources and were, when needed, of analytical grade quality. The water used was doubly distilled.

Metabolic studies. Microsomes were prepared as previously described [13] from livers of male Spraque–Dawley rats (200–250 g).

Induced enzyme activities were obtained by pretreatment of the rats for four days with either sodium phenobarbital in saline (3 × 80 mg/kg + 1 × 40 mg/kg i.p.) or β-naphthoflavone in corn oil (3 × 70 mg/kg + 1 × 35 mg/kg i.p.) before sacrifice.

Microsomal protein [14] and cytochrome P-450 content [15] were determined by the standard procedures at cited.

Standard incubations were carried out in 25 ml Erlenmeyer flasks at 37°. Microsomal suspension equivalent to 0.6 g wet liver (13.0 ± 1.3 mg protein for induced microsomes and 10.0 ± 0.7 mg for control microsomes) was incubated under air in a total volume of 5 ml of 0.15 M potassium phosphate buffer (pH 7.5) containing 1.15% KCl, 1.5 μmoles of glucose-6-phosphate, 20 μmoles of $MgCl_2$, 5 I.U. of glucose 6-phosphate dehydrogenase, PARG and other compounds as stated. The incubations were started by addition of the microsomes.

When gas mixtures were used, reactions were run in stoppered flasks flushed with the gas mixture. In the latter cases, the liver preparations and the cofactors were allowed to equilibrate with the gas for 7 min after which the incubation was started by the addition of substrate. Gas flow was continued during the incubation.

When the cytochrome P-450 metabolic intermediate complex-forming *N*-hydroxylamine [*N*(1-benzyl-2-methylpropyl)hydroxylamine] was included in the studies, this hydroxylamine was preincubated for 5 min before the addition of pargyline.

Extraction and analysis. The incubations were terminated by transferring the incubation mixtures to centrifuge tubes containing 0.5 ml of a 20% zinc sulphate solution. After centrifugation at 3000 rpm for 4 min, the supernatant was transferred to another centrifuge tube. The precipitated protein was washed with 2 ml 1.15% KCl and the pH of the combined supernatant and washing solution was adjusted to 6. The solutions were then processed through the analytical assay as previously described [9] with all the amine metabolites being analysed as isobutyl chloroformate derivatives. In this assay PARG and MPA form the same isobutyl chloroformates and are thus quantitatively separated by liquid–liquid distribution before derivatization. Also, PNO is reduced to PARG after separation from the substrate.

In one set of experiments *N*-demethylation of PARG was monitored by formadehyde production, measured by the method of Nash [16].

Stability of pargyline N-oxide (PNO) in buffer solution of pH 7.5. Potassium phosphate buffer solutions (0.5 mM, pH 7.5) containing synthetic PNO (50 μM) was kept at 37° for up to 24 hr. Samples (5 ml) were taken at various times and extracted with dichloromethane (5 ml) containing 1% isobutyl

chloroformate and *N*-methyl-*N*(1-phenyl-2-propyl) carbamate as an internal standard. The *N*-oxide was then analysed as the *N*-benzyl-*N*-methylcarbamate as described in the analytical assay [9].

RESULTS

The oxidative functionalization of PARG is associated with the microsomal fraction of the liver, requires oxygen and is NADPH-dependent. No metabolites were found in derivatized extracts of the soluble fraction or when cofactors were omitted. The overall metabolism of PARG is rapid. At an initial concentration of 100 μM most of the substrate is consumed within the first 5 min in microsomes from untreated rats (Fig. 2A) and, if microsomes from PB-pretreated rats are used (Fig. 2B), essentially all the substrate is consumed within the first few minutes.

In liver microsomes from untreated rats *N*-oxidation (PNO) was the most important reaction and at an initial substrate concentration of 100 μM, corresponded to about 30% of consumed substrate within the first minutes of incubation (Fig. 2A). The *N*-depropargylation (BMA) amounted to about 19% followed by *N*-demethylation (BPA, 12%) and *N*-debenzylation (MPA, 3.5%).

Phenobarbital pretreatment of the animals resulted in a marked increase in BPA, BMA and MPA levels, but did not effect the *N*-oxidation (Fig. 2B). The effect is more notable at a higher (1 mM) less limiting substrate concentration (Fig. 3). Also, BPA was markedly further metabolized (Fig. 2B). Thus, when demethylation was monitored by formadehyde production, 1, 2 and 5 min values of 9.8, 12.8 and 15.2 nmoles/mg protein were obtained, which are to be compared with the BPA levels depicted in Fig. 2B.

β-Naphthoflavone pretreatment (data not shown) did not effect either the *N*-oxidation or any of the *N*-dealkylating reactions.

Carbon monoxide (Table 1) inhibited all the *N*-dealkylating reactions but had no effect on the *N*-oxidation in microsomes from untreated rats. The most pronounced effect of carbon monoxide was seen on the *N*-demethylation (BPA). When using microsomes from PB-pretreated rats, carbon monoxide had an inhibitory effect also on the *N*-oxidation, but the effect was marginal.

The inhibitory effects of 25 μM DPEA and 500 μM N-(1-benzyl-2-methylpropyl)hydroxylamine on the metabolism of PARG is shown in Fig. 3. It should be noted that DPEA was added as a competing substrate while the *N*-hydroxylamine was preincubated for 5 min before the addition of pargyline. 2,4-dichloro-6-phenylphenoxyethylamine was an effective inhibitor of the *N*-depropargylation (BMA) and *N*-debenzylation (MPA) but not of the *N*-demethylation (BPA) at a PARG concentration of 1 mM. However, at a substrate concentration of 100 μM (data not shown), DPEA also inhibited *N*-demethylation (BPA). The *N*-oxidation (PNO) was

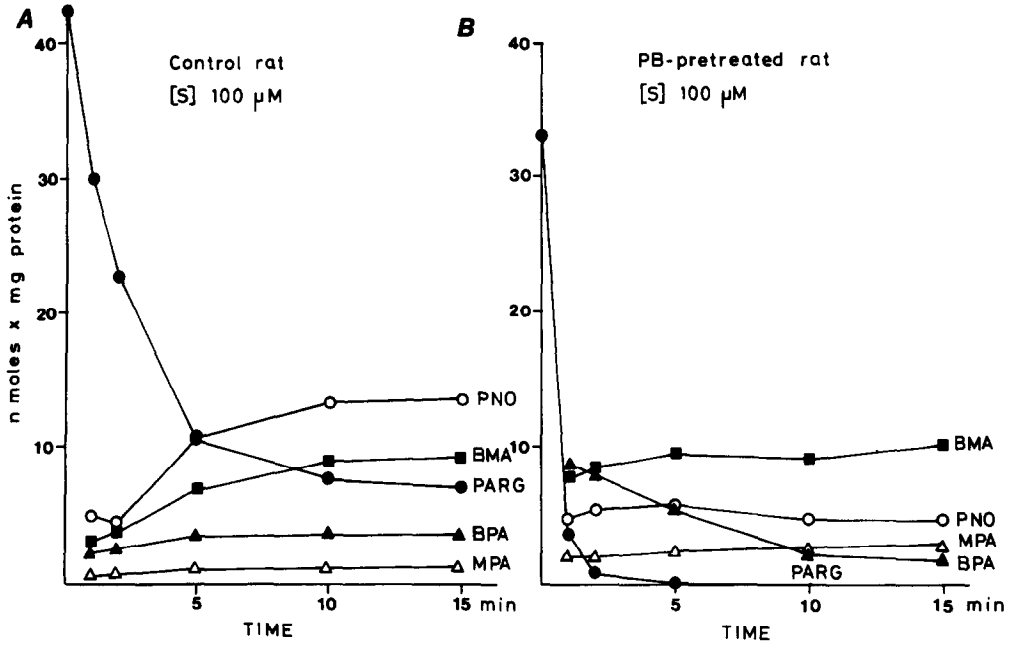

Fig. 2. Levels of PARG (●——●), BPA (▲——▲), BMA (■——■), MPA (△——△) and PNO (○——○) as a function of time in liver microsomes from untreated (A) and phenobarbital pretreated (B) rats. The initial substrate concentration was 100 μM (approx 32 nmoles/mg protein). The data points represent the means of at least six experiments comprising three different microsomal preparations which could be reproduced within ±10%. The material balance after 1 min shows that the five metabolites analysed account for (A) 69 and (B) 65%, respectively, of consumed substrate. The greater discrepancies between formed metabolites and consumed substrate seen at later time points are mainly because of further metabolism, especially of BPA and BMA, undergoing *N*-depropargylation, *p*-hydroxylation (BPA) and *N*-demethylation (BMA) [9, 21].

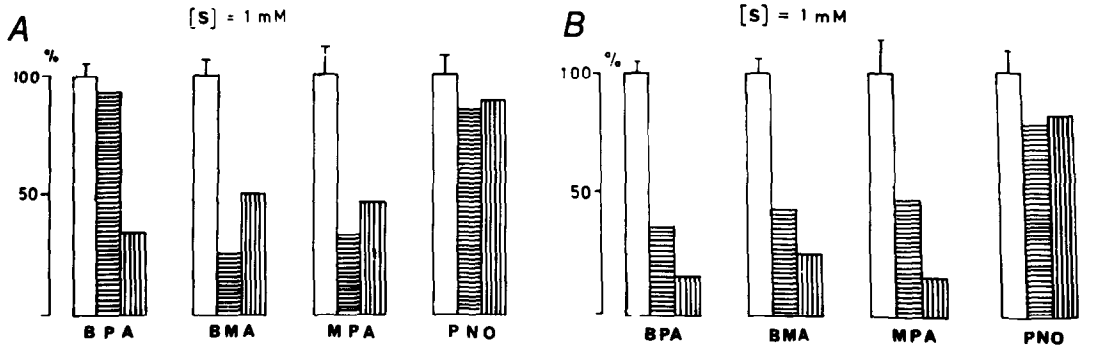

Fig. 3. Effects of 25 μM DPEA (▤) and 500 μM *N*-(1-benzyl-2-methylpropyl)hydroxylamine (▥) on the formation of pargyline metabolites in liver microsomes from untreated (A) and phenobarbital-prepreated (B) rats. The bars included in the controls denote the S.E.M. values and represent the means of at least six experiments comprising three different microsomal preparations. Levels of metabolites in the controls were BPA 11, BMA 16, MPA 7.6 and PNO 19 nmoles/mg protein and 5 min for microsomes from untreated rats and BPA 14, BMA 14, MPA 11 and PNO 7.3 nmoles/mg protein and min for microsomes from PB-pretreated rats. For further experimental details see Material and Methods.

only marginally affected by DPEA in microsomes from untreated rats. In general DPEA exhibited greater inhibitory effects in microsomes from PB-pretreated than from untreated rats. The inhibitory effects seen with 100 μM metyrapone (data not shown) were essentially the same as those seen with DPEA.

Preincubation with the metabolic intermediary complex-forming *N*-hydroxylamine [12] resulted in an inhibition of metabolism. The inhibitory effect of the hydroxylamine was most pronounced using microsomes from PB-pretreated rats with none of the *N*-dealkylating reactions exceeding 25% of the controls. The *N*-oxidation (PNO) was, dependent on the substrate concentration, inhibited to 20–50%. The presence of 500 μM azide (data not shown) had a positive effect on the formation of all metabolites and was most pronounced when microsomes from untreated rats were used, where the increase in the production of the various metabolites ranged from 34–100%.

Attempts to determine kinetic parameters by applying Michaelis–Menten kinetics to the various metabolic reactions were, except in one case—the formation of BMA—less successful. Poor reproducibility was obtained and the results suggested that kinetically different enzymes were operating simultaneously. One contributing factor is also the rapid metabolism of PARG implicating that the kinetics are not linear even for 1 min at lower substrate concentrations. Lineweaver–Burke plots with acceptable correlations were, however, obtained for the formation of BMA in the substrate range 25 μM–1 mM. The $K_{m(app)}$ (and $V_{max(obs)}$) values for the formation of BMA in microsomes from untreated and PB-pretreated rats are given in Fig. 4.

Pargyline *N*-oxide is chemically unstable and in certain aprotic solvents is prone to undergo what is known as the Meisenheimer rearrangement [8, 17]. However, from the stability studies performed in the present investigation, PNO appeared to be stable in aqueous solution of neutral pH, with less than 5% decomposition occurring in 24 hr. This is probably due to the fact that hydration of the *N*-oxide will hamper intramolecular rearrangements.

DISCUSSION

Pargyline is a drug which undergoes very rapid metabolism in microsomal preparations. Other examples of such rapidly cleared drugs are the acetylenic amine, oxybutynine [6] and lidocaine [18]. All three drugs are tertiary amines characterized by low pK_a-values—those of PARG [8] and oxybutynine [5] both being about 6.5, that of lidocaine being somewhat higher, 7.8 [19]. Thus at pH 7.5

Table 1. Effect of carbon monoxide on the formation of pargyline metabolites in liver microsomes*

Substrate concentration (1 mM)	BPA†	BMA†	MPA†	PNO†
Control	26 ± 6	54 ± 5	37 ± 10	112 ± 8
PB-pretreated	27 ± 6	43 ± 7	36 ± 7.5	75 ± 2.4

* Abbreviations: BPA, *N*-benzylpropargylamine; BMA, *N*-benzylmethylamine; MPA, *N*-methylpropargyline; PNO, pargyline-*N*-oxide; PB, phentobarbital.

† Mean values ± S.E.M. of at least three experiments expressed in per cent of the amount of metabolite formed in the presence of carbon monoxide (CO–O_2–N_2, 40:4:56) as compared to a reference gas (O_2–N_2, 4:96). The incubation time was 10 min.

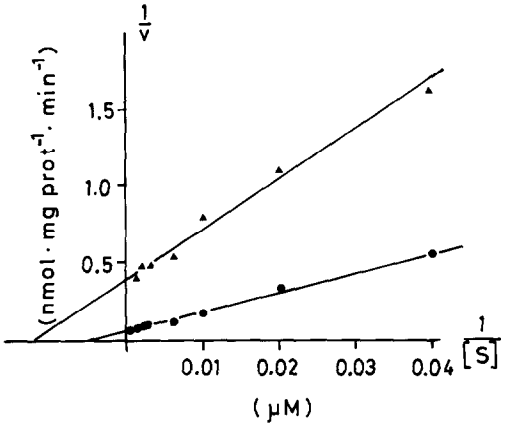

Fig. 4. Rate of formation of BMA as a function of substrate concentration in liver microsomes from untreated (▲) and phenobarbital pretreated (●) rats, displayed as Lineweaver–Burke plots. Each line represents the mean values of three different experiments. The derived kinetics constants are $K_{m(app)} = 59 \pm 3$ μM, $V_{max(obs)} = 2.0 \pm 0.1$ nmoles/mg protein and min and $K_{m(app)} = 188 \pm 23$ μM, $V_{max(obs)} = 17.4 \pm 1.5$ nmoles/protein and min for the control and induced preparation, respectively.

PARG exists predominantly as the free base. A good positive correlation with lipid solubility and negative correlation with base strength have been found for the metabolism of many amines by membrane-bound enzymes [20]. The rapid metabolism of PARG is thus consistent with the notion that lipid-soluble neutral forms of the amines are the species which undergo the biotransformation.

The metabolic transformations that pargyline can undergo comprise three optional *N*-dealkylating reactions, *N*-oxidation (Fig. 1) and conceivably also aromatic hydroxylation. Significant amounts of *p*-hydroxypargyline were, however, not found in the present study and it has been shown previously [21] that aromatic hydroxylation occurs secondary to *N*-demethylation. The three *N*-dealkylating reactions involve attack at different types of aliphatic carbons. Of these *N*-demethylation (BPA) is well known [20] as a most efficient metabolic transformation. During the formation of BMA and MPA, oxidative attack occurs at a carbon next to either a sp- or a sp^2-hybridized unsaturated carbon system, respectively. Both of these types of carbons are generally considered as being activated toward enzymatic hydroxylation [22]. To our knowledge there are no previous reports, comparing the relative efficacy of *N*-dealkylating reactions involving oxidations at such carbons.

In the case of pargyline PARG, *N*-depropargylation (BMA) and *N*-demethylation (BPA) are much more efficient than *N*-debenzylation (MPA) (Figs 2 and 3). The MPA levels equal those of BPA and BMA first at a substrate concentration of 1 mM and above, and appear to be catalysed by a different kinetic class of microsomal enzymes with a comparable low-substrate affinity.

At limiting substrate concentrations, BPA is further metabolized at a greater rate than it is being formed (Fig. 2B) in microsomes from PB-pretreated rats. A major secondary metabolic reaction of pargyline is *N*-depropargylation of BPA, yielding benzylamine [21], which although not quantitated has been identified as its isobutyl chloroformate derivative [9].

That DPEA was an effective inhibitor of the *N*-demethylation (BPA) at a PARG concentration of 100 μM but was essentially without effect at a concentration of 1 mM (Fig. 3A), shows that PARG efficiently competes with DPEA for the enzyme or binding sites generating BPA. Moreover, although BPA and BMA formation appear similar in many respects, the apparent differences in their response to the presence of DPEA, would indicate that they are catalysed by different cytochrome P-450 enzymes or that two catalytically different binding sites are involved. Similar conclusions can be drawn from the experiments including the metabolic intermediary complex-forming *N*-hydroxylamine. That the inhibitory effect of the *N*-hydroxylamine is directed towards PB-induced forms of cytochrome P-450 is consistent with the notion [23] that complex formation is most pronounced in these enzymes.

The results of the present study suggest that the major part of PNO formed in rat liver microsomes is produced by cytochrome P-450-independent pathways. An additional enzyme likely to be responsible for the *N*-oxidation is the microsomal NADPH-dependent amine oxidase originally described by Ziegler *et al.* [24, 25]. In a previous study [8] it was indicated that also cytochrome P-450 was of great significance in PNO formation, which is contradictory to the present results. One likely reason for the discrepancies could, however, be sought in the peroxidative function of cytochrome P-450 [26], by which metabolically produced hydrogen peroxide could promote the *N*-oxidation. Indeed, results from experiments in progress show that PARG undergoes hydrogen peroxide-dependent *N*-oxidation as well as *N*-dealkylation reactions.

There is a potential for the formation of toxic metabolites during the metabolism of PARG. In this respect both BMA and PNO formation must be considered. Thus propiolaldehyde [27] is formed concomitant to BMA (Fig. 1) and decomposition of PNO [8, 17] can under certain conditions generate acrolein [28]. The latter has, however, so far not been isolated during the metabolism of PARG [29], but if PNO, which due to hydration is stabilized in aqueous environment, undergoes a Meisenheimer type rearrangement [17] within the lipophilic membrane of the microsomes [30] has yet to be assessed.

Animal pretreatment and co-incubation with PARG has been reported to reduce rat hepatic microsomal ethylmorphine *N*-demethylase activity [31] as well as the ring hydroxylation of aniline [32], but the effects are moderate. Bélanger and Atitsé-Gbeassor [32] also investigated the binding of PARG to total oxidized microsomal cytochrome P-450 of rat liver and obtained a modified type II spectrum with a rather high K_S of 10 mM. Such a low affinity for the hemoproteins is not consistent with the results of the present study (e.g. Fig. 4) which show a very efficient metabolism of the drug. One would thus anticipate that the observed K_S reported by these authors is composed of multiple affinity constants

which also reflect the presence of high-affinity enzymes. They also stated that PARG is mainly ionized at neutral pH which is incorrect. Having a pK_a of 6.6 [8] the ratio of the free base of pargyline to its protonated form will be 7.9:1 at pH 7.5.

As an amplification of previous reports on PARG metabolism [8, 9, 21, 27, 31, 33–35] this study has shown that PARG is very rapidly metabolized in rat liver microsomes and comparisons between the various metabolic routes have shown that those leading to metabolites with toxic potentials are actually among the quantitatively most important. The study has also established that with this substrate, *N*-depropargylation (BMA) by far exceeds *N*-debenzylation (MPA) [22], which is consistent with the hypothesis [20] that when there are options in *N*-dealkylation the largest amine is preferentially formed.

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