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The microsomal *N*-oxidation of phentermine

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The microsomal *N*-oxidation of phentermine (Ia) to *N*-hydroxyphentermine (Ib) and to α,α-dimethyl-α-nitroso-β-phenylethane (Ic) was investigated. Maximum activities were obtained with microsomal (9000 *g* supernatant and microsomes) fractions of rabbit liver in the presence of an NADPH generating system. Incubation of Ia with hepatic washed microsomes from a phenobarbitone pretreated rabbit increased the formation of Ib and decreased that of Ic but the total amount of *N*-oxidized metabolites (i.e. Ib + Ic) was not affected. The ratio of the metabolically produced Ic to Ib but not the total amount of *N*-oxygenated metabolites varied greatly depending of the liver microsomal fractions used in the incubation mixtures of Ia; more Ib was produced from Ia using 9000 *g* supernatant and conversely, more Ic was formed using the washed microsomes of the same liver. The nitroso compound (Ic) was metabolically reduced to Ib and Ib to Ia by the hepatic 9000 *g* supernatant and soluble fraction; under the same conditions, washed microsomes had only limited reductive properties towards Ic and Ib. *N*-Hydroxyphentermine (Ib) was not metabolically oxidized to Ic when incubated with washed microsomes from rabbit liver. The use of known carbon-oxidation inhibitors showed that cytochrome P-450 is not involved in the incorporation of oxygen at the nitrogen centre of Ia. The metabolic characteristics and kinetic behaviour of the microsomal *N*-oxidation of Ia supported a recently proposed mechanism explaining the independent formation of Ib and Ic from a common precursor resulting from metabolic *N*-oxidation of Ia.

N-Hydroxyphentermine (Ib) and α,α-dimethyl-α-nitroso-β-phenylethane (Ic) were identified as the major *N*-oxygenated metabolites of the anorexic drug phentermine (Ia) when incubated with hepatic microsomal fractions preparations from rabbit and guinea-pig (Beckett & Bélanger, 1974a). They have also been found in human urine following a single dose of Ia (Beckett & Bélanger, 1974b); *p*-hydroxyphentermine has also been identified in the urine of rats (Weischer & Opitz, 1967) and man (Cho, 1974) after dosing with Ia. A two-enzyme model system including a zero-order rate and a substrate-dependent saturable system has been recently proposed to describe the *in vitro* formation of *N*-hydroxyphentermine (Ib) from phentermine (Ia) (Cho, Lindeke & Sum, 1974). Previously, the same group reported the identification of Ib as a metabolite of Ia using a microsomal fraction from rabbit liver (Cho, Lindeke & Hodshon, 1972).

The *in vitro* metabolic characteristics of the *N*-oxidation of phentermine (Ia) to *N*-hydroxyphentermine (Ib) and to α,α-dimethyl-α-nitroso-β-phenylethane (Ic) are now reported.

MATERIALS AND METHODS

Compounds and reagents

Phentermine hydrochloride was provided by Riker Laboratories (England). *N*-Hydroxyphentermine (Ib) as the oxalate, α,α-dimethyl-α-nitroso-β-phenylethane (Ic) and α,α-dimethyl-α-nitro-β-phenylethane (Id) were prepared as described by Beckett & Bélanger (1974a). The following compounds were used: 1,10-phenanthroline hydrate, 4-chloromercuribenzoic acid, nicotinamide, *N*-ethylmaleimide, sodium cyanide, sodium azide, dithiothreitol, from BDH, potassium iodide and ethylenediaminetetraacetate disodium salt ($EDTANa_2$), from May & Baker Ltd. $NADPNa_2$, $NADH_2$, glucose-6-phosphate disodium salt and glucose-6-phosphate dehydrogenase were obtained from Boehringer. Catalase from bovine liver (as purified powder) was purchased from Sigma Chemical Co.

Preparation of the liver homogenates

Young adult animals [New Zealand white rabbits (Great Toteas, Buckstead), 1·5 - 3·0 kg; Albino Dunkin Hartley guinea-pig (Redfern Animal Supplies), 750 g; Laca mouse (Tuck & Son, Southend), 30 g; South Down Warren chick (South Down Hatcherie's, Uckfield), 100 g; Syrian hamster (Ex-Chester Beatty), 100 g and Wistar rat (CFHB Carworth), 350 g] were killed, the livers rapidly removed and the 9000 *g* supernatant, soluble fraction and microsomes prepared as described (Beckett & Bélanger, 1974a). Washed microsomes were prepared by resuspending the microsomal pellets in 0·25 M tris-KCl buffer (pH 7·4) and centrifuging at 140 000 *g* for 1 h at 0°. Washed microsomes from rabbit lungs and kidneys were similarly pre-

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pared. All homogenate fractions were resuspended in fresh isotonic tris-KCl buffer at a final homogenate concentration equivalent to 0·5 g of liver ml^{-1}. In other experiments phenobarbitone (80 mg kg^{-1}, i.p.) was injected daily to rabbits for three days before killing (on the fourth day) and the hepatic homogenates prepared as described above.

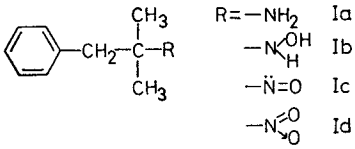
Incubation experiments

Incubations were in 25 ml conical flasks in a water bath at 37° with shaking. Unless otherwise stated, each incubation mixture contained NADP (as disodium salt, 3·4 mg, 4 μmol), glucose-6-phosphate (as disodium salt, 6 mg, 10 μmol), nicotinamide (0·1 ml of a 0·6 M solution in water, 60 μmol), $MgCl_2$ (0·2 ml of a 0·01 M solution in water, 20 μmol) all added in water to a final volume of 1 ml, phosphate buffer pH 7·4 (British Pharmacopoeia, 1968, p. 1362; 3 ml), homogenate fraction (1 ml, equivalent to 0·5 g of original liver) and substrate in water (1 ml), giving a total incubation volume of 6 ml. Glucose-6-phosphate dehydrogenase (2 units per flask) was added to the incubation mixture when microsomes or washed microsomes were used. In one experiment, various concentrations of homogenate equivalent to 0·25, 0·5, 0·75 and 1 g of liver were made up by diluting the washed microsomes with the phosphate buffer pH 7·4. When compounds having a potential inhibiting action (with a final concentration of 1 mM in each flask) were added to the incubation mixtures, they were dissolved in a phosphate buffer (pH 7·4) immediately before the experiment. Unless otherwise stated, incubations were carried out with freshly prepared homogenates at pH 7·4. In all cases, the incubation mixtures were incubated for 5 min at 37° with shaking before the addition of substrate; after addition, the incubations were carried out for various periods of time as described in Results. Using these conditions, phentermine (Ia, as HCl, 0·25–10 μmol in water, 1 ml), *N*-hydroxyphentermine as oxalate (Ib; 0·17, 0·22, 0·34 and 0·5 μmol base in water, 1 ml; freshly prepared solutions) and α,α-dimethyl-α-nitroso-β-phenylethane (Ic; 0·4, 0·411, 0·5, 0·54 and 0·68 μmol of the monomer in methanol, 0·1 ml; freshly prepared solutions) were incubated with homogenates. When not used, the homogenates were stored at 4°.

The apparent Km and the maximum rates of metabolism (Vmax) for the microsomal *N*-oxidation of phentermine (Ia) were determined over the concentration range of 0·25 to 10 μmol per 6 ml (0·0416–1·666 mM) using rabbit 9000 *g* supernatant and washed microsomes of the same liver prepared the same day. The data were plotted according to the Lineweaver-Burk (1/V *vs* 1/S) and Hofstee (V *vs* V/S) methods and subjected to regression analysis to give the appropriate Km and Vmax values.

Analysis of phentermine (Ia) and its metabolic products

After completion of the incubation, the metabolic reaction was stopped by rapidly placing the flask in an ice tray at 0°. Phentermine (Ia), its metabolic products Ib and Ic and the total *N*-oxygenated



metabolites were determined by gas-liquid chromatography using the methods described for the analysis of mephentermine and its metabolic products (Beckett & Bélanger, 1975). Phentermine (Ia) and *N*-hydroxyphentermine (Ib) were analysed quantitatively as the trifluoroacetyl and trimethylsilyl derivatives respectively. The total *N*-oxidized metabolites of Ia, i.e. *N*-hydroxyphentermine (Ib) and α,α-dimethyl-α-nitroso-β-phenylethane (Ic), were determined by the amount of α,α-dimethyl-α-nitro-β-phenylethane (Id) present in the ethereal extract of the incubation mixture (6 ml) after oxidation with potassium permanganate (1% solution, 1 ml) since it has been established that Ib and Ic, but not Ia, are oxidized quantitatively to the nitro compound (Id) under these conditions (Beckett & Bélanger, 1975). The amount of the nitroso compound (Ic) present in the incubation mixture was determined by subtracting the amount of *N*-hydroxyphentermine (Ib) from that of total *N*-oxidized metabolites of Ia.

Determination of protein and cytochromes P-450 and b_5 *contents*

The protein content of the homogenates was determined by the method of Lowry, Rosebrough & others (1951) using serum bovine albumin as standard. The concentrations of cytochromes P-450 and b_5 in homogenates were determined by the methods of Omura & Sato (1964) using an extinction coefficient of 91 $mM^{-1}\,cm^{-1}$ for the difference in absorbance of the reduced carbon monoxide-cytochrome P-450 complex between 450 and 490 nm

and an extinction coefficient of 171 $mM^{-1} cm^{-1}$ for the difference in absorbance of the reduced cytochrome b_5 between 424 and 480 nm.

RESULTS AND DISCUSSION

When phentermine (Ia) was incubated with homogenate from different organs of rabbit in the presence of a NADPH generating system, the activity to *N*-oxidize this substrate to *N*-hydroxyphentermine (Ib) and α,α-dimethyl-α-nitroso-β-phenylethane (Ic) was mainly localized in the liver microsomal fractions (Table 1). Little metabolism occurred in the liver soluble fraction. Lung and kidney washed microsomes had roughly 20% of the specific activity of the liver washed microsomes under the same conditions (Table 1) although the former are known to *N*-oxidize some aromatic primary and secondary amines at greater or equal rates than hepatic microsomes (Uehleke, 1973).

The amounts of unchanged phentermine (Ia) and total *N*-oxidized metabolites, i.e. Ib and Ic, determined after incubation of Ia for 60 min with liver 9000 *g* supernatant and washed microsomes were 4·7 and 0·26 (±0·06) μmol respectively; the total recovery of substrate moiety was 99·2% (4·96 μmol). Therefore, phentermine (Ia) was not metabolized by routes other than metabolic *N*-oxidation to the hydroxylamino (Ib) and C-nitroso (Ic) compounds under the present conditions of incubation.

The microsomal *N*-oxidation of phentermine (Ia) required reduced pyridine cofactors for maximum activity; without a NADPH generating system, liver microsomal fractions catalysed the *N*-oxidation of Ia only to a slight extent (Table 1). Doubling the amount of NADP of the standard cofactor solution (see experimental) did not increase the amount of total *N*-oxidized metabolites (Bélanger, 1975).

Of the species tested, the best *N*-oxidizing activity of Ia was found in rabbit liver microsomes, since microsomal preparations from guinea-pig, mouse, chick, hamster and rat livers have activities of 10% and less of rabbit liver under the same conditions of incubation (Bélanger, 1975). Therefore, hepatic microsomal fractions from rabbit were subsequently used in all experiments.

The 9000 *g* liver supernatants gave relative amounts of the hydroxylamino (Ib) and nitroso (Ic) compounds different from those using microsomes (Table 1) but the total amounts of *N*-oxygenated metabolites were similar. Much more hydroxylamine (Ib) was formed when phentermine (Ia) was incubated with the 9000 *g* supernatant but more nitroso (Ic) from the microsomes of the same liver under the same conditions of incubation. Washing the hepatic microsomes led to a loss of activity but altered only slightly the ratio of the nitroso compound (Ic) to *N*-hydroxyphentermine (Ib). However, considerable inter-experiment variations occurred in the ratio of the metabolites Ic to Ib.

Incubation of Ia with phenobarbitone-induced washed microsomes from rabbit liver did not affect the total *N*-oxidation of Ia but altered the ratio of the nitroso (Ic) to the hydroxylamino (Ib) compounds because formation of Ic was decreased by 85% while that of Ib was increased by 400% (Table 1). In these preparations, the amount of protein and that of cytochrome P-450 per mg of protein were roughly double those of the non-induced washed microsomes; the amount of cytochrome b_5 per mg of protein was not significantly affected by the phenobarbitone pretreatment. The increase in amount of Ib formed did not parallel that of cytochrome P-450.

Incubation of α,α-dimethyl-α-nitroso-β-phenylethane (Ic) with liver microsomal fraction from rabbits

Because the ratio of the metabolically produced nitroso (Ic) to *N*-hydroxyphentermine (Ib) varied greatly between experiments, the separate metabolism of Ic and Ib under the same conditions was investigated. α,α-Dimethyl-α-nitroso-β-phenylethane (Ic) is metabolically reduced mainly to Ib (45 ± 12%) when incubated for 1 h with hepatic 9000 *g* supernatants from rabbit under aerobic conditions but only to a small extent (8·7 ± 2%) using washed microsomes (means of 4 experiments). In absence of an NADPH generating system, only small amounts of Ib (2·5%) were formed using washed microsomes. Thus, this reduction step is enzymic and is mainly localized in the soluble fraction. The enzymic reduction of aromatic nitro compounds to their corresponding amines occurs in the soluble fraction (cytosol) and microsomes from the liver of rabbit and many other species but anaerobic conditions are considered essential (Mitchard, 1971; Poirier & Weisburger, 1974; Symms & Juchau, 1974). However, aromatic C-nitroso and hydroxylamino compounds have been shown to be intermediates in the course of the enzymic reduction of aromatic nitro compounds to the corresponding amines (Gillette, 1963; Uehleke, 1963; Uehleke & Nestel, 1967; Gillette, Kamm & Sasame, 1968).

No increase in the amounts of *N*-hydroxyphentermine (Ib, 8·8 ± 3·3% of the amounts of Ic) formed was obtained when Ic was incubated for

1 h with washed hepatic microsomes from rabbits pretreated with phenobarbitone. In fact, the specific activity of the microsomal C-nitroso reductase must have decreased by 50% because the amount of protein in the phenobarbitone-induced washed microsomes was roughly double that present in the normal (non-induced) washed liver microsomes. The difference in the rate of reduction of Ic to Ib may account for the differences in the amounts of Ib and Ic detected in the incubation mixtures of Ia using 9000 *g* supernatants or washed microsomes (Table 1) and for the different kinetic characteristics observed (see later).

Incubation of N-*hydroxyphentermine (Ib) with liver homogenate fractions from rabbit*

N-Hydroxyphentermine (Ib) is mainly metabolized by reduction to phentermine (Ia) when incubated for 1 h with rabbit liver homogenates fortified with an NADPH generating system under aerobic conditions. The rate of reduction of Ib to Ia was fast (81·6 ± 2·7%) using the 9000 *g* supernatants and very slow (7·3 ± 1·3%) using the washed microsomes. Under the same conditions, a significant amount of phentermine (Ia, 22·2% of the amount of Ib incubated) was produced when Ib was incubated with the liver soluble fraction and the results suggest that both the microsomal and the soluble fraction combined together (9000 *g* supernatant) are needed for optimum reduction. However, Ib was not further oxidized and was recovered almost completely unchanged (95·6 ± 2·7%) when incubated for 1 h with washed liver microsomes under the same conditions that catalysed the metabolic *N*-oxidation of Ia to Ib and Ic. Thus, the nitroso compound (Ic) is not derived from the microsomal oxidation of *N*-hydroxyphentermine (Ib) in the present conditions of incubation of phentermine (Ia).

A microsomal enzyme which catalyses the reduction of various hydroxylamines has been recently purified from pig liver microsomes (Kadlubar, McKee & Ziegler, 1973; Kadlubar & Ziegler, 1974). The same group reported hydroxylamine oxidase activity in pork and rat liver microsomes (Kadlubar & others, 1973; Poulsen, Kadlubar & Ziegler, 1974). However, the hydroxylamine oxidase activity was only specific for secondary hydroxylamines under specific kinetic conditions and many primary hydroxylamino compounds including *N*-hydroxyamphetamine, a metabolite of amphetamine (Beckett & Al-Sarraj, 1972), were not oxidized by this enzyme. The rapid reduction of Ib in the liver 9000 *g* supernatant may also be responsible for the variation in the apparent rate of formation of Ib upon incubation of Ia.

In vitro *kinetic studies*

The rates of *N*-oxidation of Ia to Ib and Ic when incubated with hepatic washed microsomes from

Table 1. *The* in vitro *metabolism of phentermine (5 μmol per 6 ml) with rabbit homogenate fractions from different organs at 37° in the presence or not of a NADPH generating system.* Results are expressed as a mean value ± mean deviation; the number of experiments is in parentheses. R is the ratio of the metabolically produced nitroso (Ic) to the hydroxylamine (Ib) compounds calculated by dividing the amounts of Ic (nmol) by that of Ib (nmol). nmol mg^{-1}: nmol mg^{-1} of protein.

Fractions	Incubation time (min)	Amounts of metabolites formed: Total *N*-oxidized metabolites (nmol)	Total *N*-oxidized metabolites (nmol mg^{-1})	*N*-Hydroxyphentermine (Ib) (nmol)	*N*-Hydroxyphentermine (Ib) (nmol mg^{-1})	α,α-Dimethyl-α-nitroso-β-phenylethane (Ic) (nmol)	α,α-Dimethyl-α-nitroso-β-phenylethane (Ic) (nmol mg^{-1})	R
Liver 9000 *g* supernatant	60	320 ± 40 (6)*a*	11·5 ± 2·3 (6)	119·6 ± 23·9 (7)	5·0 ± 1·8 (7)	130·1 ± 36·4 (7)	3·8 ± 0·9 (7)	1·1
Liver soluble fraction	60	33 ± 12·5 (4)	1·1 ± 0·4 (4)	28 ± 9·9 (3)	0·9 ± 0·2 (3)	13·2 ± 0·3 (2)	0·6 ± 0·2 (2)	0·5
Liver microsomes	60	303 ± 0 (2)	55·1 ± 0 (2)	67·5 ± 12·5 (2)	12·3 ± 2·3 (2)	235·5 ± 12·5 (2)	42·8 ± 2·3 (2)	3·5
Liver washed microsomes	60	200·0 ± 16·7 (13)*b*	26·4 ± 3 (13)	47·8 ± 5·6 (14)	6·4 ± 0·9 (14)	142·1 ± 18·8 (9)	16·6 ± 1·7 (9)	3·0
Liver washed microsomes*c*	60		2·9 ± 0·4 (2)		1·1 ± 0·5 (2)		1·9 ± 0·1 (2)	
Liver PB washed microsomes*d*	60		28·3 ± 1·8 (2)		27·4 ± 5·3 (2)		2·3 ± 2 (2)	0·08
Liver washed microsomes	30	145·6 ± 18 (2)	21·4 ± 2·7 (2)	35·1 ± 4·5 (2)	5·2 ± 0·7 (2)	110·5 ± 13·6 (2)	16·3 ± 2 (2)	3·1
Lung washed microsomes	30	6·9 ± 0·1 (2)	3·9 ± 0 (2)	3·6 ± 0·2 (2)	2·1 ± 0·2 (2)	3·3 ± 0·2 (2)	1·9 ± 0·2 (2)	1
Kidney washed microsomes	30	13·4 ± 2·3 (2)	3·3 ± 0·6 (2)	3·7 ± 1·4 (2)	0·9 ± 0·3 (2)	9·7 ± 3·7 (2)	2·4 ± 0·9 (2)	2·9

a and *b*: The recovery of unchanged phentermine using 9000 *g* supernatant (mean of 4 experiments) and washed microsomes (mean of 6 experiments) was 4·7 μmol in each case.
c: NADP was omitted from the cofactor solution.
d: phenobarbitone pretreated rabbit.

rabbit were linear with time to at least 30 min (Fig. 1) and linear with microsomal concentrations over the range of 6·6 to 26·4 mg of protein equivalent to 0·25–1 g of original liver (Bélanger, 1975). However, the rates of formation of Ib and Ic upon incubation of Ia with the 9000 *g* supernatant of the same liver were not linear with time (Fig. 1).

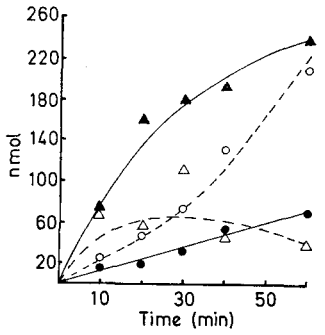


FIG. 1. The effect of duration of incubation time (min) on the *in vitro* *N*-oxidation of phentermine (Ia, 5 μmol per 6 ml) to *N*-hydroxyphentermine (Ib, ○ and ●) and α,α-dimethyl-α-nitroso-β-phenylethane (Ic, △ and ▲) using 9000 *g* supernatant (dotted line) and washed microsomes (solid line) from the same rabbit liver. Each point is the mean value of two incubations. Abscissa-amounts of metabolites formed (nmol).

When reaction rates were plotted as a function of substrate concentration from 0·25 to 10 μmol per 6 ml (0·041–1·666 mM), a typical saturation curve was obtained for the formation of total *N*-oxidized metabolites of Ia when incubated with hepatic washed microsomes from rabbit (Fig. 2A); an atypical curve suggesting substrate activation or more complex kinetic behaviour was obtained for the total *N*-oxidation of Ia using the 9000 *g* supernatant of the same liver under the same conditions (Fig. 2B). It should be noted that the ratio of the metabolites Ic to Ib varied greatly with respect to the substrate concentration incubated, the time of incubation and the microsomal fraction used (Figs 1 and 2). Good fitting curves were obtained when the data of the total *N*-oxidized metabolites formed upon incubation of Ia with the hepatic 9000 *g* supernatant and washed microsomes were plotted according to Lineweaver-Burk method (Table 2) thus suggesting the presence of a single enzyme system. The Km value for the total *N*-oxidation of Ia using the 9000 *g* supernatant is roughly double that obtained with the washed microsomes of the same liver; this discrepancy is explained by the fact that washing the microsomes results in 50% loss of the specific activity for the total microsomal *N*-oxidation of Ia (see Table 1). The amounts of

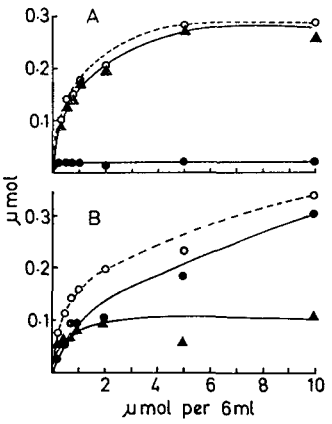


FIG. 2. The effect of substrate (phentermine) concentration (μmol per 6 ml) on the formations of total *N*-oxidized metabolites (–○–), *N*-hydroxyphentermine (Ib, –●–) and α,α-dimethyl-α-nitroso-β-phenylethane (Ic, ▲) using rabbit washed microsomes (A) and 9000 *g* supernatant (B) from the same liver. The incubation time was 60 min. Each point is the mean value of two incubations. Abscissa—amounts of metabolites formed (μmol).

Ib formed from Ia with hepatic washed microsomes were independent (zero order) of the substrate concentrations used (Fig. 2A); the major *N*-oxygenated metabolite, the nitroso compound (Ic), gave Km and Vm values closely related to those of the total *N*-oxidation of Ia (Table 2). Under identical conditions, different kinetic behaviour was observed when different concentrations of

Table 2. *Enzyme-substrate characteristics (Km and Vmax) for the total* N-*oxidation of phentermine (Ia), the formation of* N-*hydroxyphentermine (Ib) and that of α,α-dimethyl-α-nitroso-β-phenylethane (Ic) by rabbit washed microsomes and 9000* g *supernatant from the same liver.* The incubation time was 60 min. Data were plotted according to Lineweaver-Burk method and submitted to regression analysis to give the appropriate Km and Vmax values. Km and Vmax represent μmol per 6 ml and μmol per 0·5 g of liver per 60 min respectively.

Metabolic reaction	No. of data	Corr. coeff.	% of fit of curve	Km	Vmax
Washed microsomes					
Total *N*-oxidized metabolites	13	+0·98	96·3	0·38	0·26
Formation of Ib	7	−0·45	20·1	−0·05	0·01
Formation of Ic	7	+0·99	97·8	0·50	0·26
9000 *g* supernatant					
Total *N*-oxidized metabolites	14	+0·99	97·6	0·73	0·28
Formation of Ib	14	+0·97	94·2	3·4	0·38
Formation of Ic	12	+0·34	11·8	0·11	0·07

Ia were incubated with the 9000 *g* supernatant of the same liver (Fig. 2B). Poor correlations were obtained when the appropriate kinetic data of the rates of formation of Ic were plotted (Table 2). The rate of formation of Ib was not zero-order with respect to the substrate concentrations incubated; the Hofstee plot (V *vs* V/S) of the data yielded a biphasic curve as recently reported for the formation of Ib with rabbit liver microsomes (Cho & others, 1974) suggesting a multi-enzyme system.

The kinetics of formation of Ib and Ic upon incubation of Ia with hepatic microsomal fractions from rabbits suggested that Ib and Ic were formed independently since no lag period for the formation of these two *N*-oxidized metabolites of Ia was obtained (Fig. 1). Furthermore, the separate incubation of Ib with liver washed microsomes under the same conditions did not give Ic although the latter is metabolically reduced to Ib; the rate was very slow using the washed microsomes and thus this reaction is unlikely to be responsible for the metabolic formation of Ib especially at the early stage of incubation of Ia with the microsomes (Fig. 1).

The kinetics of the microsomal formation of total *N*-oxidized metabolites of phentermine (Ia) suggest that a single enzyme system catalyses the incorporation of oxygen at the nitrogen centre of Ia to give both Ib and Ic, i.e. that Ib and Ic are derived from the same metabolic precursor. Furthermore, the independent direct formation of C-nitroso (Ic) and hydroxylamino (Ib) compounds from phentermine (Ia) indicates the final involvement of one oxygen atom in the immediate precursor of Ib and two in the case of Ic and is explicable in terms of a mechanism of microsomal *N*-oxidation involving formation of an oxygen radical/flavoprotein anion associated with the nitrogen radical cation in a complex as proposed recently by Beckett & Bélanger (1974c). The rate of dissociation to a hydroperoxide versus the metabolic reduction of this complex controls the formation of α,α-dimethyl-α-nitroso-β-phenylethane (Ic) and *N*-hydroxyphentermine (Ib) respectively. The present results indicate that the rate of metabolic reduction of the precursor complex of Ib and Ic (Beckett & Bélanger, 1974c) is increased by phenobarbitone pretreatment of the rabbit to favour the formation of Ib instead of Ic from Ia.

Effects of potential inhibitory compounds on the microsomal N-*oxidation of phentermine (Ia)*

The effects of the compounds on the microsomal *N*-oxidation of Ia to Ib and Ic and on the microsomal reduction of Ic to Ib are listed in Table 3.

Sodium cyanide, sodium azide, and ethylenediaminetetraacetate disodium ($EDTANa_2$) increased the total *N*-oxidation of Ia when present at concentration of 1 mM in the incubation mixtures; nicotinamide, potassium iodide, *N*-ethylmalaimide and dithiothreitol had no significant effect on the *in vitro* metabolism of Ia. Sodium cyanide and sodium azide are known inhibitors of microsomal alcohol

Table 3. *The effect of potential inhibitors (1* mM*) and catalase (100 μg per 6 ml) on (A) the* in vitro N-*oxidation of phentermine (Ia, 2 μmol per 6 ml) and (B) the* in vitro *reduction of* α,α-*dimethyl-*α-*nitroso-*β-*phenylethane (Ic, 0·54 μmol per 6 ml incubated) to* N-*hydroxyphentermine (Ib) with hepatic washed microsome from rabbit.* The incubation time was 60 min. Results are expressed as a mean percentage value ± mean deviation of duplicate experiments compared to control incubation of Ia (100 %) in A and Ic (100 %) in B.

Potential inhibitors	Total *N*-oxidized metabolites	A % formation of *N*-hydroxy-phentermine	α,α-Dimethyl-α-nitroso-β-phenyl-ethane	B % reduction to *N*-hydroxy-phentermine
None (control)	100	100	100	100
Sodium cyanide	138 ± 10·1	179·8 ± 12	128 ± 9·5	140 ± 25
Sodium azide	128 ± 8·5	352·1 ± 7·5	70·5 ± 29·4	263·8 ± 6·1
Ethylenediaminetetraacetate disodium ($EDTANa_2$)	114 ± 6·4	270·1 ± 4·4	47·7 ± 0	510·2 ± 1·6
Nicotinamide	97 ± 2	95·2 ± 2	100·7 ± 7	—
Potassium iodide	101·8 ± 0·1	111·4 ± 18	83 ± 32	—
N-Ethylmaleimide	107·6 ± 7·1	118·4 ± 24	105 ± 16	137 ± 17
Dithiothreitol	94·7 ± 1·3	96 ± 15	95·2 ± 2·4	—
1,10-Phenanthroline hydrate	81·4 ± 3·4	84·5 ± 13	81·7 ± 1	139 ± 31
4-Chloromercuribenzoic acid	0 ± 0	0 ± 0	0 ± 0	—
Catalase	94 ± 3	93 ± 1	94 ± 4	—

mixed-function oxidase (Orme-Johnson & Ziegler, 1965) but had no effect on a purified amine oxidase (Zeigler & Petit, 1966). Ethylenediaminetetra-acetate (EDTA), an inhibitor of lipid peroxidation in rabbit microsomes (Jacobson, Levin & others, 1973), increases the microsomal *N*-oxidation of imipramine (Gigon & Bickel, 1971). Cyanide is a potent inhibitor of microsomal carbon-oxidation systems (Mitoma, Posner & others, 1956; Creaven, Parke & Williams, 1965) and binds to cytochrome P-450 (Jefcoate, Gaylor & Calabrese, 1969) in rabbit liver; also iodine under the same conditions used herein destroys cytochrome P-450 (Imai & Sato, 1967; Ullrich, 1969). Because these compounds do not decrease the *N*-oxidation of Ia (Table 3), cytochrome P-450 cannot be involved in the microsomal *N*-oxidation of Ia.

Nicotinamide is widely used *in vitro* in incubation mixtures to inhibit the microsomal nucleotidase of liver which destroys NADP (Mann & Quastel, 1941) although it inhibits some microsomal carbon-oxidation systems (Schenkman, Ball & Estabrook, 1967; Sasame & Gillette, 1970). Under the present experimental conditions, addition of an excess of nicotinamide (1 mM) to that present in the NADPH generating system did not affect the *N*-oxidation of Ia or modify the ratio of the metabolites Ic to Ib. However, Cho & others (1974) reported an increase of the amount of Ib following incubation of Ia (in the presence of 16·7 mM of nicotinamide) but, as recently reported, the concentration of nicotinamide added is an important factor in determining its effects on microsomal catalysed reactions (Parli & Mannering, 1971). The *N*-oxidation of Ia was inhibited slightly by 1,10-phenanthroline and completely by 4-chloromercuribenzoic acid.

More significant was the effect of sodium cyanide, sodium azide and EDTA on the ratio of the nitroso (Ic) to the hydroxylamino (Ib) compounds apparently greatly favouring the formation of Ib from Ia. The interpretation is complicated because separate incubation of Ic in the presence of the above compounds led to an increase in the rate of metabolic reduction of Ic to Ib. Catalase had only a slight inhibitory effect on the microsomal *N*-oxidation of Ia to Ib and Ic, thus precluding a role for hydrogen peroxide as the endogeneous oxidant of Ia.

In conclusion, the described properties of the microsomal *N*-oxidation of the primary amine phentermine (Ia) supported a recently proposed mechanism (Beckett & Bélanger, 1974c) explaining the independent formation of α,α-dimethyl-α-nitroso-β-phenylethane (Ic) and *N*-hydroxyphentermine (Ib) from a common precursor resulting from metabolic *N*-oxidation attack of Ia. The failure of some known microsomal carbon-oxidation inhibitors to effect the *in vitro* *N*-oxidation of phentermine (Ia) supports the conclusion that *N*-oxidation and carbon-oxidation involve separate metabolic pathways (Beckett, 1971) and that cytochrome P-450 is not involved in the incorporation of oxygen at the nitrogen centre.

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