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METABOLISM OF THE PHENOTHIAZINE DRUG PERAZINE BY LIVER AND LUNG MICROSOMES FROM VARIOUS SPECIES

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Abstract—The oxidative metabolism of perazine was studied *in vitro*, using solvent extraction and thin layer chromatography followed by spectrophotometry for determination of the metabolites. Liver microsomes from rats, rabbits, pigs, guinea-pigs and cats and lung microsomes from rats, rabbits and pigs served as the enzyme sources.

Kinetics of *N*-oxidation, *N*-demethylation, sulfoxidation and aromatic hydroxylation were measured with liver microsomes. Demethylation, sulfoxidation and aromatic hydroxylation underlie a substrate inhibition already at a perazine concentration of 1 mM, whereas *N*-oxidation usually is maximal with 2 mM perazine and starts to be inhibited at 4 mM perazine. In the concentration range tested (0·25–8 mM perazine) the *N*-oxide is always the major metabolite. Excessive *N*-oxidation has been observed in liver microsomes from individual pigs.

Lung microsomes form substantial quantities of perazine *N*-oxide only, while other metabolites are produced to a negligible extent. An extremely high capacity for perazine *N*-oxidation was observed with rabbit lung microsomes, whereas microsomal preparations from rat and pig lungs *N*-oxidize perazine at a slower rate.

OF THE series of tricyclic psychopharmacological agents, chlorpromazine and imipramine have been subjected to intensive studies of their metabolism by microsomal preparations from animal tissues.[1–6] A qualitatively similar metabolite pattern resulted as that observed in investigations of urinary excretion following application of these drugs (e.g. cf. Refs. 7–9).

In previous papers[10,11] we reported on the pattern of metabolites excreted by psychiatric patients treated with perazine (10-[3′-(4″-methyl-piperazinyl)-propyl]-phenothiazine). The present study is dealing with the *in vitro* metabolism of perazine. Liver microsomes from male rats were primarily used as the enzyme source in order to obtain a basis for investigations on the influence of inducing agents on the *in vitro* transformations of perazine.[12] The metabolites formed were the products of *N*-oxidation, *N*-demethylation, sulfoxidation and aromatic hydroxylation of the perazine molecule and thus corresponded to those found in human urine.[10] Since one-dimensional thin layer chromatography allows for separation and simultaneous determination of all four types of metabolic products, perazine seems to be a suitable model compound for following the various types of metabolic conversions on the same molecule. The study was therefore extended to liver microsomes from other species besides rats and in some cases also to lung microsomes.

Part of the results have been the subject of a preliminary report.[13]

MATERIALS AND METHODS

Chemicals. Organic solvents were of ordinary grade purity and were redistilled before use. NADP and isocitrate dehydrogenase were purchased from Boehringer u. Soehne, Mannheim, Germany, D,L-isocitrate, trisodium salt, from Serva, Heidelberg, Germany. Perazine and desmethyl perazine (DMP) as dimalonates were kindly supplied by Chemische Fabrik Promonta, Hamburg, Germany. Perazine sulfoxide (Per-SO), perazine *N*-oxide (Per-NO) and hydroxy-perazine (OH-Per) were compared with the metabolites isolated from human urine according to Breyer.[10]

Animals. Male Wistar rats, 200–300 g were used. They were fed a standard laboratory diet (Altromin R®) and tap water *ad lib.* until the time of sacrifice. Female rabbits (2–4 kg), male guinea-pigs (350–500 g) and male cats (1·3 and 3 kg) were of unspecified breed. Pig liver and lung were obtained as slaughterhouse material about 20–30 min after killing of adult female pigs.

Preparation of microsomes. After killing the laboratory animals by decapitation or exsanguination the livers and lungs were removed as quickly as possible and chilled in ice-cold 0·9% NaCl. After weighing they were cut and washed free from blood in 0·1 M sodium phosphate buffer pH 7·4. Specimens of tissue were homogenized with 4 vol. of buffer for 30 sec with an Ultra-Turrax (Janke & Kunkel, Staufen, Germany). The homogenates were centrifuged for 10 min at 100 *g* followed by 1 min at 1000 *g*. The supernatant was decanted and the mitochondrial fraction removed by centrifuging for 10 min at 5000 *g* and 10 min at 10,000 *g*. From the resulting supernatant the microsomes were sedimented in a Spinco model L ultracentrifuge for 60 min at 40,000 rev/min. The pellet was suspended in phosphate buffer and resedimented. The washed microsomes were homogenized with Krebs–Ringer solution: 0·1 M phosphate buffer (3 : 1, adjusted to pH 7·4) in a Potter–Elvehjem homogenizer and made up to a volume of approximately 1·5 ml/g of fresh liver or 0·3 ml/g of fresh lung, respectively. All operations were carried out at 0–4°.

Protein was measured according to Lowry *et al.*[14] using total serum protein as standard (Lab-Trol®, DADE Division, Miami, Florida, U.S.A.). Cytochrome P-450 determinations were carried out according to Omura and Sato.[15]

Incubations. Unless stated otherwise, incubations were for 10 min with shaking under air at 37° in centrifuge tubes with wide diameter. The incubation mixture contained per ml: 2 mg of microsomal protein, 1 μmole NADP, 8 μmoles D,L-isocitrate, 5 μmoles $MgCl_2$, 50 μg isocitrate dehydrogenase, 0·85 ml Krebs–Ringer solution: phosphate buffer (3 : 1) and varying amounts of perazine. The incubation volume was 2 ml. The reaction was started after a 5 min pre-incubation period by adding the substrate as a neutralized solution in 0·1 ml of water. Inhibitors were added in 0·1 ml of water immediately before the substrate. The reaction was stopped by the addition of 0·35 ml 25% NH_3 solution and 2 ml 1·2-dichloroethane and shaking. Control samples were run with microsomes inactivated by heating to 60° for 3 min.

Isolation and determination of metabolites. The incubation mixtures were extracted with successive portions of 2 ml, 1 ml and 1 ml dichloroethane, phases being separated by centrifugation. The organic layers were removed with a Pasteur pipette, combined and evaporated under reduced pressure. The residue was transferred quantitatively to a thin layer chromatographic plate by washing the glass three times with 0·12 ml chloroform–methanol (2 : 1, v/v) and pipetting the solution on a band of 3·5 cm width. As adsorbent freshly activated Kieselgel GF_{254} (E. Merck, Darmstadt, Germany) was

used in a thickness of 0·4 mm. The plate was run in isopropanol–chloroform–water–25% NH_3 (40 : 20 : 2·5 : 2, by vol.) to a height of 13–14 cm above the application line. After drying in a hood for 3 min, the plate was sprayed with water, and u.v.-absorbing bands were marked under 254 nm light. These were removed and extracted according to Breyer[10] using one 2-ml portion and two 1-ml portions of dichloroethane. The combined organic phases were taken to dryness in a rotary evaporator at 30° under reduced pressure.

Perazine, DMP, Per-NO and Per-SO were measured by dissolving the chromatographically isolated material in 4 ml 0·1 N HCl and reading the spectrum from 230 to 380 nm on a Zeiss PMQ II spectrophotometer. OH-Per was dissolved in 3 ml 0·1 N HCl and subjected to the coupling procedure described previously.[10] Calculations of absolute amounts were based on the absorption coefficients given by Breyer[10] and on the assumption that the molar absorption coefficients are equal in a series of closely related substances.[16] This assumption could be verified for perazine and its sulfoxide[10] and now for DMP, too.

All values were corrected for recoveries of the single metabolites. These were 60 ± 6 per cent for OH-Per, 92 ± 4 per cent for Per-SO, 91 ± 1 per cent for DMP and 79 ± 3 per cent for Per-NO (means ± standard deviations, n = 7–11). Perazine could be recovered to 92–95 per cent in four experiments.

RESULTS

Metabolite pattern

Thin layer chromatography of extracts from short term incubations of liver microsomes with perazine revealed hydroxy perazine, perazine sulfoxide, desmethyl perazine, perazine *N*-oxide and negligible amounts of further metabolites besides the starting material. R_f values of the major products in the solvent system used are given in Table 1. The substances recovered in these bands proved to be homogeneous upon rechromatography in other solvent systems.[10]

TABLE 1. R_f VALUES OF PERAZINE AND ITS MAIN METABOLITES IN ISOPROPANOL–CHLOROFORM–WATER–25% AMMONIA (40 : 20 : 2·5 : 2 by vol.) ON THIN LAYER PLATES OF KIESELGEL GF_{254}

Substance	R_f
Perazine	0·74
Hydroxy perazine (OH-Per)	0·60
Perazine sulfoxide (Per-SO)	0·45
Desmethyl perazine (DMP)	0·37
Perazine *N*-oxide (Per-NO)	0·25

In several experiments with 0·5 mM substrate the residual perazine was determined in addition to the four major metabolites. The sum of these five compounds was 93–99 per cent of the initial substrate added.

In incubations with heat-inactivated microsomes only traces of Per-SO were formed.

Time and cofactor dependence

As illustrated in Fig. 1, the *N*-oxidation of perazine by liver microsomes from male rats proceeds linearly over a time interval of at least 15 min, whereas the rate of the demethylation, sulfoxidation and aromatic hydroxylation decreases already after 5 min. However, in order to obtain a sufficient quantity of material for an exact determination of metabolites, incubations were usually carried out over 10 min. Only those samples with an initial perazine concentration of 0·25 mM were incubated for 5 min, since about 50 per cent of the substrate would have been used up within 10 min.

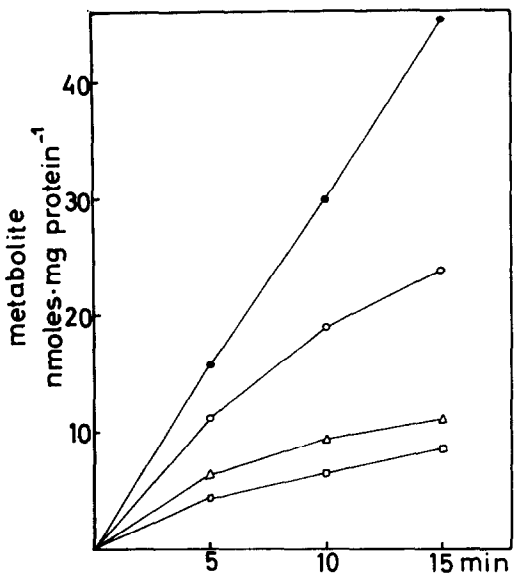

FIG. 1. Time course of the formation of perazine metabolites in rat liver microsomes (substrate concentration 0·5 mM). □——□ hydroxy perazine; ○——○ desmethyl perazine; △——△ perazine sulfoxide; ●——● perazine *N*-oxide.

The demethylation and *N*-oxidation were only slightly influenced by varying the magnesium concentration (Fig. 2). The conventionally used concentration of 6 mM Mg proved optimal for *N*-oxide formation, while a small increase in the DMP yield could be achieved by raising the Mg concentration to 11 or 21 mM.

Doubling of the NADPH-regenerating system did not accelerate DMP or Per-NO formation from perazine in an initial concentration between 0·5 and 8 mM.

Reduction of the O_2 pressure by factors of 2 and 4 led to a decrease in the *N*-oxidation rate only.

Dependence on substrate concentration

Substrate concentrations of 0·5 mM and higher caused clouding of the microsomal suspensions. This is not due to insolubility of the drug at pH 7·4, since perazine solutions can be adjusted to this pH value without precipitation of the free base. Instead the adsorption of the phenothiazine to the microsomes apparently leads to an aggregation of the particles.

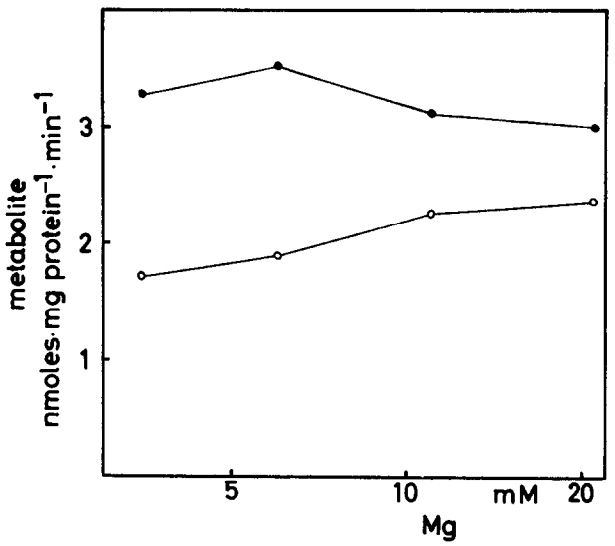

FIG. 2. Dependence of the demethylation (○——○) and the *N*-oxidation (●——●) of 0·5 mM perazine in rat liver microsomes on the Mg concentration of the incubation system.

Rat liver microsomes. *N*-Demethylation, sulfoxidation and aromatic hydroxylation proceeded fastest with the lowest substrate concentrations tested, i.e. 0·25 mM or 0·5 mM perazine, respectively (Fig. 3). With perazine concentrations of 1 mM and

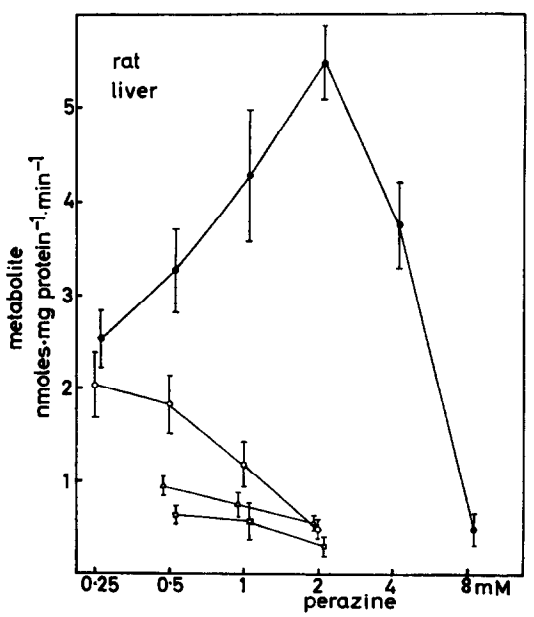

FIG. 3. Formation of perazine metabolites in rat liver microsomes as a function of the substrate concentration. Vertical bars represent standard deviations. □——□ hydroxy perazine; ○——○ desmethyl perazine; △——△ perazine sulfoxide; ●——● perazine *N*-oxide.

higher these three reactions are clearly substrate-inhibited, and above 2 mM perazine they become too slow for reliable determination of their products. In contrast, the *N*-oxidation reaches its maximal velocity with 2 mM and is still detectable with 8 mM perazine. Though the *N*-oxidation rate decreases considerably when substrate concentrations are reduced below 2 mM, this reaction is the fastest one even with 0·25 mM perazine. From the concentration dependence between 0·25 and 2 mM substrate, one can roughly estimate the apparent K_m value of the perazine *N*-oxidation to be around 0·5 mM.

Rabbit liver microsomes. As depicted in Fig. 4, *N*-demethylation and *N*-oxidation are less influenced by varying the substrate concentration than with rat liver microsomes, though the dependence is still recognizable. The *N*-oxidation proceeds faster than with microsomal preparations from the rat at all substrate levels tested.

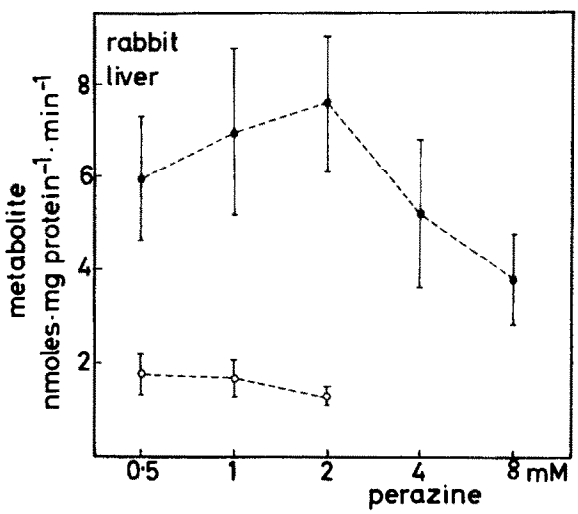

FIG. 4. Formation of desmethyl perazine (○----○) and perazine *N*-oxide (●----●) in rabbit liver microsomes as a function of the substrate concentration. Means ± standard deviations.

Pig liver microsomes (Fig. 5). These proved to possess a very low activity for demethylation. The *N*-oxidation rate varied considerably. In microsomal preparations from two of the four animals investigated it was higher than in liver microsomes from any other species. The most active sample exhibited a maximal *N*-oxidation rate with 4 mM perazine, whereas increasing the substrate concentration from 2 to 4 mM resulted in a slight decrease in reaction velocity in the remaining three preparations. Because of the wide individual variations in the amount of *N*-oxide formed, no mean values were calculated, but the concentration dependence of *N*-oxidation rate is illustrated in Fig. 5 for each animal separately.

Guinea-pig and cat liver microsomes. Preparations from guinea-pigs resembled those from rats in all respects. Cat liver exhibited very low activities in all reactions tested.

Table 2 summarizes the results concerning all four types of metabolites when incubations were carried out with 0·5 mM perazine. It appears from these results that sulfoxidation and hydroxylation are very slow reactions in liver microsomes from all species tested.

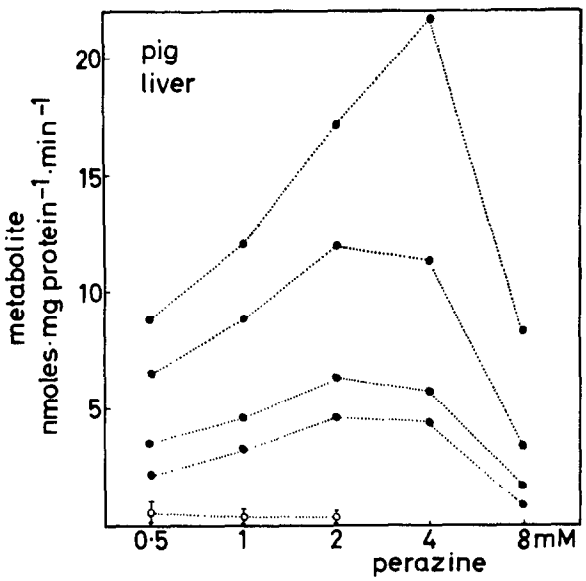

FIG. 5. Formation of desmethyl perazine (○.....○) and perazine *N*-oxide (●.....●) in pig liver microsomes as a function of the substrate concentration. For demethylation, means ± standard deviations are given for four animals, *N*-oxidation rates are depicted for each animal separately.

TABLE 2. CYTOCHROME P-450 CONCENTRATIONS AND FORMATION OF PERAZINE METABOLITES IN LIVER MICROSOMES FROM VARIOUS SPECIES (SUBSTRATE CONCENTRATION 0·5 mM, EXPERIMENTAL CONDITIONS AS DESCRIBED IN MATERIALS AND METHODS)

Species, sex	*n*	Cytochrome P-450 (nmoles.mg protein^{-1})	Perazine metabolites formed (nmoles.mg protein^{-1}.min^{-1}) OH-Per	Per-SO	DMP	Per-NO
Rat, male	22 × 2*	1·45 ± 0·17	0·64 ± 0·10	0·95 ± 0·11	1·8 ± 0·3	3·3 ± 0·4
Rabbit, female	4	1·93 ± 0·04	0·70 ± 0·17	1·12 ± 0·28	1·8 ± 0·4	6·0 ± 1·3
Pig, female	4	0·84 ± 0·10	0·5; 0·5; 0·8	0·3; 0·6	0·4 ± 0·1	see Fig. 6
Guinea-pig, male	4	1·64 ± 0·07	0·45 ± 0·24	0·52 ± 0·06	1·3 ± 0·2	3·1 ± 0·9
Cat, male	2	0·5; 0·22	<0·2	0·3	0·14; 0·06	0·35; 0·32

* For each experiment livers from two rats were pooled.

Mean values ± standard deviations.

Lung microsomes from rats, rabbits and pigs. The results are illustrated in Fig. 6. The only reaction proceeding at a measurable rate is the *N*-oxidation. A striking feature is the extremely high *N*-oxidizing capacity of rabbit lung microsomes which is not even reduced by perazine concentrations up to 8 mM. Per-NO formation by pig lung microsomes, too, is not effectively inhibited by increasing the substrate concentration. Lung microsomes from rats exhibit a lower activity in perazine *N*-oxidation than do liver microsomes; the substrate inhibition is similar with particles from both tissues.

DMP was detectable only after incubation of rabbit lung microsomes with 0·5 mM perazine. The demethylation rate never exceeded 0·2 nmole. mg protein^{-1}.min^{-1}.

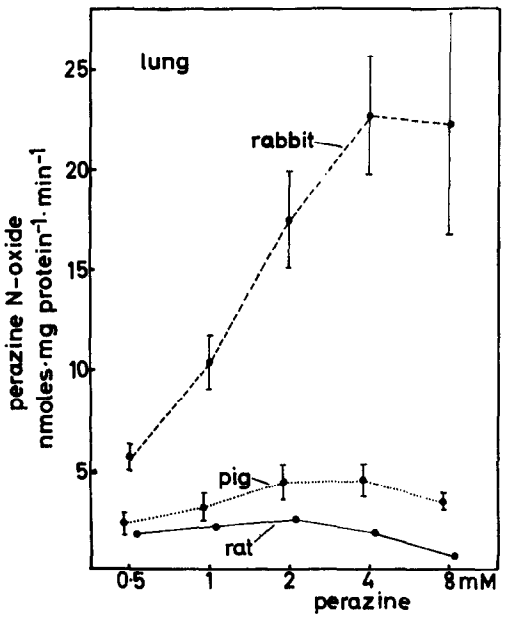

FIG. 6. Formation of perazine *N*-oxide in lung microsomes from rats (●——●), rabbits (●- - - -●) and pigs (●.....●) as a function of the substrate concentration. Vertical bars represent standard deviations.

Other tissues. Microsomes from rabbit renal cortex formed Per-NO from 0·5 mM perazine with an initial rate of 0·55 nmole. mg protein^{-1}. min^{-1}, which is about one tenth of the rate observed with rabbit liver microsomes. In contrast to lung microsomes, the kidney microsomal preparation exhibited a similar dependence of the *N*-oxidation rate on the substrate concentration as was observed with liver microsomes.

Rat brain microsomes did not form any perazine metabolites to a measurable extent.

Effect of inhibitors of drug metabolism

From Table 3 it is clear that inhibitors, which are assumed to affect cytochrome P-450 dependent drug oxidations, decrease the formation of OH-Per, Per-SO and DMP by rat liver microsomes, while the *N*-oxidation of perazine remains virtually unaltered.

DISCUSSION

The metabolite pattern found in the present experiments qualitatively corresponds to that observed in investigations on chlorpromazine[1–4] and imipramine.[5,6] The same types of reactions are inhibited by SKF 525-A using chlorpromazine[2] and perazine as substrates. Quantitatively, however, there are certain differences.

When comparing the results obtained with rat liver microsomal preparations, the high capacity for *N*-oxide formation from perazine seems remarkable. An important contribution from this metabolic pathway has also been found by Beckett and Hewick,[3]

TABLE 3. INFLUENCE OF SKF 525-A AND METYRAPONE ON THE FORMATION OF METABOLITES FROM PERAZINE (0·5 mM) BY RAT LIVER MICROSOMES

Inhibitor	Perazine metabolites formed (nmoles.mg protein^{-1}.min^{-1}) OH-Per	Per-SO	DMP	Per-NO
None	0·52	0·87	1·4	3·4
SKF 525-A, 0·1 mM	0·24	0·44	0·7	3·7
None	0·64	1·09	2·0	2·9
Metyrapone, 0·5 mM	0·44	0·70	1·0	3·0

Experimental conditions as described in Materials and Methods.

who reacted chlorpromazine with fortified rat liver microsomes, and by Nakazawa,[6] who incubated rat liver 9000 *g* supernatant with ^{14}C-imipramine. In contrast, Bickel and Baggiolini[5] described desmethyl imipramine to be the major metabolite formed from imipramine by rat liver microsomes, while the *N*-oxide was produced in far smaller amounts. Coccia and Westerfeld[2] in their study on chlorpromazine metabolism reported on analogous results. Besides, aromatic hydroxylation has been found to be a fast reaction with imipramine as the substrate.[5]

The relative contributions of the individual metabolic pathways are apparently highly dependent upon the experimental conditions, e.g. use of postmitochondrial supernatant or washed microsomes, various buffer systems, amounts of cofactors, incubation time and nature and concentration of the substrate.

In the studies on imipramine[5,6] and chlorpromazine[1,2,4] metabolism, incubations were carried out for 25–60 min (Beckett and Hewick[3] do not specify their incubation time). In the present study microsomes were incubated for 10 min only in order to obtain reaction rates close to initial reaction velocities. From the data depicted in Fig. 1 it can be seen that prolonged incubations would have favoured *N*-oxidation since this reaction shows closer linearity with time than the other transformations. Due to the short incubation time, secondary reaction products (e.g. desmethyl perazine sulfoxide or desmethyl hydroxy perazine[10]) did not occur in measurable amounts, whereas Forrest and Brookes[4] isolated considerable quantities of the analogous chlorpromazine metabolites after incubating for 45–60 min.

As already pointed out by Nakazawa,[6] the dependence of the metabolic rates on the magnesium concentration seems to be a function of the buffer system employed. With the one used here containing 22 mM phosphate in the final incubation mixture, no obvious changes in reaction velocities with Mg concentration could be observed, as have been described in studies on imipramine.[5,6]

A limitation of the reaction rate by the amount of the NADPH-regenerating system, as was found by Bickel and Baggiolini,[5] could not be demonstrated in this study, probably because of the shorter duration of incubations.

It was shown here that an important factor, which has not been intensively studied until now in investigations on tricyclic drug metabolism, is the substrate concentration.

Bickel and Baggiolini[5] reported on a decrease in the demethylation rate with imipramine concentrations above 0·4–0·6 mM (microsomal protein: 1 and 2 mg/ml) which is in good agreement with our data. Most of their experiments have been carried out

with 0·4 mM imipramine, and similar concentrations of chlorpromazine have been used by other investigators.[1–4] These substrate concentrations are near-optimal for demethylation, sulfoxidation and aromatic hydroxylation judging from the experimental evidence presented in Fig. 3, since these reactions are already subject to pronounced substrate inhibition at 1 mM perazine. In correspondence with this substrate inhibition, perazine[13] and imipramine[17] are also able to inhibit the microsomal hydroxylation of other drugs.

In contrast, the *N*-oxidation of perazine could be accelerated in all tissue preparations by increasing the substrate concentration to 2 mM and in some cases even further by using 4 mM perazine. The physiological significance of these results is questionable since comparable tissue levels will hardly be achieved *in vivo*. The finding of excessive *N*-oxidation of 2–4 mM perazine by rabbit lung microsomes in the absence of other metabolic transformations may, however, be of interest for production of this metabolite on a milligram scale, since chemical oxidation of perazine will result in a mixture of different *N*-oxides and *N*-oxide sulfoxides along with the sulfoxide.[10] Extrapolation of the curves shown in Fig. 3 to smaller substrate concentrations leads to the conclusion that at low perazine levels *N*-oxidation will become slower than demethylation and possibly even than sulfoxidation and aromatic hydroxylation. In addition, it must be kept in mind that a lower oxygen pressure as is present in liver tissue will cause lower *N*-oxidation rates compared to conditions of atmospheric oxygen pressure.

An inter-species comparison of *in vitro* metabolism of chlorpromazine has been carried out by Forrest and Brookes.[4] As far as the same species (cat, rabbit, guinea-pig) have been used, there are considerable differences between the findings on chlorpromazine and those on perazine metabolism. These are certainly due not only to differences in experimental procedure and substrate but also to strain differences, as already pointed out by the former authors. A common observation is the poor metabolic capacity of cat liver.

Metabolic conversions of tricyclic psychopharmacological agents by extrahepatic tissues have been studied using imipramine as a model only. Bickel *et al.*[18] found imipramine demethylation and *N*-oxidation to be negligible with extrahepatic microsomes; Crammer and Rolfe[19] detected some demethylation, aromatic hydroxylation and side chain removal upon incubation of tissue slices with imipramine. A comparison with the findings presented here shows that perazine apparently is a particularly suitable substrate for *N*-oxide formation not only with hepatic but also with lung microsomes, for the *N*-oxide was formed with a rate in some cases exceeding that observed with liver microsomes.

Studies on kinetics and species and organ distribution of enzymatic activity for dimethylaniline *N*-oxidation[20–23] revealed results in many respects analogous to those presented here for perazine *N*-oxidation.

When comparing the results of these *in vitro* studies on perazine metabolism with those obtained by analyzing the urinary excretion pattern in humans[11] and rats[24] it appears that aromatic hydroxylation and sulfoxidation play a relatively minor role in microsomal systems, whereas they give rise to the major urinary metabolites in the living organism. This discrepancy could be due to the fact that phenolic metabolites *in vivo* are rapidly conjugated with glucuronic acid and that the resulting conjugates as well as the sulfoxides quickly leave the endoplasmic reticulum by equilibration with other compartments of the cell and are disposed by excretion. Desmethyl perazine,

however, is lipophilic enough to remain adsorbed to intracellular membranes and can be subject to further metabolic degradation.

Whether perazine *N*-oxide is reduced *in vivo* to perazine remains to be elucidated. Bickel[25] reviewed the mechanisms by which *N*-oxides have been claimed to be reduced to the corresponding tertiary amines in parenchymatous tissues and in blood. A further site for this reaction has been described by Beckett *et al.*[26] who could trace the reduction of nicotine *N*-oxide to the intestinal flora. Such sequences of *N*-oxidation and reduction would allow for several reaction cycles the amount of *N*-oxide available for urinary excretion becoming progressively smaller.

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