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

THE ROLE OF CYTOCHROME *P*-450 AND MIXED-FUNCTION AMINE OXIDASE IN THE *N*-OXIDATION OF *NN*-DIMETHYLANILINE

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Evidence is established for the existence of alternative metabolic routes of *N*-oxidation of *NN*-dimethylaniline in rabbit liver microsomal fraction. One pathway involves the participation of two types of cytochrome *P*-450 with different sensitivities towards heat. Both types may represent distinct haemoprotein species or two physical forms of a single pigment. The other pathway is represented by the mixed-function amine oxidase. The enzyme lacks NADPH dehydrogenase activity and is insensitive to treatment with 2-bromo-4′-nitroacetophenone and steapsin: it catalyses *N*-oxidation of imipramine, trimethylamine and *NN*-dimethylaniline in molar proportions considerably different from those of the cytochrome *P*-450-supported reactions. Cytochrome *P*-450 is estimated to account for the formation of at least 50–60% of the total *NN*-dimethylaniline *N*-oxide formed in the intact rabbit liver microsomal fraction, the remainder arising from the action of the mixed-function amine oxidase.

The metabolic route of *N*-oxidation of a wide variety of tertiary amines to produce *N*-oxides has received increasing attention during the past decade. Some of the reasons for this increased interest are the discovery of the natural occurrence of *N*-oxides in plant and animal tissues, the use of certain *N*-oxides in therapy and the observation that a series of tertiary-amine drugs undergoes biotransformation to the *N*-oxides. The subject has been extensively reviewed by Bickel (1969).

Attempts have been made to isolate and characterize the enzyme system(s) responsible for the *N*-oxidation of tertiary-amine substrates. An *NN*-dimethylaniline mono-oxygenase (*N*-oxide-forming, EC 1.14.13.8), also designated mixed-function amine oxidase, has been purified to homogeneity from pig liver microsomal fraction; the oxidase is a flavoprotein containing 6mol of FAD/mol of enzyme and has a mol.wt. of 474000 (Masters & Ziegler, 1971). The enzyme is devoid of cytochrome *P*-450 (EC 1.14.14.1) and NADPH–cytochrome *c* reductase (EC 1.6.2.4) (Ziegler & Mitchell, 1972). A large number of tertiary and some secondary amines are *N*-oxidized by this system.

On the other hand, evidence has accumulated hinting at the participation of the liver microsomal cytochrome *P*-450 in the *N*-oxidation of tertiary amines (White & Mattocks, 1971; Hlavica, 1971, 1972; Hill *et al.*, 1972; Gorrod, 1973; Hlavica & Kehl, 1974).

The further characterization of the systems supporting *N*-oxidation of tertiary-amine compounds is the aim of the present paper. We report experiments designed to probe the NADPH–cytochrome *c* reductase and cytochrome *P*-450 segment of the hepatic microsomal electron-transport chain with respect to involvement in the *N*-oxidation of *NN*-dimethylaniline. Moreover, the mixed-function amine oxidase-dependent *N*-oxidation of the arylamine will be described in detail.

Experimental

Chemicals

Chemicals used in this study were obtained from the following sources: NADH, $NADP^+$, glucose 6-phosphate, cytochrome *c*, oxidized glutathione, glucose 6-phosphate dehydrogenase (EC 1.1.1.49), glutathione reductase (EC 1.6.4.2), xanthine oxidase (EC 1.2.3.2), catalase (EC 1.11.1.6) and trypsin (EC 3.4.21.4) from Boehringer (Mannheim, Germany); menadione, L-histidine, guanidine hydrochloride, sodium cholate, *NN*-dimethylaniline hydrochloride and silica gel $HF_{254-366}$ from Merck A.G. (Darmstadt, Germany); hypoxanthine and protamine sulphate from Serva (Heidelberg, Germany); dithiothreitol from EGA-Chemie (Steinheim, Germany); 2-bromo-4′-nitroacetophenone from Eastman Organic Chemicals (Rochester, NY, U.S.A.); Triton X-45 and Triton X-102 from Sigma Chemical Co. (St. Louis, MO, U.S.A.); crude pancreatic lipase (steapsin) (EC 3.1.1.3) and trimethylamine hydro-

chloride from Fluka A.G. (Buchs SG, Switzerland); [^{14}C]imipramine hydrochloride [*N*-(3-dimethylaminopropyl)iminodi[*methylene*-^{14}C]benzyl hydrochloride] from Amersham Buchler (Wenden, Germany). The reference compounds imipramine, imipramine *N*-oxide and desmethylimipramine were generously provided by Ciba–Geigy A.G. (Wehr, Germany). Pregnenolone 16α-carbonitrile was kindly given by Searle Laboratories (Chicago, IL, U.S.A.).

Preparation of microsomal fractions

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 lib.*, were prepared by the method of Jagow *et al.* (1965). Haemoglobin was removed from the microsomal suspensions by two washings with 0.15M-KCl. For routine assays, the microsomal pellets were suspended in 0.15M-KH_2PO_4/Na_2HPO_4, pH7.4.

For some experiments, liver microsomal fractions from Sprague–Dawley rats, beagle dogs, cats, pigs and guinea pigs were prepared analogously.

Extraction of lipid from microsomal fractions

Freeze-dried rabbit liver microsomal fractions (250 mg) were homogenized in 25ml of ice-cold butan-1-ol and centrifuged at 35000*g* for 5min. The precipitate was resuspended in 25ml of butan-1-ol and treated as described above. The pellet was rinsed twice with 25ml of ice-cold acetone, and the final acetone suspension was filtered on a Büchner funnel. The fine cream-coloured powder was dried in a desiccator under vacuum and stored at −20°C.

Purification of cytochrome P-450 and mixed-function amine oxidase

Cytochrome *P*-450 from untreated animals was partially solubilized from liver microsomal fractions as indicated by Autor *et al.* (1973). In general, 2-fold purification of the pigment was achieved. The final preparations were stored at −20°C without significant loss of activity.

The mixed-function amine oxidase was purified from rabbit liver by the method of Ziegler & Mitchell (1972). As a routine, the fraction precipitating between 40 and 45% saturation on addition of a saturated solution of $(NH_4)_2SO_4$, pH4.8, was used. All preparations were devoid of detectable amounts of cytochrome *P*-450, but still contained traces of cytochrome b_5 and NADPH–cytochrome *c* reductase. The content of acid-extractable flavin was 4.2 (±0.2 S.E.M.) nmol/mg of protein ($n = 3$). The specific activity of the preparations varied between 34 and 102nmol of *NN*-dimethylaniline *N*-oxide formed/min per mg of protein. The K_m for *NN*-dimethylaniline was determined to be 89μM (pH7.4; 37°C). *n*-Octylamine (final concentration 1.0mM), a potent activator of the pig liver oxidase (Ziegler & Mitchell, 1972), failed to affect the rabbit liver enzyme. The oxidase was fairly stable when stored at −20°C.

Treatment of enzyme preparations with 2-bromo-4′-nitroacetophenone

To test the enzyme preparations for the presence of NADPH–cytochrome *c* reductase, rabbit liver microsomal fraction, partially solubilized cytochrome *P*-450 or mixed-function amine oxidase was diluted to a protein concentration of 2mg/ml in 0.15M-KH_2PO_4/Na_2HPO_4, pH7.4. 2-Bromo-4′-nitroacetophenone (final concentration 0.1mM) was added in a small volume of methanol. The samples were incubated for 10min at 37°C. Alkylation was stopped by addition of a freshly prepared aqueous solution of histidine (final concentration 0.02M), and the microsomal fraction was spun down at 78000*g* for 1h. The pellet was rinsed twice with ice-cold 0.15M-KCl. Cytochrome *P*-450 and mixed-function amine oxidase were precipitated with $(NH_4)_2SO_4$ (final saturation 50 and 45% respectively) and dialysed overnight against 0.15M-KH_2PO_4/Na_2HPO_4, pH7.4.

Treatment of enzyme preparations with steapsin

Digestion of rabbit liver microsomal fraction or mixed-function amine oxidase with steapsin was performed in 0.05M-KH_2PO_4/Na_2HPO_4, pH7.7: 1mg of steapsin was added/5mg of enzyme protein, and the mixtures were incubated for 5min at 37°C. After incubation, the microsomal fraction was centrifuged at 78000*g* for 1h. The pellet was washed twice with ice-cold 0.15M-KCl. Samples of digested mixed-function amine oxidase were taken for enzymic determinations without further treatment.

Enzyme determinations

The standard incubation mixture for measuring *N*-oxidation of the amines contained: 0.15M-KH_2PO_4/Na_2HPO_4, pH7.4; 1.2mM-$NADP^+$; 10mM-glucose 6-phosphate; 6mM-$MgCl_2$; 5μg (0.7unit) of glucose 6-phosphate dehydrogenase/ml of mixture; amine substrate {1.0mM-*NN*-dimethylaniline, 0.1mM-trimethylamine or 0.1mM-[^{14}C]imipramine (10.5mCi/mmol)}; enzyme (2mg of rabbit liver microsomal protein/ml, 0.14mg of mixed-function amine oxidase/ml or 1.43nmol of cytochrome *P*-450/ml). Before addition of the amine substrates the mixtures were equilibrated for 10min under air. Reactions were carried out at 37°C for 10min. During this period, the rates of *N*-oxidation remained constant.

The incubation mixtures for measuring the peroxidatic formation of *NN*-dimethylaniline *N*-oxide by catalase contained the following components in a final volume of 7.0ml: 0.15M-KH_2PO_4/Na_2HPO_4, pH7.4; 14mg of rabbit liver microsomal protein; 1.0mM-*NN*-dimethylaniline; 1.5mM-hypoxanthine; 2mg (0.8unit) of xanthine oxidase and 1mg (70000

units) of catalase. The reaction was carried out at 37°C for 10min.

NADPH–cytochrome *c* reductase was assayed as described by Masters *et al.* (1968) by using $\varepsilon_{550} = 27700 \text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Paul, 1969). One unit is defined as 1 nmol of cytochrome *c* reduced/min.

Analytical procedures

NN-Dimethylaniline *N*-oxide was determined by the method of Ziegler & Pettit (1964). Trimethylamine *N*-oxide was measured as described by Fok & Ziegler (1970). Imipramine *N*-oxide was determined by t.l.c. as described by Bickel & Gigon (1971): the spot corresponding to imipramine *N*-oxide was scraped from the plate, and radioactivity was measured by liquid-scintillation spectrometry (Bray, 1960).

Cytochromes *P*-450 and *P*-420 were determined by the method of Omura & Sato (1964*b*) by using $\Delta\varepsilon_{450-490} = 91000 \text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ and $\Delta\varepsilon_{420-490} = 111000 \text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$. Cytochrome b_5 was measured with NADH by using $\Delta\varepsilon_{424-409} = 185000 \text{litre} \cdot \text{mol}^{-1} \cdot \text{cm}^{-1}$ (Omura & Sato, 1964*a*). Insufficient NADH–cytochrome b_5 reductase was present in the partially solubilized preparations to permit quantification of cytochrome b_5. Hence crude reductase was prepared from microsomal fractions as described by Levin *et al.* (1972) and added to the assay mixtures.

NADPH was assayed by following oxidation of the nucleotide in the presence of glutathione reductase (Ciotti & Kaplan, 1969). Acid-extractable flavin was measured as indicated by Green *et al.* (1955). Formaldehyde was assayed as described by Nash (1953). Protein was determined by the method of Gornall *et al.* (1949) as modified by Szarkowska & Klingenberg (1963).

Pseudo-first-order rate constants for inactivation were computed from $k = 0.693/t_{0.5}$, where $t_{0.5}$ is the period in which 50% of activity is lost. Statistical evaluation was performed by Student's *t* test.

Results

Effect of treatment of microsomal fraction and purified mixed-function amine oxidase with 2-bromo-4′-nitroacetophenone on the N-oxidation of NN-dimethylaniline

Aromatic nitro compounds have been shown to undergo nitroreduction catalysed by NADPH–cytochrome *c* reductase (Feller *et al.*, 1971). The effect of 2-bromo-4′-nitroacetophenone, a substrate analogue of nitrobenzene, on the microsomal NADPH–cytochrome *c* reductase was studied, since treatment of enzymes with halomethyl ketone derivatives of substrates has been shown to effect inactivation due to alkylation of functional groups (Shaw & Ruscica, 1971; Heymann & Krisch, 1972).

Table 1 shows that treatment of rabbit liver microsomal fractions with 0.1mM-2-bromo-4′-nitroacetophenone causes partial inhibition of the particle-bound reductase. Similarly, the rate of microsomal *N*-oxide formation from *NN*-dimethylaniline is considerably decreased. Alkylation, however, does not modify the content of microsomal cytochrome *P*-450.

This could mean that at least part of the *N*-oxide formed results from a reaction supported by NADPH–cytochrome *c* reductase. This view is consistent with the inability of 0.1mM-2-bromo-4′-nitroacetophenone to impair *N*-oxidation of *NN*-dimethylaniline mediated by the purified mixed-function amine oxidase (Table 1).

Effect of treatment of microsomal fraction and purified mixed-function amine oxidase with steapsin on the N-oxidation of NN-dimethylaniline

Steapsin treatment of rabbit liver microsomal fraction depletes the particles of NADPH–cytochrome *c* reductase, the reductase activity appearing in the supernatant fraction obtained after high-speed centrifugation (Table 2). Solubilization of the microsomal reductase is accompanied by partial inhibition of the microsomal amine oxide formation from *NN*-dimethylaniline (Table 2). There is also some loss of microsomal cytochrome *P*-450 due to conversion of the haemoprotein into the cytochrome *P*-420 form (Table 2).

On the other hand, steapsin treatment does not impair the catalytic capacity of the purified mixed-function amine oxidase or solubilize the flavoprotein from the microsomal fraction (Table 2).

Effect of menadione on the microsomal and mixed-function amine oxidase-catalysed N-oxidation of NN-dimethylaniline

Switching of the NADPH–cytochrome *c* reductase-directed electron transport from drug oxidation to menadione has been shown to block cytochrome *P*-450-dependent mixed-function oxidation reactions (Wills, 1972).

Table 3 summarizes the effect of 0.1mM-menadione on the *N*-oxidation of *NN*-dimethylaniline. The naphthaquinone almost completely blocks the cytochrome *P*-450-dependent *N*-dealkylation of the amine in rabbit liver microsomal fractions, whereas the microsomal *N*-oxidation of the arylamine is inhibited by only 60%. Inhibition is not simulated by poor stability of the *N*-oxide formed in the presence of menadione, since known amounts of *NN*-dimethylaniline *N*-oxide added to the incubation mixtures in the presence of the naphthaquinone were recovered almost quantitatively (Table 3). Neither is the inhibitory effect due to consumption of NADPH during incubation. Although menadione decreases the concentration of NADPH in the incubation mixtures from 0.78 to 0.46mM (Table 3), the latter

Table 1. *Treatment of rabbit liver microsomal fraction and solubilized mixed-function amine oxidase with 2-bromo-4'-nitroacetophenone: effect on cytochrome P-450, NADPH–cytochrome c reductase and N-oxygenating activity*

Rabbit liver microsomal fraction and purified mixed-function amine oxidase were diluted to a protein concentration of 2mg/ml of mixture in 0.15M-KH_2PO_4/Na_2HPO_4, pH7.4, and treated with 0.1mM-2-bromo-4'-nitroacetophenone as described in the Experimental section. The results are expressed as means ± S.E.M. for the numbers of preparations given in parentheses. As a routine, preparation started from the pooled livers of three to seven animals.

	Native system	2-Bromo-4'-nitro-acetophenone-treated system
(*a*) Microsomal fraction		
Cytochrome *P*-450 (nmol/mg of protein)	1.60± 0.15 (7)	1.49±0.09 (7)
NADPH–cytochrome *c* reductase (units/mg of protein)	86.7 ± 3.80 (7)	39.6 ±3.80 (7)
N-Oxidation of *NN*-dimethyl-aniline (nmol/min per mg of protein)	5.20± 0.21 (4)	1.93±0.16 (4)
(*b*) Purified mixed-function amine oxidase		
N-Oxidation of *NN*-dimethylaniline (nmol/min per mg of protein)	59.4 ±10.3 (4)	53.4±8.50 (4)

Table 2. *Treatment of rabbit liver microsomal fraction and solubilized mixed-function amine oxidase with steapsin: effect on cytochrome P-450, NADPH–cytochrome c reductase and N-oxygenating activity*

Digestion of rabbit liver microsomal fraction and mixed-function amine oxidase with steapsin was performed for 5min at 37°C in 0.15M-KH_2PO_4/Na_2HPO_4, pH7.7, as described in the Experimental section. 'Steapsin supernatant' is the supernatant fraction obtained after centrifugation of steapsin-treated microsomal fraction at 78000*g*. The results are expressed as means ± S.E.M. for the number of preparations given in parentheses. As a routine, preparation was started from the pooled livers of three to seven animals.

	Native system	Steapsin-treated system	'Steapsin supernatant'
(*a*) Microsomal fraction			
Cytochrome *P*-450 (nmol/mg of protein)	2.53± 0.32 (5)	1.83± 0.24 (5)	—
NADPH–cytochrome *c* reductase (units/mg of protein)	86.6 ±11.7 (5)	9.90± 1.10 (5)	264.5±31.5 (5)
N-Oxidation of *NN*-dimethylaniline (nmol/min per mg of protein)	5.80± 0.30 (5)	2.60± 0.30 (5)	0 (5)
(*b*) Purified mixed-function amine oxidase			
N-Oxidation of *NN*-dimethylaniline (nmol/min per mg of protein)	102.9 ±35.7 (3)	95.6 ±34.1 (3)	—

concentration, which is five times the K_m for NADPH (P. Hlavica & M. Kehl, unpublished work), still suffices to saturate the system.

On the other hand, 0.1mM-menadione does not affect the *N*-oxidation of *NN*-dimethylaniline catalysed by the purified mixed-function amine oxidase (Table 3). This finding is in accord with previous reports on the pig liver amine oxidase, which has been shown to be devoid of detectable NADPH dehydrogenase activity (EC 1.6.99.2) (Ziegler & Mitchell, 1972).

Effect of pretreatment of rabbits with $CoCl_2$ on the microsomal amine oxide formation from NN-dimethylaniline

Administration of $CoCl_2$ to animals has been shown to inhibit synthesis of certain haemoproteins, such as cytochrome *P*-450, associated with inhibition of mixed-function oxidations dependent on this pigment (Tephly & Hibbeln, 1971).

Treatment of rabbits with $CoCl_2$ decreases the content of hepatic cytochrome *P*-450 and rate of *N*-oxide formation from *NN*-dimethylaniline (Table

Table 3. *Effect of menadione on the N-oxidation of NN-dimethylaniline catalysed by microsomal fraction and mixed-function amine oxidase*

Oxygenating activity was assayed as described in the Experimental section. Menadione (final concentration 0.1 mM) was added to the incubation mixtures in a small volume of methanol. In some experiments, *NN*-dimethylaniline *N*-oxide (final concentration 0.2 mM) was added to the reaction mixtures; recovery of the *N*-oxide was measured after 10 min incubation. The NADPH content of the assay media was determined as a routine at the end of the incubation period. The results are expressed as means ± S.E.M. for the numbers of preparations given in parentheses.

	Additions to the basic assay media ... None	Menadione (0.1 mM)
(*a*) Microsomal fraction		
N-Demethylation of *NN*-dimethylaniline (nmol/min per mg of protein)	6.62 ± 0.51 (5)	0.65 ± 0.28 (5)
N-Oxidation of *NN*-dimethylaniline (nmol/min per mg of protein)	8.32 ± 0.45 (5)	3.34 ± 0.33 (5)
NADPH content of the assay media (mM)	0.78 ± 0.02 (5)	0.46 ± 0.03 (5)
Recovery of *NN*-dimethylaniline *N*-oxide (%)	100 (6)	89.3 ± 4.10 (6)
(*b*) Purified mixed-function amine oxidase		
N-Oxidation of *NN*-dimethylaniline (nmol/min per mg of protein)	36.7 ± 6.80 (7)	34.3 ± 7.20 (7)

Table 4. *Effect of pretreatment of rabbits with $CoCl_2$ on the microsomal N-oxidation of NN-dimethylaniline*

Rabbits were injected subcutaneously with $CoCl_2$,-$6H_2O$ (60 mg/kg body wt.) at 72, 48 and 24 h before death. Controls received an equal volume of 0.9% NaCl. Liver microsomal fraction was prepared as described in the Experimental section. The results are expressed as means ± S.E.M. for eight rabbits. * $P < 0.001$ compared with untreated controls.

Treatment	Cytochrome *P*-450 (nmol/mg of protein)	*N*-Oxidation of *NN*-dimethylaniline (nmol/min per mg of protein)
Controls	1.79 ± 0.15	6.77 ± 0.59
$CoCl_2$-treated rabbits	0.52 ± 0.08	3.58 ± 0.33*

4). Addition of 0.1 mM-$CoCl_2$ to control microsomal fractions, however, does not affect *N*-oxidation of the amine.

Catalase, another haemoprotein usually contaminating the microsomal fraction, has been reported to oxidize amine substrates through a peroxidatic reaction (Kadlubar *et al.*, 1973). Hence the observed decrease in *N*-oxide formation could be equally well due to a cobalt-induced lack of this enzyme. However, rabbit liver microsomal fraction supplemented with catalase and a H_2O_2-generating system produces only insignificant amounts of *NN*-dimethylaniline *N*-oxide [0.17 (±0.1 S.E.M.) nmol/min per mg of protein; $n = 6$]. Further, 1.0 mM-NaN_3 does not inhibit the microsomal *N*-oxidation of *NN*-dimethylaniline.

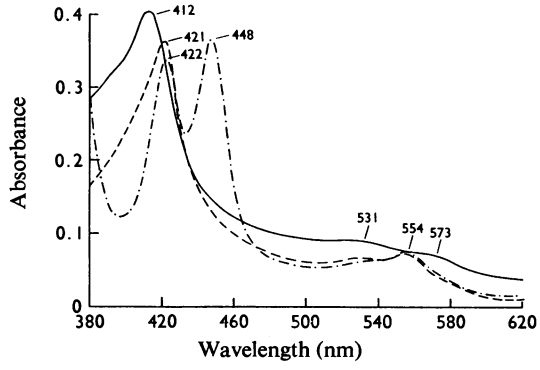

Fig. 1. *Absolute spectra of partially solubilized cytochrome P-450 from the rabbit*

Cytochrome *P*-450, prepared as described in the Experimental section, was diluted with 0.15 M-KH_2PO_4/Na_2HPO_4, pH 7.4, to a final concentration of 2.24 μM in the sample cuvette; buffer was in the reference cuvette. Spectra were recorded with a Cary 118 spectrophotometer: optical path length was 1 cm. ——, Oxidized form; – – – –, dithionite-reduced form; –·–·, CO adduct of the dithionite-reduced form.

Effect of pretreatment of rabbits with pregnenolone 16α-carbonitrile on the microsomal N-oxidation of NN-dimethylaniline

Pretreatment of rabbits with phenobarbital or 3-methylcholanthrene does not stimulate hepatic

Table 5. *Effect of pretreatment of rabbits with pregnenolone 16α-carbonitrile on the microsomal N-oxidation of NN-dimethylaniline*

Pregnenolone 16α-carbonitrile (20mg/kg body wt.) was administered to rabbits five times, at 12h intervals. Each dose was given in 5ml of water containing Tween 80 (one drop/10ml). Controls received an equal amount of Tween 80 in water. At 12h after the last treatment, the animals were killed and liver microsomal fractions prepared as described in the Experimental section. The results are expressed as means ± S.E.M. for four rabbits. * $P < 0.005$ compared with the controls; ** $P < 0.025$ compared with the controls.

Treatment	Cytochrome *P*-450 (nmol/mg of protein)	NADPH–cytochrome *c* reductase (units/mg of protein)	*N*-Oxidation of *NN*-dimethylaniline (nmol/min per mg of protein)
Controls	1.52±0.19	86.2±14.5	6.59±0.83
Pregnenolone 16α-carbonitrile-treated rabbits	2.62±0.17*	168.3±43.2	10.30±0.95**

Table 6. *Cytochromes P-450 and P-420, cytochrome b_5 and NADPH–cytochrome c reductase in preparations solubilized from rabbit liver microsomal fraction: influence of modification of the individual components on the N-oxygenating activity*

Solubilization of cytochrome *P*-450 and extraction of lipid from microsomal fractions was performed as described in the Experimental section. Digestion of microsomal fractions with steapsin was carried out at 37°C for 30min. *N*-Oxygenating activity was assayed with 1.43μM-cytochrome *P*-450 or *P*-420 in 0.15M-KH_2PO_4/Na_2HPO_4, pH7.4. The results are expressed as means ± S.E.M. of three to seven preparations.

Composition of solubilized system	Pretreatment of microsomal fraction before the solubilization procedure ... None	Extraction with butanol/acetone	Steapsin digestion
Cytochrome *P*-450 (nmol/mg of protein)	2.57± 0.18	0.68± 0.12	0
Cytochrome *P*-420 (nmol/mg of protein)	0	0.08± 0.02	4.08±1.36
Cytochrome b_5 (nmol/mg of protein)	0.67± 0.09	0.57± 0.11	1.13±0.65
NADPH–cytochrome *c* reductase (units/mg of protein)	158.3 ±35.0	83.2 ±12.6	1.77±0.23
N-Oxidation of *NN*-dimethylaniline (nmol/min per mg of protein)	7.00± 0.58	0.41± 0.08	0
N-Oxidation of *NN*-dimethylaniline (nmol/min per nmol of cytochrome *P*-450)	2.74± 0.19	0.59± 0.05	—

microsomal *N*-oxidation of *NN*-dimethylaniline (Hlavica & Kiese, 1969). We therefore decided to investigate the characteristics of pregnenolone 16α-carbonitrile induction, since the latter has been recognized as a new type of inducer.

Administration of the steroid to rabbits increases the content of hepatic microsomal cytochrome *P*-450, elevates NADPH–cytochrome *c* reductase activity and enhances the microsomal *N*-oxidation of *NN*-dimethylaniline to comparable extents (Table 5).

N-Oxidation of NN-dimethylaniline by partially solubilized cytochrome P-450 from liver microsomal fraction

To assess the role of cytochrome *P*-450 in the *N*-oxidation of *NN*-dimethylaniline more precisely, the haemoprotein was partially solubilized from liver microsomal fraction of untreated animals and subjected to catalytic rate studies.

Fig. 1 gives the absolute spectrum of a typical preparation of solubilized cytochrome *P*-450 from

Table 7. *Cytochrome P-450, cytochrome b_5, NADPH–cytochrome c reductase and N-oxygenating activity in preparations solubilized from liver microsomal fractions of various animals*

Solubilization of cytochrome *P*-450 and enzyme assays were carried out as described in the Experimental section. The results are expressed as means ± S.E.M. of three to six preparations. The cytochrome *P*-450 content of the intact microsomal fraction is given in parentheses.

Species	Cytochrome *P*-450 (nmol/mg of protein)	Cytochrome b_5 (nmol/nmol of cytochrome *P*-450)	NADPH–cytochrome *c* reductase (units/nmol of cytochrome *P*-450)	*N*-Oxidation of *NN*-dimethylaniline (nmol/min per nmol of cytochrome *P*-450)
Guinea pig	2.48 ± 0.27 (1.33)	0.21 ± 0.03	47.1 ± 2.84	2.76 ± 0.61
Rat	1.47 ± 0.14 (0.82)	0.16 ± 0.01	26.5 ± 7.16	2.43 ± 0.45
Cat	0.51 ± 0.02 (0.20)	0.75 ± 0.01	31.0 ± 0.02	3.64 ± 0.06
Dog	0.80 ± 0.10 (0.48)	0.42 ± 0.16	11.7 ± 2.83	6.27 ± 2.82
Pig	0.77 ± 0.10 (0.60)	0.71 ± 0.04	89.1 ± 20.6	20.13 ± 1.84

the rabbit; the absorption band at 422nm, appearing in the dithionite-reduced sample on addition of CO, is probably due to the presence of small amounts of cytochrome b_5. As shown in Table 6, cytochrome b_5 constitutes about 20% of the total haemoprotein in the purified preparations, but no cytochrome *P*-420 was detected. The final preparations still contain sufficient amounts of NADPH–cytochrome *c* reductase to support *N*-oxidation of *NN*-dimethylaniline without the addition of exogenous reductase. The reaction requires the presence of intact cytochrome *P*-450, NADPH–cytochrome *c* reductase and lipid, as evidenced by impaired amine oxidation after modification of these factors (Table 6).

Cytochrome *P*-450 solubilized from the livers of other species was found to catalyse amine oxide formation from *NN*-dimethylaniline, too (Table 7). 1-(1-Naphthyl)-2-thiourea (0.1mM), a selective inhibitor of the mixed-function amine oxidase (Ziegler & Mitchell, 1972), was used to probe the preparations for the presence of contaminating oxidase; the compound, however, had only little effect on the *N*-oxidation reaction.

Although the widely differing NADPH–cytochrome *c* reductase contents of the preparations prevent direct comparison of the apparent molecular activities with each other, it seems that there are species-dependent differences in the catalytic capacity of the individual haemoproteins (Table 7). It is interesting to note that the pig, which has particularly high mixed-function amine oxidase activity, also appears to have high cytochrome *P*-450 activity for the *N*-oxidation of *NN*-dimethylaniline.

Influence of temperature on the N-oxygenating activity of rabbit liver microsomal fraction, solubilized cytochrome P-450 and mixed-function amine oxidase

Previous experiments demonstrated that preincubation of rabbit liver microsomal fraction at 37°C in the absence of NADPH rapidly destroys the enzyme activity for the *N*-oxidation of *NN*-dimethylaniline (Uehleke, 1973).

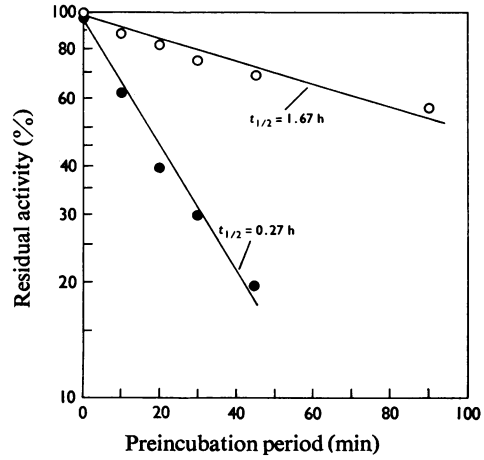

Fig. 2. *Effect of preincubating rabbit liver microsomal fraction and mixed-function amine oxidase in the absence of NADPH on the N-oxidation of NN-dimethylaniline*

Rabbit liver microsomal fraction and mixed-function amine oxidase were diluted with 0.15M-KH_2PO_4/Na_2HPO_4, pH7.4, to protein concentrations of 2mg/ml and 0.14mg/ml respectively. The samples were preincubated at 37°C in the absence of NADPH for the times indicated. At the end of the preincubation periods, the mixtures were supplemented with NADPH-generating system, and *N*-Oxidation was started by the addition of *NN*-dimethylaniline (final concentration 1.0mM). ●, *N*-oxide formed by microsomal fraction; ○, *N*-oxide formed by the purified mixed-function amine oxidase. The experimental points represent the means of four to seven determinations.

Preincubation of the purified mixed-function amine oxidase from rabbit liver at 37°C in the absence of the cofactor resulted in a slow loss of *N*-oxygenating activity for *NN*-dimethylaniline, the initial rate of inactivation being pseudo-first-order (Fig. 2), with a calculated rate constant of 0.41 h^{-1}.

When solubilized cytochrome *P*-450 from the rabbit was preincubated at 37°C in the absence of NADPH, a biphasic decrease in the *N*-oxygenating activity for *NN*-dimethylaniline was observed (Fig. 3), with rate constants of 5.78 h^{-1} and 0.19 h^{-1} calculated for the fast-phase and slow-phase component of the system. The ratio of the fast-phase to the slow-phase component was 1.4:1. The observed decrease in *N*-oxygenating activity is not the consequence of a heat-induced destruction of cytochrome *P*-450 (Fig 3).

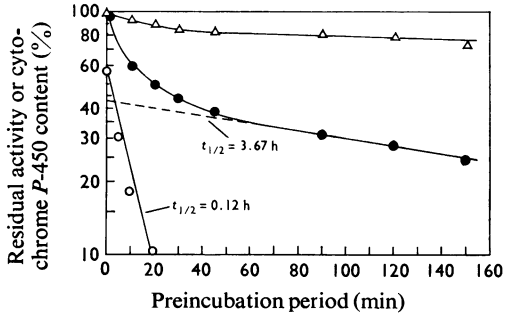

Fig. 3. *Effect of preincubating solubilized cytochrome P-450 from the rabbit in the absence of NADPH on the N-oxidation of NN-dimethylaniline*

Solubilized cytochrome *P*-450 was diluted with 0.15 M-KH_2PO_4/Na_2HPO_4, pH 7.4, to a final concentration of 1.43 μM and treated as described in the legend to Fig. 2. At the end of the preincubation periods, part of the mixtures was taken for measuring the decay of cytochrome *P*-450. ●, *N*-Oxide formed by solubilized cytochrome *P*-450; ○, corrected values for the fast-phase portion of the inactivation curve; △, cytochrome *P*-450 content of the assay media. The experimental points are the means of four determinations.

As the rate constant for heat-inactivation of the slow-phase component is close to that for inactivation of the mixed-function amine oxidase, experiments were conducted to rule out identity of both systems. Susceptibility of the slow-phase component, prepared by incubating samples of native cytochrome *P*-450 for five half-lives of the fast-phase component (35 min) at 37°C, to 0.1 mM-2-bromo-4′-nitroacetophenone was used as a probe (cf. Table 1). The inhibitor decreased the rate of the oxidation reaction catalysed by the slow-phase component from 2.1 (±0.3 S.E.M.) nmol of *N*-oxide formed/min per mg of protein ($n = 4$) to 1.2 (±0.2 S.E.M.) nmol of *N*-oxide formed/min per mg of protein ($n = 4$).

The biphasic decrease in *N*-oxygenating activity illustrated in Fig. 3 is not due to the presence in the cytochrome *P*-450 fraction of a family of NADPH–cytochrome *c* reductases with different sensitivity to heat, since exposure of the reductase to 37°C for 35 min did not affect its catalytic capacity.

Preincubation of rabbit liver microsomal fraction at 37°C in the absence of NADPH caused the complex system catalysing *N*-oxidation of *NN*-dimethylaniline to lose activity with a calculated rate constant of 2.61 h^{-1} (Fig. 2).

Comparative studies on the N-oxidation of NN-dimethylaniline, imipramine and trimethylamine by solubilized cytochrome P-450 and mixed-function amine oxidase

A concept has been developed suggesting that strongly basic amines are *N*-oxidized by the mixed-function amine oxidase, whereas less-basic amines may undergo *N*-oxidation by both the cytochrome *P*-450 system and the amine oxidase (Gorrod, 1973). To test this hypothesis, *NN*-dimethylaniline, imipramine and trimethylamine were compared with respect to rate of conversion into the corresponding *N*-oxides by these systems. The amines were selected because of their widely differing pK_a values.

Our data show that strongly basic amines are not

Table 8. *N-Oxidation of NN-dimethylaniline, imipramine and trimethylamine by solubilized cytochrome P-450 and mixed-function amine oxidase from the rabbit: influence of pK_a of substrate on the metabolic rate*

Assay conditions were as described in the Experimental section. In this set of experiments, the concentration of the amines was uniformly 0.1 mM. The results are expressed as means ± S.E.M. of six preparations.

		System	
Substrate	pK_a	Cytochrome *P*-450 (nmol of *N*-oxide formed/min per nmol of cytochrome *P*-450)	Mixed-function amine oxidase (nmol of *N*-oxide formed/min per nmol of flavin)
NN-Dimethylaniline	5.1	1.53 ± 0.03	4.45 ± 0.10
Imipramine	8.0	0.51 ± 0.04	0.36 ± 0.04
Trimethylamine	9.7	0.63 ± 0.11	1.72 ± 0.42

excluded from being *N*-oxidized by the cytochrome *P*-450 system (Table 8). *N*-oxidation of the alkylamines proceeds at a lower rate than *N*-oxide formation from the arylamine. The amine oxidase appears to have a higher *N*-oxygenating capacity as compared with the haemoprotein system. The *N*-oxidation products formed from imipramine, trimethylamine and *NN*-dimethylaniline by cytochrome *P*-450-dependent reactions were calculated to be in the molar proportions 1:1.2:3. The mixed-function amine oxidase was found to mediate *N*-oxidation of these compounds in the molar proportions 1:4.7:12.3.

Discussion

Our data establish unequivocal evidence for the existence of alternative metabolic routes of *N*-oxidation of *NN*-dimethylaniline in rabbit liver microsomal fraction.

One pathway involves the participation of the cytochrome *P*-450 system. Thus cytochrome *P*-450 solubilized from liver of rabbit and other species mediates *N*-oxidation of the tertiary arylamine (Tables 6 and 7). The data suggest the involvement of at least two types of the pigment in this *N*-oxidation process, characterized by differing sensitivity to heat (Fig. 3). The molar ratio of the two types of haemoprotein was calculated to be 1.4:1. This is close to 1.9:1 found for the biphasic incorporation of radioactivity from δ-amino[^{3}H]laevulinic acid into the liver microsomal cytochrome *P*-450 of adult male rats (Levin *et al.*, 1975). It remains unclear whether both types of cytochrome *P*-450 represent separate species of the haemoprotein independently synthesized in the rabbit liver, or two physical forms of a single pigment. The experiments with pregnenolone 16α-carbonitrile (Table 5) would favour the former view; previous results, however, demonstrated conversion of *NN*-dimethylaniline into the *N*-oxide by two interconvertible forms of a CO-sensitive monooxygenase (Hlavica, 1972; Hlavica & Kehl, 1974).

The other pathway is represented by the mixed-function amine oxidase. The enzyme differs from the cytochrome *P*-450 system with respect to sensitivity to heat (cf. Figs. 2 and 3), 2-bromo-4′-nitroacetophenone (Table 1) and steapsin (Table 2). It lacks NADPH dehydrogenase activity (Table 3) and catalyses *N*-oxidation of imipramine, trimethylamine and *NN*-dimethylaniline in molar proportions considerably different from those of the cytochrome *P*-450-supported reactions (Table 8).

The concept of two enzymes catalysing *N*-oxidation of *NN*-dimethylaniline is consistent with previous findings. Thus oxidation of the arylamine involves formation of two enzyme–*NN*-dimethylaniline complexes (Hlavica, 1970). Similarly, two enzyme–O_2 complexes are formed, the pK_a of only one of them being affected by the addition of CO (Hlavica & Kehl, 1974).

The pK_a value of amine substrates has been proposed to determine which of the *N*-oxygenating systems might act on these compounds (Gorrod, 1973). The data presented here clearly show that, at least in our case, differentiation by means of pK_a is not feasible (Table 8). Similarly, *N*-oxidation of the strong base dibenzylamine (pK_a 8.6) has been shown to implicate catalysis by both oxygenating systems (Beckett & Gibson, 1975). It seems that steric effects may not be disregarded in discussing interaction of the amines with the oxidases.

To what extent do both oxygenating systems contribute to the total turnover of *NN*-dimethylaniline giving rise to *N*-oxide formation? From the incomplete inhibition of amine oxide formation observed after treatment of rabbit liver microsomal fraction with 2-bromo-4′-nitroacetophenone, steapsin and menadione (Tables 1, 2 and 3) or pretreatment of rabbits with $CoCl_2$ (Table 4), the cytochrome *P*-450 system might be roughly estimated to account for at least 50–60% of the total amount of *NN*-dimethylaniline *N*-oxide formed, the remainder depending on the mixed-function amine oxidase. This view is strongly supported by the inactivation kinetics of rabbit liver microsomal fraction exposed to heat in the absence of NADPH, which closely resemble those of the fast-phase component of solubilized cytochrome *P*-450 and differ considerably from those of the mixed-function amine oxidase (cf. Figs. 2 and 3). However, it should be kept in mind that *NN*-dimethylaniline may undergo a number of other metabolic fates, such as *C*-oxidation. This could influence the quantitative basis of the argument on the activity of cytochrome *P*-450, since heat and the other modifiers might affect *N*-oxidation of the amine by changing the rates of some of the *C*-oxidation reactions.

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