Studies on the Mechanism of Hepatic Microsomal *N*-Oxide Formation

N-OXIDATION OF *NN*-DIMETHYLANILINE BY A RECONSTITUTED RABBIT LIVER MICROSOMAL CYTOCHROME *P*-448 ENZYME SYSTEM

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(Received 25 January 1979)

The *N*-oxidation of *NN*-dimethylaniline was studied by using a reconstituted rabbit liver microsomal enzyme system consisting of highly purified cytochrome *P*-448, NADPH-cytochrome *c* reductase and lipid factor. Both cytochrome *P*-448 and NADPH-cytochrome *c* reductase were required for optimum *N*-oxygenating activity; the catalytic capacity of the reductase fraction for supporting *N*-oxide formation varied with the isolation procedure applied. Addition of microsomal lipids to the assay media stimulated *N*-oxidation of the arylamine. *N*-Oxide formation appeared to be not generally controlled by electron transfer from cytochrome *b*₅ to cytochrome *P*-448. The present work confirms that cytochrome *P*-448 can mediate about 44% of the rabbit liver microsomal *N*-oxidation of *NN*-dimethylaniline, thus reinforcing the existence of at least two distinct tertiary amine *N*-oxidases, i.e. haemoprotein and flavoprotein oxidase, in liver microsomal fractions.

The metabolic conversion of tertiary-amine compounds into *N*-oxides, some of which exhibit carcinogenic activity (Bickel, 1969), has been generally believed to proceed by a cytochrome *P*-450-independent pathway, represented by a flavo-protein oxidase (EC 1.14.13.8) that has been purified from pig liver microsomal fraction (Ziegler & Mitchell, 1972).

However, by using *NN*-dimethylaniline as a probe, careful analysis of the kinetics of *N*-oxidation of the arylamine by the mixed-function oxidase system revealed the participation of a CO-sensitive component in this process (Hlavica, 1972; Hlavica & Kehl, 1974). Further, a close relationship was observed between the cytochrome *P*-450-dependent *N*-hydroxylation of aniline and the *N*-oxide formation from *NN*-dimethylaniline with respect to sensitivity to photo-oxidation, reactivity to protective substrates and susceptibility to diethyl pyrocarbonate (Hlavica & Kehl, 1976). By using partially solubilized cytochrome *P*-450, two types of this pigment with different sensitivities towards heat were recognized to mediate *N*-oxidation of the tertiary amine (Hlavica & Kehl, 1977); binding of the *N*-oxidized intermediate to the haemoprotein was found to be associated with the formation of a ferrohaemochrome absorbing at 448 nm (Hlavica & Aichinger, 1978*a,b*).

These findings were taken as an indication of the presence of two distinct tertiary amine *N*-oxidases, i.e. cytochrome *P*-450 and flavoprotein oxidase, in

the microsomal fraction. The present paper supports this concept by providing evidence for the ability of a reconstituted system containing highly purified cytochrome *P*-448, NADPH-cytochrome *c* reductase and lipid factor to catalyse *N*-oxide formation from *NN*-dimethylaniline.

Experimental

Chemicals

Chemicals used in the present study were obtained from the following sources: NADP⁺, NADPH, NADH, horse heart cytochrome *c*, glucose 6-phosphate, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glucose oxidase (EC 1.1.3.4), catalase (EC 1.11.1.6) and standard proteins from Boehringer (Mannheim, Germany); *NN*-dimethylaniline hydrochloride, sodium cholate, glycerol, poly(ethylene glycol)s 6000 and 20000 from Merck A.-G. (Darmstadt, Germany); dithiothreitol from EGA-Chemie (Steinheim, Germany); Triton N-101 from Sigma Chemie G.m.b.H. München (Neubiberg, Germany); Emulgen 911 from Kao-Atlas Co. (Tokyo, Japan); DE-52 and CM-52 cellulose from Whatman (Maidstone, Kent, U.K.); adenosine 2',5'-bisphosphate-Sepharose 4B from Deutsche Pharmacia G.m.b.H. (Freiburg, Germany); calcium phosphate gel and all reagents used for sodium dodecyl sulphate/polyacrylamide-gel electrophoresis from Bio-Rad Laboratories G.m.b.H. (München, Germany).

Preparation of microsomal fraction

Microsomal fractions from the livers of adult male and female Chinchilla rabbits, fed on a standard diet obtained from Altromin (Lage, Germany) and water *ad libitum*, were prepared by the method of Jagow *et al.* (1965). Haemoglobin was removed from the microsomal suspensions by two washings with 0.15M-KCl. As a routine, the microsomal pellets were suspended in 0.15M-KH₂PO₄/Na₂HPO₄ (pH 7.4) and stored at -20°C until use.

Purification of cytochrome P-450, NADPH-cytochrome c reductase, flavoprotein oxidase and lipid fraction

All procedures were carried out at 4°C. Frozen rabbit liver microsomal fractions were thawed and diluted to a final protein concentration of 6–10 mg/ml in 0.1M-KH₂PO₄/Na₂HPO₄ (pH 7.4) containing 0.1 mM-dithiothreitol and 30% (v/v) glycerol. Sodium cholate was added to yield a concentration of 3 mg/mg of protein, and the mixture was stirred for 1 h. The solubilized preparation was fractionated with poly(ethylene glycol); the fraction precipitated by 10–20% (w/v) poly(ethylene glycol) was collected by centrifugation and dissolved in a small volume of 5 mM-KH₂PO₄/Na₂HPO₄ (pH 7.6) containing 0.1 mM-dithiothreitol, 30% (v/v) glycerol and 0.1% (v/v) Emulgen 911. The clear red solution was applied to a DE-52 DEAE-cellulose column (4 cm × 30 cm) previously equilibrated with the same buffer. Cytochrome P-450, which did not bind to the cellulose, eluted in the void volume of the column (fraction A). Two additional cytochrome fractions (B and C) were eluted by sequentially increasing the phosphate concentration of the buffer to 20 and 50 mM. Fractions A–C were concentrated by dialysis against 0.1M-KH₂PO₄/Na₂HPO₄ (pH 7.4) containing 30% (v/v) glycerol and 30% (w/v) poly(ethylene glycol) 20000. The concentrated fractions were dialysed against 5 mM-KH₂PO₄/Na₂HPO₄ (pH 6.5) containing 0.1 mM-dithiothreitol, 30% (v/v) glycerol and 0.1% (v/v) Emulgen 911 and applied to separate CM-52 cellulose columns (2 cm × 30 cm) previously equilibrated with the same buffer. Cytochrome P-450 fractions were eluted by sequentially increasing the phosphate concentration of the buffer to 20, 50 and 100 mM. To remove excess Emulgen 911, the eluates were treated with calcium phosphate gel (2 mg/mg of protein). The final preparations were stored at -20°C.

For preparation of NADPH-cytochrome c reductase, rabbit liver microsomal fractions were suspended in 10 mM-KH₂PO₄/Na₂HPO₄ (pH 7.6). Triton N-101 was added to give a concentration of 1.3 mg/mg of protein, and the suspension was stirred overnight. The mixture was centrifuged at 105 000g for 1 h and the precipitate discarded. The supernatant fraction was made 10% (v/v) in glycerol and applied to a DE-52 DEAE-cellulose column

(4 cm × 20 cm) previously equilibrated with 10 mM-KH₂PO₄/Na₂HPO₄ (pH 7.6) containing 10% (v/v) glycerol and 0.25% (v/v) Triton N-101 (buffer A). Buffer A (300 ml) containing 0.13 M-KCl was passed through the column to remove most of the cytochromes and phospholipids. Subsequently, the column was washed with 300 ml of buffer A without Triton N-101 to remove the detergent. The reductase was then eluted with a linear gradient of 0.13 to 0.5 M-KCl in buffer A without Triton N-101. The pooled reductase fractions were applied to an adenosine 2',5'-bisphosphate-Sepharose 4B column (1 cm × 13 cm) previously equilibrated with 5 mM-KH₂PO₄/Na₂HPO₄ (pH 7.6) containing 0.1 mM-dithiothreitol, 20% (v/v) glycerol and 0.1% (v/v) Emulgen 911 (buffer B). The column was washed with 100 ml of buffer B. The reductase was then eluted with a linear gradient of 0 to 1 mM-NADP⁺ in buffer B. The reductase fractions were combined, treated with calcium phosphate gel, concentrated as described above and stored at -20°C.

Flavoprotein oxidase was prepared by the method of Ziegler & Mitchell (1972).

Total lipids were isolated from rabbit liver microsomal fractions by the procedure of Folch *et al.* (1957). The final chloroform/methanol extract was concentrated to near-dryness in a rotary evaporator and stored at -20°C until use. For preparation of liposomes, 5 mg of the material was suspended in 1 ml of 0.15M-KH₂PO₄/Na₂HPO₄ pH 7.4, and treated for two 15 s intervals in an ultrasonic disintegrator.

Gel electrophoresis

Polyacrylamide-gel electrophoresis was performed in the presence of 0.1% (w/v) sodium dodecyl sulphate on 7.5% (w/v) separating slab gels as described by Laemmli (1970); samples were heated for 5 min at 100°C before application to the discontinuous-gel system. After electrophoresis at 4°C and a constant current of 3 mA/gel, the gels were stained with Coomassie Brilliant Blue R-250 by the method of Fairbanks *et al.* (1971). Apparent molecular weights were estimated by using standard proteins as described previously (Weber & Osborn, 1969).

Enzyme determinations

The reconstituted system used for measuring the N-oxidation of *NN*-dimethylaniline contained 0.15M-KH₂PO₄/Na₂HPO₄ (pH 7.4), 1.7 mM-NADP⁺, 14 mM-glucose 6-phosphate, 8.4 mM-MgCl₂, 16 nkat of glucose 6-phosphate dehydrogenase/ml of mixture, 0.8 μM-cytochrome P-450, appropriate amounts of NADPH-cytochrome c reductase and lipid as indicated in the legends to the Figures, and 1.4 mM-*NN*-dimethylaniline in a total volume of 2.5 ml. Before addition of the amine substrate, the mixture

was equilibrated for 5 min at 37°C; the *N*-oxidation reaction was carried out for 20 min.

The assay media for measuring the reduction of cytochrome *P*-450 by NADPH-cytochrome *c* reductase contained the following components in a final volume of 1.75 ml: 0.15 M-KH₂PO₄/Na₂HPO₄ (pH 7.4), 5.7 mM-glucose, 50 nkat of glucose oxidase/ml of mixture, 25 μ kat of catalase/ml of mixture, 71 μ g of lipid/ml of mixture, 1.1 μ M-cytochrome *P*-450, and appropriate amounts of NADPH-cytochrome *c* reductase as indicated elsewhere in the text. The samples were gassed with CO for 2 min. NADPH (final concentration 0.37 mM) was added and the absorbance change between 450 and 500 nm recorded at 25°C; the optical path length was 1 cm.

NADPH-cytochrome *c* reductase (EC 1.6.2.4) was assayed as described by Masters *et al.* (1968). NADH-cytochrome *b*₅ reductase (EC 1.6.2.2) was measured by the method of Strittmatter (1968) with K₃Fe(CN)₆ as electron acceptor.

Analytical procedures

NN-Dimethylaniline *N*-oxide was determined by the method of Ziegler & Pettit (1964). Cytochrome *P*-450 and *b*₅ were measured as described by Omura & Sato (1964*a,b*). Protein was determined by the method of Gornall *et al.* (1949) as modified by Szarkowska & Klingenberg (1963).

Results and Discussion

Cytochrome *P*-450 recovered from the DE-52 DEAE-cellulose column (fractions A–C) completely separated into at least two subfractions on CM-52 cellulose chromatography. When the position of the Soret band of the ferrocycytochrome–CO complex is plotted as a function of purity of the individual haemoprotein subfractions, it appears that with increasing purification of the pigment the Soret maximum is shifted from 450 to 448 nm (Fig. 1). This spectral shift might result from separation of multiple forms of cytochrome *P*-450, but can as well be argued to arise from alterations in the properties of a single cytochrome during the isolation procedure.

As shown in Fig. 1, rabbit liver cytochrome *P*-448 was purified to a specific content of 15–16 nmol/mg of protein, based on the protein determination method of Gornall *et al.* (1949). The final preparations were devoid of detectable amounts of cytochrome *b*₅ and NADPH-cytochrome *c* reductase, but still contained traces of NADH-cytochrome *b*₅ reductase. Polyacrylamide-gel electrophoresis of the cytochrome in the presence of sodium dodecyl sulphate shows one major polypeptide having an apparent mol.wt. of about 53 000 (Fig. 2).

The absolute spectrum of the oxidized form of purified cytochrome *P*-448 displays a Soret maximum at 414 nm and poorly resolved α - and β -bands

Fig. 1. Spectral characteristics of the CO adduct of rabbit liver microsomal ferrocycytochrome *P*-450 as a function of enzyme purity

Rabbit liver cytochrome *P*-450 was purified as described in the Experimental section to yield six to seven final subfractions. Each fraction was analysed for the position of the Soret maximum of the ferrocycytochrome–CO complex, and λ_{max} was plotted as a function of the specific haemoprotein content. The filled circle indicates the corresponding result for the intact liver microsomal fraction.

between 500 and 564 nm; it also has high-spin characteristics as evidenced by a shoulder at 396 nm. The absolute spectrum of the reduced form of the haemoprotein exhibits absorption peaks at 411 and 553 nm.

In summary, our cytochrome preparation closely resembles a mixture of low-spin and high-spin cytochrome *P*-450 LM₄, as previously isolated from control rabbits (Haugen & Coon, 1976; Philpot &

Fig. 2. Polyacrylamide slab-gel electrophoresis of highly purified cytochrome *P*-448, flavoprotein oxidase and NADPH-cytochrome *c* reductase

Gel electrophoresis was carried out in the presence of 0.1% sodium dodecyl sulphate in a discontinuous system as described in the Experimental section. The samples contained the following proteins: (A) standard proteins (phosphorylase *b*, bovine serum albumin, ovalbumin, carbonic anhydrase), 1 μ g of each; (B) rabbit liver microsomal fraction, 22 μ g; (C) cytochrome *P*-448, 6 μ g; (D) flavoprotein oxidase, 5 μ g; (E) NADPH-cytochrome *c* reductase, 2 μ g; (F) mixture of (C), (D) and (E). The molecular weights of the proteins are indicated on the left of the gels corresponding to their mobilities within the gels.

Arinc, 1976) or animals pretreated with either 3-methylcholanthrene (Kawalek *et al.*, 1975) or β -naphthoflavone (Haugen & Coon, 1976; Guengerich, 1977a).

NADPH-cytochrome *c* reductase was purified by affinity chromatography to a specific activity of 200–360 nkat/mg of protein for cytochrome *c* as electron acceptor and estimated to have an apparent subunit mol.wt. of 79 000 (Fig. 2). As shown in Fig. 3, the enzyme retains activity for the reduction of its native acceptor, cytochrome *P*-450; reductase preparations recovered after DE-52 DEAE-cellulose or adenosine 2',5'-bisphosphate-Sepharese 4B chromatography (see the Experimental section) exhibit different catalytic capacities for haemoprotein reduction (Fig. 3).

Preparations of cytochrome *P*-448 and NADPH-cytochrome *c* reductase were devoid of detectable amounts of flavoprotein oxidase (Fig. 2), the latter having an apparent subunit mol.wt. of about 59 000,

Fig. 3. NADPH-cytochrome *c* reductase-dependent reduction of purified cytochrome *P*-448

All experiments were carried out under anaerobic conditions in a medium containing 0.15 M-KH₂PO₄/Na₂HPO₄, pH 7.4, 1.1 μ M-cytochrome *P*-448, 0.37 mM-NADPH and 71 μ g of lipid/ml of mixture. The reactions were started by the addition of either 1.6 nkat of NADPH-cytochrome *c* reductase/ml of mixture prepared by DE-52 DEAE-cellulose chromatography (—; specific activity 17 nkat/mg of protein for cytochrome *c* reduction) or 14 nkat of NADPH-cytochrome *c* reductase/ml of mixture prepared by affinity chromatography on adenosine 2',5'-bisphosphate-Sepharese 4B (----; specific activity 360 nkat/mg of protein for cytochrome *c* reduction).

which is close to the value of 60 000 reported for the pig liver oxidase (Ziegler & Poulsen, 1978).

When reconstituted, cytochrome *P*-448 and NADPH-cytochrome *c* reductase are active in supporting *N*-oxidation of *NN*-dimethylaniline; oxygenating activity is completely dependent on the presence of both components (Table 1). The rate of *N*-oxide formation from the parent amine is a function of concentration and purity of NADPH-cytochrome *c* reductase (Fig. 4): there is a progressive transformation with increasing purity of the reductase to a species with decreased catalytic capacity for the reduction of cytochrome *P*-450 but high affinity for cytochrome *c* (cf. Fig. 3).

Based on a limiting concentration of cytochrome *P*-448 in the presence of the other components, an apparent catalytic-centre activity of 0.023–0.028 mol of *N*-oxide formed/s per mol of cytochrome *P*-448 can be taken from Table 1 and Fig. 4. This value is within the range of data reported for a large number

of other mixed-function oxidation reactions mediated by the reconstituted rabbit liver cytochrome *P*-448 system (Philpot & Arinc, 1976; Guengerich, 1977a; Johnson & Müller-Eberhard, 1977); when compared with the total turnover of *NN*-dimethylaniline *N*-oxide in the intact liver microsomal fraction (Hlavica & Kehl, 1977), about 44% of the microsomal *N*-oxidation of the arylamine can be calculated to result from haemoprotein catalysis.

N-Oxidation of *NN*-dimethylaniline requires the presence of lipid; maximal stimulation, however, is only observable within a narrow range of lipid concentrations (Fig. 5). In some experiments the effect of lipid was only marginal or even absent. This is believed to result from varying efficiency in removing Emulgen 911 from the final enzyme preparations: non-ionic detergents are well known to substitute for lipid in reconstituted systems (Lu *et al.*, 1974).

The question arises as to whether cytochrome *b*₅-directed electron transfer to cytochrome *P*-450 is of importance in the *N*-oxidation of *NN*-dimethylaniline. Since the catalytic-centre activity for the *N*-oxidation reaction catalysed by a cytochrome

Table 1. Requirements of various microsomal fractions from rabbit liver for the *N*-oxidation of *NN*-dimethylaniline

Reactions were carried out at 37°C in 0.15 M-KH₂PO₄/Na₂HPO₄, pH 7.4, containing 1.4 mM-*NN*-dimethylaniline, NADPH-generating system and 40 µg of lipid/ml of mixture. Where indicated, cytochrome *P*-448 (final concentration 0.5 µM) and/or NADPH-cytochrome *c* reductase (13 nkat/ml of mixture) were incorporated into the assay media. + and - indicate presence or absence from the medium respectively.

Cytochrome <i>P</i> -448 fraction added	NADPH-cytochrome <i>c</i> reductase fraction added	<i>N</i> -Oxidation of <i>NN</i> -dimethylaniline (nmol/min per nmol of cytochrome <i>P</i> -448)
+	-	0
-	+	0
+	+	1.4

Fig. 4. Effect of concentration of microsomal NADPH-cytochrome *c* reductase on the rate of *N*-oxidation of *NN*-dimethylaniline by cytochrome *P*-448

Reactions were carried out at 37°C in 0.15 M-KH₂PO₄/Na₂HPO₄, pH 7.4, containing 1.4 mM-*NN*-dimethylaniline, NADPH-generating system, 0.8 µM-cytochrome *P*-448, 40 µg of lipid/ml of mixture and NADPH-cytochrome *c* reductase prepared either by DE-52 DEAE-cellulose chromatography (○; specific activity 17 nkat/mg of protein for cytochrome *c* reduction) or by affinity chromatography on adenosine 2',5'-bisphosphate-Sepharose 4B (●; specific activity 360 nkat/mg of protein for cytochrome *c* reduction). The molar ratio of NADPH-cytochrome *c* reductase to cytochrome *P*-448 was calculated by assuming highly purified reductase from the rabbit to have a mol.wt. of 79000 and a specific activity of 800 nkat/mg of protein for cytochrome *c* reduction (Guengerich, 1977b).

Fig. 5. Effect of concentration of microsomal lipids on the rate of *N*-oxidation of *NN*-dimethylaniline by cytochrome *P*-448

Reactions were carried out at 37°C in 0.15 M-KH₂PO₄/Na₂HPO₄, pH 7.4, containing 1.4 mM-*NN*-dimethylaniline, NADPH-generating system, 0.8 µM-cytochrome *P*-448, 1.6 nkat of NADPH-cytochrome *c* reductase/ml of mixture and lipid as indicated in the Figure.

*b*₅-free system (see above) agrees reasonably well with that for the liver microsomal process (Hlavica & Kehl, 1977), it appears that this pathway is not generally controlled by cytochrome *b*₅. This view is supported by the lack of NADH-synergism in the *N*-oxidation of the arylamine (Hlavica, 1972).

In summary, our results establish further evidence for the ability of cytochrome *P*-450 to mediate *N*-oxidation of *NN*-dimethylaniline. This finding is at variance with observations of Gorrod *et al.* (1975) who by comparing the effects of inhibitors and

modifiers of drug metabolism on the rabbit liver microsomal C- and N-oxidation of the N-ethyl-N-methyl analogue of NN-dimethylaniline concluded that cytochrome P-450 was not involved in the N-oxidation of this amine. Critical evaluation of the experimental data, however, allows alternative interpretations: assuming NN-dimethylaniline to be N-oxidized by two distinct mixed-function oxidases, i.e. cytochrome P-450 and flavoprotein oxidase, the extent to which either of these systems contributes to the total amount of N-oxide formed in the intact liver microsomal fraction mainly depends on the catalytic capacities of the individual oxidases and the molar ratio of the two enzymes present. Taking cytochrome P-450 to account for about 44% of the total turnover of NN-dimethylaniline giving rise to N-oxide formation in the rabbit liver microsomal fraction (see above) and considering that the haemoprotein-dependent N-oxidation is likely to be only partially inhibited even by its specific inhibitors, total microsomal N-oxide formation is likely to be affected only to a minor extent by these agents. This precludes direct comparison of inhibition data obtained for the liver microsomal C- and N-oxidation of NN-dialkylanilines, the former reaction mainly depending on catalysis by cytochrome P-450 (Gorrod *et al.*, 1975; Prough & Ziegler, 1977). Significant inhibition of liver microsomal N-oxidation of tertiary amines by modifiers of the cytochrome P-450 system, therefore, can only be detected when there is no appreciable contribution of the flavoprotein oxidase to the total amount of N-oxide formed. This appears to be the case with pyridines: the purified flavoprotein oxidase does not N-oxidize N-methylnicotinamide to a measurable extent (Ziegler *et al.*, 1969). Consequently, liver microsomal N-oxide formation from 3-substituted pyridines is inhibitable by CO and SKF 525-A (Gorrod, 1978).

Apparent differences in the enzymology of conversion of NN-dialkylanilines and pyridines to the corresponding N-oxides have been attributed to differences in the electronic states of the individual amines and types of hybridization involved (Gorrod, 1978). Our results would suggest that nucleophilicity does not generally determine whether the preferred route of N-oxidation is via cytochrome P-450 or flavoprotein oxidase.

As regards the effect of CO on the metabolism of NN-dimethylaniline, N-oxidation of the amine has been found to be stimulated by the inhibitor; this has been interpreted to result from inhibition of the CO-sensitive liver microsomal dimethylaniline N-oxide aldolase (EC 4.1.2.24; Ziegler & Pettit, 1966). Aldolase activity, however, has been shown to be negligible in the rabbit liver microsomal fraction (Hlavica & Kiese, 1969; Gorrod *et al.*, 1975). Subsequent experiments suggested that the enhancement of N-oxide formation from NN-dimethyl-

aniline by CO resulted from displacement of the equilibrium between two interconvertible forms of cytochrome P-450 to the species with increased affinity for O₂; stimulation was shown to be superimposed on an inhibitory effect (Hlavica, 1972; Hlavica & Kehl, 1974). This phenomenon is remarkably similar to that produced by metyrapone: this compound, eliciting a CO-like difference spectrum with reduced cytochrome P-450, has been proposed to effect transition between two functionally different forms of the haemoprotein associated with stimulation or inhibition of certain types of mixed-function oxidation reactions (Hildebrandt *et al.*, 1969). This mechanism could be operative in the N-oxidation of NN-dimethylaniline, too, since intermediate concentrations of metyrapone have been observed to increase the rate of N-oxide formation from NN-dialkylanilines in rabbit liver microsomal fractions (Uehleke *et al.*, 1971; Gorrod *et al.*, 1975) without affecting this process when catalysed by the purified flavoprotein oxidase (Ziegler *et al.*, 1973).

Several laboratories have reported differences in the sensitivities of rabbit liver microsomal C- and N-oxidation of NN-dialkylanilines towards mercurials (Devereux & Fouts, 1974; Gorrod *et al.*, 1975). Assuming these agents mainly interact with components of the cytochrome P-450-dependent electron-transport chain, these findings were interpreted to mean separate metabolic routes for these reactions (Gorrod *et al.*, 1975). Since solubilized rabbit liver flavoprotein oxidase has been shown to be highly sensitive to mercury (Devereux *et al.*, 1977), this explanation evidently does not hold.

Some primary amines, including n-octylamine, have been found to be potent activators of the pig liver flavoprotein oxidase (Ziegler & Mitchell, 1972). There exist, however, marked species differences in the activation of N-oxidation of NN-dimethylaniline by n-octylamine: in rat and rabbit liver microsomal fractions, even at high concentrations of the amine, stimulation is insignificant (Poulsen *et al.*, 1974). Similarly, the purified rabbit liver flavoprotein oxidase is insensitive to 1.0 mM-n-octylamine (Hlavica & Kehl, 1977). On the other hand, Gorrod (1978) reported stimulation by n-octylamine of rabbit liver microsomal N-oxidation of NN-dialkylanilines, whereas N-oxide formation from pyridines was inhibited. This could be due to participation in these N-oxidation processes of multiple forms of cytochrome P-450 with different sensitivities towards n-octylamine: by virtue of its ability to strongly bind to some haemoprotein species predominantly catalysing N-oxidation of pyridines, n-octylamine could shunt electrons away from this species to another one characterized by very low affinity for the inhibitor but high catalytic capacity for the N-oxidation of NN-dialkylanilines; this results in stimulation of the

latter process. Indeed, the broad spectrum of compounds containing a tertiary nitrogen atom appear not to be metabolized by a uniform type of cytochrome *P*-450. Thus the pigment *N*-oxidizing tertiary-heterocyclic amines, such as pyrrolizidine alkaloids (Mattocks & White, 1971) or pyridines (Gorrod, 1978), bear close resemblance to that catalysing *N*-hydroxylation of primary alkylamines (Sum & Cho, 1977), primary arylamines (Hlavica & Kiese, 1969; Heinze *et al.*, 1970; Uehleke & Tabarelli, 1973; Hlavica & Kehl, 1974), secondary arylamines (Beckett & Gibson, 1975) and imines (Parli *et al.*, 1971) with respect to sensitivity to CO, SKF 525-A or induction by phenobarbital, and differ from the cytochrome *P*-448 system preferentially mediating *N*-hydroxylation of *N*-acetamides (Hinson *et al.*, 1975; Lotlikar & Zaleski, 1975) and some secondary heterocyclic amines (Jaccarini & Felice, 1978). On the other hand, cytochrome *P*-450 supporting liver microsomal *N*-oxidation of *NN*-dialkylanilines is selectively inducible by steroids (Arrhenius, 1968; Devereux & Fouts, 1975; Hlavica & Kehl, 1977).

In conclusion, the existence of at least two distinct tertiary amine *N*-oxidases, i.e. cytochrome *P*-450 and flavoprotein oxidase, proposed first by our own laboratory (Hlavica, 1971, 1972; Hlavica & Kehl, 1974, 1976, 1977; Hlavica & Aichinger, 1978*a,b*), is supported by the present results.

The financial support of the Deutsche Forschungsgemeinschaft (grant H1 1/8) is gratefully acknowledged. We thank Mrs. Jutta Mietaschke and Miss Lydia Heide for their expert technical assistance.

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