Biochemical Pharmacology, Vol. 26, pp. 1455–1462. Pergamon Press, 1977. Printed in Great Britain.

THE *IN VITRO* METABOLISM OF MEPHENTERMINE

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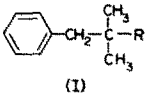
(*Received* 24 *August* 1976; *accepted* 15 *February* 1977)

Abstract—The metabolism of mephentermine (Ie) to phentermine (Ia), *N*-hydroxyphentermine (Ib) and *N*-hydroxymephentermine (If) was investigated using hepatic microsomal preparations from various species. Liver microsomes from rabbit were the best homogenate fractions to metabolise Ie. The effect of incubation times, various substrate concentrations, addition of selective inhibitors and activators and species differences suggested that the dealkylation of Ie to Ia involved a separate metabolic route than the *N*-oxidation of Ie to Ib and If and that both hydroxylamino compounds Ib and If resulted from metabolic oxidation at the nitrogen atom of Ie. A mechanism explaining the separate formation of Ib and If through a common intermediate resulting from *N*-oxidation of Ie is proposed. The synthesis and the properties of *N*-methyl-(α,α-dimethyl-β-phenethyl)nitrone (Ig) are reported.

Tertiary and secondary amines are metabolised *in vitro* and *in vivo* by oxidative dealkylation believed to involve the incorporation of atmospheric oxygen at the α-carbon atom to the nitrogen to yield unstable α-carbinolamines which spontaneously break down to secondary and primary amines respectively and a carbonyl compound usually an aldehyde [1–4]; in some cases, some stabilised α-carbinolamine intermediates have been isolated [5–7].

N-Oxidation is an important metabolic route *in vitro* and *in vivo* in animals and man [3, 8–14]. Tertiary amines yield *N*-oxides [8]; secondary amines yield hydroxylamines and nitrones, if they possess at least one hydrogen atom on the α-carbon [15], while primary amines give hydroxylamines and C-nitroso-compounds if they have no hydrogen atom on the α-carbon [13, 14]. *N*-Oxides which rearrange to α-carbinolamines by intramolecular migration of the oxygen atom have been proposed and disputed as intermediates for the oxidative dealkylation of tertiary to secondary amine [4, 16–24].

The primary amine phentermine (Ia), *N*-hydroxyphentermine (Ib) and *N*-hydroxymephentermine (If) were identified as metabolites (Fig. 1) of the secondary amine mephentermine (Ie) when incubated with hepatic microsomal fractions from rabbit [25]. The characteristics of these two metabolic routes are now reported.



(I)

Compounds	R
Phentermine (Ia, α,α-dimethylphenethylamine)	NH_2
N-Hydroxyphentermine (Ib, *N*-hydroxy-α,α-dimethylphenethylamine)	
α,α-Dimethyl-α-nitroso-β-phenylethane (Ic)	N=O
α,α-Dimethyl-α-nitro-β-phenylethane (Id)	
Mephentermine (Ie, *N*-methyl-α,α-dimethylphenethylamine)	
N-Hydroxymephentermine (If, *N*-hydroxy-*N*-methyl-α,α-dimethyl-β-phenethylamine)	
N-Methyl-(α,α-dimethyl-β-phenethyl) nitrone (Ig)	

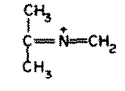


Fig. 1. Structure of mephentermine, its metabolic products, related compounds and characteristic mass fragment ion (i) of *N*-methyl-(α,α-dimethyl-β-phenethyl) nitrone.

MATERIALS AND METHODS

Compounds. Phentermine hydrochloride and mephentermine sulfate were kindly supplied by Riker Laboratories (Loughborough) and John Wyeth and Brother (Berkshire) respectively. *N*-Hydroxyphentermine (Ib), *N*-hydroxymephentermine (If), α-α-dimethyl-α-nitroso-β-phenylethane (Ic) and α,α-dimethyl-α-nitro-β-phenylethane (Id) were prepared as described [13, 25]. All the drugs and their metabolic products were checked for purity by thin-layer and gas–liquid chromatography. The following compounds were used: sodium cyanide, sodium azide, 4-chloromercuribenzoic acid, dithiothreitol, *N*-ethylmaleimide, 2-mercaptoethylammonium chloride (cysteamine hydrochloride), all from B.D.H., potassium iodide, ethylenediaminetetraacetate disodium salt (EDTA Na_2) formaldehyde solution (40% in water) from May and Baker Ltd. NADP Na_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.

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Synthesis of N-methyl-(α,α-dimethyl-β-phenethyl) nitrone (Ig). Nitrones can be prepared chemically in good yield by condensation of primary hydroxylamines with a carbonyl compound [26]. *N*-Hydroxyphentermine (Ib) was extracted with ether from a freshly prepared solution of the oxalate (54 mg) in phosphate buffer pH 7.4, the organic extract dried over $MgSO_4$ (anhyd.), filtered, evaporated in vacuo and the residue dissolved in benzene (50 ml) in a round bottom flask. Formaldehyde solution (40% in water, 1 ml) was added and the mixture refluxed for 4 hr, then the solvent evaporated off, the residue dissolved in ether, the solution dried ($MgSO_4$ anhyd.), filtered and evaporated to yield a solid residue having a strong smell of formaldehyde. This residue was devoid of any detectable amount of compounds Ib and If by previously described gas–liquid and thin-layer chromatography system [25]. The nitrone structure, Ig (Fig. 1), of this residue was proved by the following: the infra-red spectrum showed bands at 1560 (medium, —N=CH_2) and 1150 strong, N→O) cm^{-1} common in all spectra of nitrones [26]; the p.m.r. characteristics in $CDCl_3$ were 5H (singlet, Ar) τ2.8, 2H (two doublets, N = CH_2), τ3.4–4.0, 2H (singlet, CH_2), τ6.95, 6H (singlet, C-$(CH_3)_2$), τ8.6, the splitting of the methylene protons into two doublets is characteristic of α-unsubstituted nitrones [27]; the g.c.-mass spectrum of this residue gave the following mass ion fragments at m/e 91(100%), 32(66%), 55(28%), 117(25%), 70(21%), 45(21%), 162(19%), 105(19%), 132(18%), 41(16%), 39(14%), 104(14%), 65(12%), 43(12%), 133(10%), and 92(10%). The ion at m/e 70 corresponds to structure i (Fig. 1) and has been shown to be a characteristic fragment of various nitrone compounds related to phenethylamines [28].

T.l.c. and g.l.c. analysis of an ethereal solution of the nitrone compound (Ig) gave a single spot at R_f 0.29 using solvent system 1 and one peak at R_t 24.4 min (170°) when injected on column C [25]. Futhermore, oxidation of Ig in phosphate buffer pH 7.4 with potassium manganate ($KMnO_4$ 1%, 20 min) followed by exctraction into ether and g.l.c. analysis on column C led to complete disappearance of the peak corresponding to Ig (R_t 24.4 min, 170°) in favour to that of α,α-dimethyl-α-nitro-β-phenylethane (Id, R_t 6.4 min, 170°). However, water and formaldehyde were found to be present by p.m.r. analysis of the sample of the nitrone and attempts to purify this compound led to decomposition.

Gas–liquid and thin-layer chromatography. The columns used for g.l.c. analysis and the solvent systems used for t.l.c. analysis were described before [25] and used under the same conditions. A.W.G. Pye "Serie 104" gas chromatograph model 84 (Pye Unicam Ltd., Cambridge) with a flame ionization detector and a 1 mV Perkin-Elmer 56 recorder were used.

Physical measurements. Infra-red spectra were recorded with a Unicam SP-1000 spectrometer. Proton magnetic resonance (p.m.r.) spectra in $CDCl_3$ were recorded using a Perkin–Elmer R-10 n.m.r. spectrometer and a Northern Scientific 544 CAT with tetramethylsilane as the reference standard. All mass spectra were obtained using a Perkin–Elmer Model 270 gas chromatograph-mass spectrometer (g.c.-m.s.) system at an electron energy of 70 eV. A 2-m glass column packed with Chromosorb W (100–120 mesh size) and coated with Carbowax 20M (7.5%) was used at 170° for recording the g.c.-mass spectrum of the nitrone compound (Ig); helium (0.7 $kg\,cm^{-2}$) was the carrier gas.

Animals and preparation of the liver homogenates. Young adult animals [New Zealand white rabbits (Great Toteas, Bickstead), 1.5–3.0 kg; Albino Dunkin Hartley guinea pig (Redfern Animal Supplies), 750 g; Laca mouse (Tuck and Son, Southend), 30 g; South Down Warren chick (South Down Actuaries, Uckfield), 100 g; Syrian hamster (Ex-Chester Beatly), 100 g and Wistar rat (CFHB Carworth), 350 g] were killed, their livers rapidly removed and the 9,000 *g* supernatant, and microsomes prepared as described elsewhere [13]. 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 hr at 0° using a M.S.E. Superspeed 40 centrifuge. All homogenate fractions were resuspended in fresh isotonic Tris–KCl buffer at a final homogenate concentration equivalent of 0.5 g of liver per ml.

Incubation experiments. Incubations were carried out in 25 ml conical flasks in a water bath at 37° with shaking. Unless 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 vol. 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 vol. of 6 ml. Nicotinamide is widely used *in vitro* in incubation mixtures to inhibit the microsomal nucleotidase of liver which destroys NADP; under these experimental conditions it does not inhibit the metabolic oxidation catalysed by microsomes [30]. 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 microsomes equivalent to 0.25, 0.5, 0.75 and 1 g of liver were made up by diluting the washed microsomes with an appropriate volume of phosphate buffer. In all cases, the incubation mixtures were incubated for 5 min at 37° with shaking before the addition of substrate, then the incubations carried out for various periods of time. Mephentermine (Ie, as sulphate, 1–10 μmol in water, 1 ml) and *N*-hydroxymephentermine (If, 0.19 μmol, as oxalate in water, 1 ml; freshly prepared solution) were incubated with homogenates under the above conditions. When compounds other than Ie and If were added to the incubation mixtures (final concentration of 1 mM) they were dissolved in phosphate buffer (pH 7.4) immediately before the experiment. When not used, the homogenate preparations were stored at 4°.

The K_m and the maximum rates of metabolism (V_{max}) for the microsomal *N*-dealkylation and oxidation of mephentermine (Ie) were determined over the concentration ragne of 1 to 10 μmol per 6 ml (0.166–1.666 mM) using hepatic washed microsomes from rabbit. The data were plotted according to the Lineweaver–Burk (1/v vs 1/s) method and subjected to regression analysis to give the appropriate K_m and V_{max} values.

Table 1. The *in vitro* metabolism of mephentermine (Ie, 5 μmol per flask) using hepatic microsomal preparations from rabbit liver*

Homogenate	Amounts of metabolites formed: Phentermine (Ia) (nmol)†	Phentermine (Ia) (nmol gm^{-1})‡	*N*-Hydroxyphentermine (Ib) (nmol)	*N*-Hydroxyphentermine (Ib) (nmol mg^{-1})	*N*-Hydroxymephentermine (If) (nmol)	*N*-Hydroxymephentermine (If) (nmol mg^{-1})
9000 *g* Supernatant	794.7 ± 72	21.2 ± 2	135.3 ± 0.1	3.6 ± 0.0	78.4 ± 1	1.9 ± 0.1
Microsomes	676.2 ± 24	48.3 ± 17	91.6 ± 13	7.7 ± 1	75.0 ± 5	6.3 ± 1
Washed microsomes	431.8 ± 12	47.5 ± 1	74.6 ± 20	11.0 ± 3	57.0 ± 17	8.2 ± 2
Washed microsomes§	25 ± 2	2.7 ± 0.2	0	0	0	0

* The incubation time was 30 min for the formation of Ia and 60 min for the formation of Ib and IIb. Results are expressed as a mean value of two experiments ± mean deviation.
† nmoles.
‡ nmoles per mg of protein.
§ NADP was omitted from the cofactor solution.

Quantitative analysis. The metabolic reaction was stopped by rapidly putting the flask in an ice tray. The amount of mephentermine (Ie) and its metabolic products i.e. phentermine (Ia), *N*-hydroxyphentermine (Ib) and *N*-hydroxymephentermine (If) were determined by gas–liquid chromatography as described before [25]. The total amount of *N*-oxidised metabolites of Ie i.e. Ib and If was 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 ($KMnO_4$ 1%, 1 ml, 20 min shaking) since Ib and If, but not Ia and Ie, are oxidised quantitatively to the nitro compound (Id) under these conditions [25]. The protein content of the homogenates was determined by the method of Lowry *et al.* [29].

RESULTS AND DISCUSSION

In a preliminary experiment, mephentermine (Ie, 5 μmol per flask) was incubated with washed microsomes from a rabbit (equivalent to 0.5 g or original liver) plus the NADPH generating system for 30 min at 37° and the amounts of Ie, Ia and total *N*-oxidised metabolites were determined. The percentage of recoveries (mean value of duplicate experiments) of unchanged mephentermine (Ie), phentermine (Ia) and total *N*-oxidised metabolites were 88.4 (4.42 μmol), 8.2 (0.408 μmol) and 3.2 (0.162 μmol) respectively; the total recovery of substrate moiety was 99.8 per cent (4.99 μmol). Thus, mephentermine (Ie) is not metabolised by routes other than *N*-dealkylation and *N*-oxidation under the present conditions of incubation.

Results of the incubation of mephentermine (Ie) with various homogenate preparations from one rabbit liver showed that the activity to metabolise Ie to phentermine (Ia), *N*-hydroxyphentermine (Ib) and *N*-hydroxymephentermine (If) is mainly localised in the microsomes (Table 1). Washing the microsomes did not affect the specific activity of the dealkylation of Ie to Ia but increased that of *N*-oxidation namely formation of Ib and If. Reduced pyridine cofactor (NADPH) was necessary for maximum activity of the microsomes. In the absence of a NADPH generating system, washed microsomes from rabbit liver dealkylated Ie to significant amounts of Ia but no hydroxylamines Ib and If could be detected (Table 1). Therefore, hepatic washed microsomes plus a NADPH generating system were subsequently used in all experiments.

N-Methyl-(α,α-dimethyl-β-phenethyl)nitrone (Ig) could not be detected when neutral ethereal extracts of incubation mixtures of Ie where analysed on g.l.c. column G (170°, [25]). Nitrones are major *N*-oxidised products of metabolism of many phenethylamines such as the *N*-ethyl, *N*-propyl and *N*-butyl derivatives

Table 2. The effect of incubation time on the *in vitro* metabolism of mephentermine (Ie, 5 μmol per flask) using washed microsomes from rabbit liver

Time (min)	Amounts of metabolites formed (nmol mg^{-1})*: Phentermine (Ia)	*N*-Hydroxyphentermine (Ib)	*N*-Hydroxymephentermine (If)	Sum of Ib + If	Total *N*-oxidised metabolites
10†	24.8	0.8 ± 0.1‡	2.2	3.0	2.9
15	29.1 ± 0.7	1.8 ± 0.1	3.4 ± 0.1	5.2	6.0 ± 0.03
20	34.3	2.6 ± 0.04	4.2 ± 0.1	6.8	8.3
30	41	4.9 ± 0.7	5.3 ± 0.4	10.2	11.1 ± 0.7
40	59.2 ± 5.1	6.0 ± 0.2	5.2 ± 0.04	11.2	14.4
60	82.5 ± 3	13.6 ± 4.9	11.8 ± 2.8	25.4	23.1 ± 2.7
10§	19.1	1.6	5.4	7.0	9.4
20	27.7	7.8	8.4	16.2	13.6
40	44.6	17.5	9.4	27.2	22
60	79.4	13.6	12.8	26.2	23.1

* nmoles per mg of protein.
† Experiment 1.
‡ Results are expressed as a mean value of duplicate incubations ± mean deviation.
§ Experiment 2.

Table 3. The incubation of *N*-hydroxymephentermine acid oxalate (If, 0.19 μmol per 6 ml) with phosphate buffer at pH 7.4 (A) and with phosphate buffer containing liver washed microsomes from rabbit plus the standard NADPH generating system (B) for 60 min at 37°

System	*N*-Hydroxymephentermine (If)	Amount (μmol) recovered as *N*-Hydroxyphentermine (Ib)	Total *N*-oxidised metabolites
A	0.168 (88.6)*	0.029 (15.3)	0.189 (99.0)
B 1†	0.04 (22.8)	0.107 (56.3)	0.160 (84.4)
2‡	0.023 (12.4)	0.135 (71.4)	0.162 (85.5)

* The percentage of recoveries are in parenthesis.
† Experiment 1.
‡ Experiment 2.

of amphetamine and norfenfluramine when incubated with hepatic microsomal fraction [15].

The concentrations of *N*-hydroxyphentermine (Ib) determined in the organic extracts of incubation mixtures of mephentermine (Ie) were similar or greater to those determined when phentermine (Ia) was incubated under similar conditions indicating that the primary hydroxylamine (Ib) was derived from a route additional to that involving *N*-oxidation of the metabolically produced phentermine (Ia). Also, if *N*-oxidation of the primary amine phentermine (Ia) occurred in the incubation mixtures of Ie, then α,α-dimethyl-α-nitroso-β-phenylethane (Ic) should be present in greater amounts than Ib since Ic is the major *N*-oxidised metabolite when Ia is incubated with hepatic microsomes [30]. However, the nitroso compound (Ic) could not be detected on g.l.c. analysis (column A, 100° [13]) of a concentrated ethereal extract of three incubation mixtures of mephentermine (Ie), while phentermine (Ia, at the same concentration of If used) incubated for the same period of time (60 min) with the same microsomes (from rabbit liver) under identical conditions yielded Ic. Thus phentermine (Ia) was precluded as the metabolic precursor of *N*-hydroxyphentermine (Ib) when mephentermine (Ie) was incubated with liver microsomal fractions.

Further evidence that α,α-dimethyl-α-nitroso-β-phenylethane (Ic) was not formed in the incubation mixtures of mephentermine (Ie) was obtained from the determination of the amount of total *N*-oxidised metabolites (Table 2); these corresponded roughly to the sum of *N*-hydroxyphentermine (Ib) and *N*-hydroxymephentermine (If) determined in the incubation mixtures of Ie. The hydroxylamines Ib and If, the nitroso (Ic) and the nitrone (Ig) compounds have been shown to be quantitatively oxidised in the nitro (Id) compound by potassium permanganate ($KMnO_4$ 1%, [13, 25]); this was the basis of the estimation of total *N*-oxidised metabolites.

Thus, from the above evidence the presence of *N*-methyl-(α,α-dimethyl-β-phenethyl)nitrone (Ig) in the incubation mixtures of mephentermine (Ie) is precluded.

Incubation of N-*hydroxymephentermine* (*If*). The possibility of metabolic oxidative dealkylation of the metabolically produced *N*-hydroxymephentermine (If) to give *N*-hydroxyphentermine (Ib) in the incubation mixtures of mephentermine (Ie) was considered. The secondary hydroxylamine (If) was mainly metabolised by dealkylation to the primary hydroxylamine (Ib) when incubated with hepatic microsomes from rabbit liver plus a NADPH generating system in the absence of mephentermine (Ie) (Table 3). The amount of If incubated was roughly three times that determined when mephentermine (Ie) was incubated under identical conditions. The percentages of recoveries (mean value of duplicate experiments) of unchanged If, Ib and total *N*-oxidised products were 17, 64 and 85 respectively after 60 min of incubation of If. The rates of formation of Ib from If under these conditions (9.1 mg of protein content) were 12 and 15 nmoles per mg of protein per 60 min. The enzymic reduction of the hydroxylamines Ib and If to the corresponding amines Ia and Ie may be responsible for the missing 15 per cent of the recovery of total *N*-oxidised products (Table 3) compared with that obtained from incubation in phosphate buffer under the same conditions. Secondary hydroxylamine reductase and oxidase activities have been reported and the enzyme systems purified from pig and porcine liver microsome [31, 32]. It was established that the same enzyme system catalysed the disappearance of secondary hydroxylamines and the oxidation of

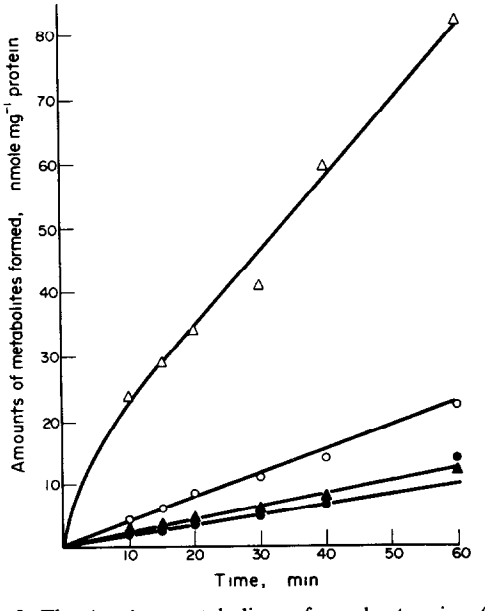

Fig. 2. The *in vitro* metabolism of mephentermine (Ie, 5 μmol) as a function of time (min) using washed microsomes from rabbit liver. Each point is the mean value of two experiments. —△— Phentermine (Ia). —○— Total *N*-oxidised metabolites. —●— *N*-Hydroxyphentermine (Ib). —▲— *N*-Hydroxymephentermine (If).

Table 4. Enzyme-substrate characteristics (K_m and V_{max}) for the *in vitro* metabolism of mephentermine (Ie) to phentermine (Ia), *N*-hydroxyphentermine (Ib), *N*-hydroxymephentermine (If) and total *N*-oxidised metabolites using washed microsomes from rabbit liver

Metabolic reaction*	Correlation coefficient†	K_m‡	V_{max}‡
Formation of Ia	0.92	0.917	0.199
Formation of Ib	0.95	3.182	0.084
Formation of If	0.96	0.81	0.11
Formation of total *N*-oxidised metabolites	0.95	1.347	0.194

* The incubation time was 5 min for the formation of Ia and 30 min for the formation of Ib, If and total *N*-oxidised metabolites. Two experiments representing two independent microsomal preparations were performed. Incubations were done in triplicate in each experiment.

† The data (6 in each case) were plotted according to Lineweaver–Burk method and submitted to regression analysis.

‡ K_m and V_{max} represent μmol per 6 ml and μmol per 0.5 g liver per 5 (for the formation of Ia) or 30 min (for the formation of Ib, If and total *N*-oxidised metabolites) respectively.

secondary amines to secondary hydroxylamines and that the two reactions are differentiated by the optimum experimental conditions [33, 34].

Kinetic studies. The rate of metabolism of mephentermine (Ie) to phertermine (Ia), *N*-hydroxyphentermine (Ib) and *N*-hydroxymephentermine (If) was linear with respect to microsomal concentration of 5.5–22.5 mg of protein equivalent to 0.25–1 g of original liver. The amount of *N*-hydroxyphentermine (Ib), *N*-hydroxymephentermine (If) and total *N*-oxidised metabolites produced were linear with time to 60 min (Fig. 2); under the same conditions, the formation of phentermine (Ia) from Ie was linear between 10 and 60 min. The deviation from the linearity obtained for the dealkylation of Ie to Ia during the first 10 min of incubation of Ie may result from the accumulation of NADPH generated prior to the addition of Ie.

When reaction rates were plotted as a function of mephentermine (Ie) concentration from 1 to 10 μmol per 6 ml, typical saturation curves were obtained for the formation of Ia, Ib, If and total *N*-oxidised metabolites. The data of the rates of formation of the metabolites Ia, Ib, If and the total *N*-oxidised metabolites were plotted according to the Lineweaver–Burk method and the enzyme-substrate characteristics (K_m and V_{max}) were calculated (Table 4). Good fitting curves were obtained for all the metabolic reactions. The maximum rate of formation of phentermine (Ia) is six times greater than that of the total *N*-oxidised metabolites although the tabulated rates are similar (Table 4); this discrepancy is explained by the fact that an incubation time of 5 min was chosen for the metabolic formation of Ia against 30 min for the measurement of total *N*-oxidised metabolites. On the other hand, the maximum rate of formation of total *N*-oxidised metabolites represents the added rates of formation of Ib and If suggesting the presence of a single enzyme system catalysing the incorporation of oxygen in mephentermine (Ie) to give *N*-hydroxyphentermine (Ib) and *N*-hydroxymephentermine (If).

Effect of foreign compounds on the in vitro *metabolism of mephentermine (Ie).* The effects of foreign compounds on the microsomal metabolism of mephentermine (Ie) to phentermine (Ia), *N*-hydroxyphentermine (Ib) and *N*-hydroxymephentermine (If) are listed in Table 5. Sodium cyanide, sodium azide and EDTA (ethylediaminetetraacetate) strongly increased the formation of the hydroxylamines Ib and If but only slightly affected the formation of Ia; these compounds also increased, though to a lesser extent, the meta-

Table 5. The effect of foreign compounds on the *in vitro* metabolism of mephentermine (Ie) using rabbit liver washed microsomes

Compounds*	Percentage formation of Phentermine (Ia)	*N*-Hydroxyphentermine (Ib)	*N*-Hydroxymephentermine (If)
None (control)	100.0 ± 0.3†	100.0 ± 0.3	100.0 ± 12
Sodium cyanide	132.5 ± 20.8	417.9 ± 9.3	528.7 ± 22.5
Sodium azide	98.0 ± 7.7	154.7 ± 18.1	209.7 ± 29.4
EDTA (ethylenediaminetetracetate)	113.2 ± 0.2	491.5 ± 6.1	684.0 ± 6.1
4-Chloromercuribenzoic acid	2.8 ± 1	0	0
N-Ethylmaleimide	80.4 ± 15.3	1.8 ± 1.8	22.6 ± 2.4
Cysteamine	93.6 ± 4.2	70.7 ± 19.4	129.6 ± 3.3
Dithiothreitol	89.5 ± 12.6	9.1 ± 6.4	8.6 ± 1.3
Potassium iodide	67.2 ± 1.4	103.4 ± 27.8	135.5 ± 0.9
Catalase	86.5 ± 8.9	95.9 ± 5	94.5 ± 14.3

* Foreign compounds (final concentration of 1 mM) and catalase (100 μg per flask) were incubated for 5 min with the microsomal fractions prior to the addition of mephentermine (5 μmol) and then the incubation carried out for 30 min.

† Results are expressed as a percentage mean value of two experiments ± mean deviation compared to control (100%) incubations.

bolic *N*-oxidation of Ia when incubated with microsomes under similar conditions [30]. The formation of phentermine (Ia) and the hydroxylamino compounds Ib and If are almost completely inhibited by the addition of 4-chloromercuribenzoic acid in the incubation mixtures of Ie in agreement with earlier results on the microsomal *N*-oxidation of Ia [30]. The metabolic formation of Ib and If were strongly inhibited by the addition of *N*-ethylamaleimide and dithiothreitol while the formation of Ia, a typical α-carbon oxidation process, was only slightly affected. Potassium iodide, a known decomposer of cytochrome P-450 [35, 36], inhibited the dealkylation of Ie to Ia but not the formation of Ib and If. Enzyme inhibitors have been used to provide evidence that *N*-oxidation and *N*-dealkylation (α-C-oxidation) involved different metabolic processes [3]. Catalase had only a slight inhibitory effect on the microsomal metabolism of mephentermine (Ie) precluding endogenous hydrogen peroxide as the *in vitro* oxidant of Ie.

Species difference in the in vitro *metabolism of mephentermine (Ie).* Microsomes from rabbit liver were found to be the best homogenate preparations to metabolise mephentermine (Ie) *in vitro* to Ia, Ib and If (Table 6). Large variations in the ratio of the amount of phentermine (Ia) formed to that of the total *N*-oxidised metabolites and in the ratio of the amount of *N*-hydroxyphentermine (Ib) to N-hydroxymephentermine (If) formed were obtained between the species investigated. Hepatic microsomes from chick are as active than those from rabbit to dealkylate Ie to Ia but formed very small amounts of Ib and If (less than 10% compared to rabbit microsomes). Inversely, liver microsomes from guinea pig showed a more profound decrease in the formation of Ia than that of Ib and If respectively. The ratio of the metabolically produced *N*-hydroxyphentermine (Ib) to *N*-hydroxymephentermine (If) increased when mephentermine (Ie) was incubated with hepatic washed microsomes from guinea pig and hamster but decreased with those from chick, cat and mouse.

Proposed mechanism for the microsomal α-carbon oxidation of mephentermine (Ie) to give phentermine (Ia) and metabolic N*-oxidation of mephentermine (Ie) to* N*-hydroxyphentermine (Ib) and* N*-hydroxymephentermine (If).* Metabolic α-C- and *N*-oxidation involve different metabolic processes [3, 9]. The rate of formation of Ia from Ie is much faster than that of the *N*-oxygenated metabolites Ib and If. *N*-Ethylmaleimide and dithiothreitol inhibit whereas EDTA, sodium cyanide and sodium azide strongly enhance the metabolic formation of Ib and If but slightly affect that of Ia when added to the incubation mixtures of Ie. Also, potassium iodide depresses the formation of Ia but not that of Ib and If from Ie. Furthermore, liver microsomes from chick dealkylate Ie and Ia but do not readily form Ib and If to the same extent as hepatic microsomes from rabbit under the same conditions of incubation; the reverse is true for microsomes from guinea pig. The results suggest that the metabolic dealkylation of Ie to Ia and the formation of the *N*-oxygenated metabolites Ib and If from Ie involve different metabolic processes namely α-carbon (α-C) and *N*-oxidation respectively.

Time study of the metabolic formation of phentermine (Ia) and *N*-hydroxyphentermine (Ib) from mephentermine (Ie) with rabbit liver microsomes gave curves suggesting the independent formation of Ia and Ib. α,α-Dimethyl-α-nitroso-β-phenylethane (Ic) was a major *N*-oxidised metabolite of Ia when incubated with rabbit liver microsomes but was not formed in the incubations of Ie. It is concluded that *N*-hydroxyphentermine (Ib) does not derive from the metabolically produced phentermine (Ia) in the incubations of mephentermine (Ie).

The kinetics of formation of the hydroxylamines Ib and If upon incubation of Ie with liver microsomes from rabbit for up to 60 min gave curves which indicated the independent formation of Ib and If from Ie. The secondary hydroxylamine If was mainly metabolised by *N*-dealkylation to Ib when incubated with rabbit liver microsomes in the absence of Ie; the rate of formation of Ib from If was too low to account for the amounts of Ib present in the incubation mixtures of Ie under the same conditions. Furthermore, in the incubation mixtures of Ie the concentration of Ie (5 μmol per 6 ml) exceeded that of the metabolically produced If by a factor of at least one hundred at the early stage of incubation (10 min) and competition for the *N*-dealkylation reaction would favour the faster rate of formation of Ia from Ie rather than Ib from If. Moreover, addition of cyanide, azide and EDTA in the incubation mixtures of Ie strongly increased the formation of both Ib and If but not Ia. Thus, under the present conditions of incubation of Ie, the primary hydroxylamine (Ib) is not produced by *N*-dealkylation i.e. not by the α-oxidation of the metabolically produced secondary hydroxylamine (If)

Table 6. Species differences in the *in vitro* metabolism of mephentermine (Ie, 5 μmol per 6 ml) when incubated with liver washed microsomes for 60 min

Species	Amounts of metabolites formed (nmol mg^{-1})*		
	Phentermine (Ia)	*N*-Hydroxyphentermine (Ib)	*N*-Hydroxymephentermine (If)
Rabbit	84.0 ± 5†	14.0 ± 5	12.0 ± 3
Guinea pig	19.0 ± 1	20.3	5.1 ± 0.2
Hamster	19.5 ± 4	5.0 ± 0.5	1.2 ± 0.1
Mouse	N.D.‡	0.1 ± 0.03	0.5 ± 0.2
Rat	15.1 ± 0.8	0.8 ± 0.1	0.4 ± 0.1
Chick	73.6	0.2 ± 0.2	1.3 ± 0.1
Cat	43.9	0.03 ± 0.01	0.4 ± 0.4

* nmoles per mg of protein

† Results are expressed as a mean value ± mean deviation of two experiments.

‡ Not determined.

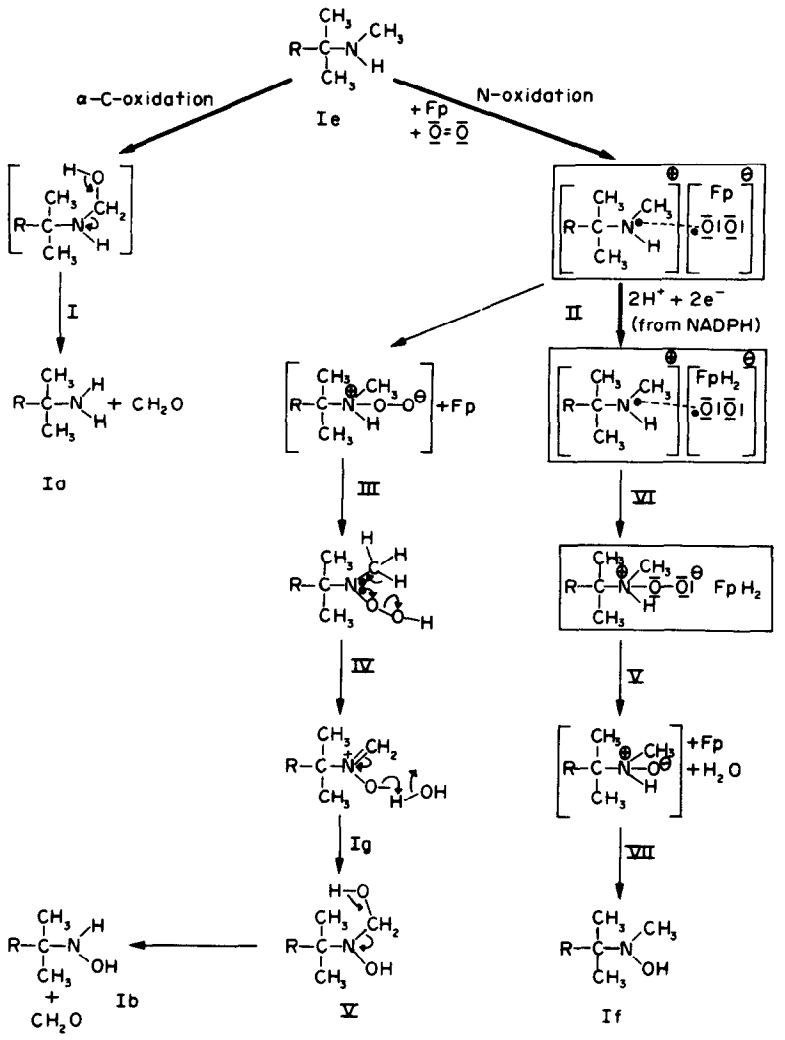

Fig. 3. Scheme for the metabolic α-C-oxidation of mephentermine to give dealkylation and of the metabolic *N*-oxidation to a complex which results in the formation of *N*-hydroxyphentermine and *N*-hydroxymephentermine. R = $ArCH_2$, Fp = flavoprotein → = metabolic route, → = chemical change.

but is produced by from *N*-oxidative metabolic attack on mephentermine (Ie).

The V_{max} of the formation of the primary hydroxylamino compound Ib from the secondary amine Ie is of the same order than that of the *N*-oxidation of Ie to If indicating that the same enzyme catalyses the incorporation of oxygen present in Ib and If.

The results of the *in vitro* oxidative metabolism of mephentermine (Ie) are rationalised in Fig. 3; use is made of the principles outlined earlier [37] to explain the formation of C-nitroso and hydroxylamino groups from the primary amine phentermine (Ia) by involving the separate formation of a primary and secondary hydroxylamino group from the secondary hydroxylamine group from the secondary amine mephentermine (Ie) through a single enzyme system.

Metabolic α-carbon oxidation of Ie yields the unstable carbinolamine (I) which readily eliminates formaldehyde to give the primary amine Ia.

Metabolic *N*-oxidation through a flavoprotein results in the formation of the free radical ion complex (II). Rapid dissociation will produce the Zwitterion (III) and free flavoprotein; proton rearrangement then occurs to give the *N*-hydroperoxide (IV) from which water is eliminated to yield the nitrone compound (Ig). Nitrones of this type are very unstable and undergo immediate hydrolysis in neutral aqueous solution to give the corresponding primary hydroxylamines (Beckett and others, unpublished findings). Therefore, hydrolysis of Ig and spontaneous breakdown of the carbinolhydroxylamine (V) gives the primary hydroxylamine (Ib) and formaldehyde. The formation of formaldehyde i.e. *N*-dealkylation is thus produced by both α-C and *N*-oxidation of Ie.

On the other hand, if rapid dissociation of the components of the radical complex (II) does not occur, the flavoprotein in the complex will be reduced to complex VI containing reduced flavoprotein (F_pH_2). The nitrogen and oxygen free radicals interact to give the Zwitterion followed by dissociation to yield flavoprotein, one molecule of water and the *N*-oxide (VII) which undergoes proton rearrangement to give

N-hydroxymephentermine (If). The oxygen atom present in the hydroxylamines Ib and If is thus derived from the atmosphere.

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