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The metabolism of dibenzylamine: identification of *NN*-dibenzylhydroxylamine as the major *in vitro* metabolic product from rabbit fortified hepatic homogenates

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A study of the *in vitro* metabolism of dibenzylamine with fortified rabbit liver 9000 *g* supernatant fractions has shown that *N*-oxidation is the major metabolic process. Of the total amount of dibenzylamine metabolized, 90% is converted to *NN*-dibenzylhydroxylamine. The primary amine, benzylamine, is formed to the extent of only 6% of total substrate metabolized. The identity of the major metabolite, *NN*-dibenzylhydroxylamine has been established by thin-layer chromatography, gas-liquid chromatography and combined gas-liquid chromatography-mass spectrometry.

In recent years, *N*-oxidation has become an increasingly important metabolic pathway in view of the toxicological properties of the *N*-oxidized metabolites formed (Bickel, 1969), especially hydroxylamine metabolites and their conjugates (Cucinell, Israili & Daiton, 1972; Beckett, 1973; Weisberger & Weisberger, 1973). However, progress in this field suffers from the disadvantage that present methods of analysis of hydroxylamines involve complicated and time-consuming procedures; in addition, hydroxylamines are extremely labile to the extraction and analytical conditions used (Beckett, 1974).

Dibenzylamine (DBA; Ia) is a compound closely related to *NN*-dibenzyl-β-chloroethylamine (dibenamine), a known adrenergic blocking agent, and is itself a potential adrenoceptor blocking agent. In this report, we present evidence that *in vitro* metabolism of DBA (Ia) gives *NN*-dibenzylhydroxylamine (Ib) as the major metabolic product, and we describe the quantitative estimation of the latter by a rapid, specific and sensitive technique involving gas-liquid chromatography.

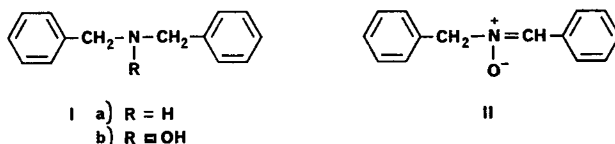

FIG. 1. Structure of *NN*-dibenzylamine and its *N*-oxidized products.

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MATERIALS AND METHODS

Materials

Glucose-6-phosphate and NADP (oxidized form), were obtained from the Boehringer Corporation, *N*-trimethylsilylimidazole and *N,O*-bis-(trimethylsilyl)-trifluoroacetamide were purchased from the Pierce Chemical Company and all other reagents were laboratory reagent grade. Dibenzylamine (Ia) was converted to its hydrochloride salt and recrystallized from an ethanol (96%)–diethyl ether mixture before use, and its purity determined by gas-liquid chromatography (g.l.c.) and thin-layer chromatography (t.l.c.). Synthetic *NN*-dibenzylhydroxylamine (Ib) was prepared (Beckett, Coutts & Ogunbona, 1973), and its purity ascertained by t.l.c., g.l.c. and elemental analysis. All column packing materials and supports were purchased from Perkin-Elmer Ltd.

Animals

Animals used were male rabbits (New Zealand White strain, 2 to 3 kg), which were fasted overnight before death. The homogenized liver 9000 *g* supernatant was prepared by the method of Beckett & Bélanger (1974).

Standard incubation system

DBA.HCl (Ia, 10 μmol base ml^{-1}) and *NN*-dibenzylhydroxylamine (Ib, 2 μmol base ml^{-1}) as the hydrochloride salt in water were incubated under aerobic conditions in a thermostatically controlled shaking water bath at 37°. Each incubate contained 1 ml of substrate, 3 ml phosphate buffer pH 7·4, 1 ml homogenized liver 9000 *g* supernatant and 1 ml cofactor solution containing glucose-6-phosphate (6 mg), NADP Na_2 (3·4 mg), $MgCl_2$ 0·1 M (0·2 ml), nicotinamide 0·6 M (0·1 ml) and distilled water (0·7 ml). The reactions were started by the addition of substrate after an initial pre-incubation period of 10 min to allow temperature equilibration and reduction of NADP, and continued for 60 min. The reactions were terminated by placing the incubation flasks in ice and the extraction was carried out as described below. All incubations were in duplicate and control incubations were performed in the absence of substrate or rabbit liver homogenate, or in some cases, in the presence of a heat-denatured enzyme preparation (90°, 15 min).

Extraction and analysis of parent compound and metabolites from the biological fluid

1. *Neutral extracts.* After incubation of DBA (Ia) with a fortified rabbit hepatic homogenate (9000 *g* supernatant), 2 ml aliquots of the incubation mixture (pH 7·4) were extracted for 30 min into freshly distilled, peroxide-free diethyl ether (2 × 15 ml) after the addition of 187 μg *p*-chlorophenylcyclopropylketone in water (1 ml) as internal marker. The extract was centrifuged between each extraction, the combined ether extracts were transferred to a tapered test-tube and evaporated to dryness in a water-bath at 42°. The hydroxylamine (Ib) thus extracted was dissolved in 60–100 μl of dry acetonitrile (redistilled from P_2O_5). When *N*-trimethylsilylimidazole (30–50 μl) was added with thorough mixing, the trimethylsilyl (TMS) derivative of the hydroxylamine (Ib) was formed quantitatively. The amount of hydroxylamine (Ib) present was determined by the "internal marker" method (Beckett & Rowland, 1965), with reference to a standard curve by analysis on g.l.c. system C (see p. 662).

2. *Alkaline extracts.* After incubation, the incubation mixture was rendered alkaline by the addition of 1 ml 5N NaOH. For the determination of unchanged DBA (Ia), 1 ml aliquots were extracted for 20 min into diethyl ether (3 × 15 ml) after the addition of 400 μg *N*-(*o*-chlorobenzyl)amphetamine HCl in water (1 ml) as internal marker. The extracts were centrifuged as before and the ether extracts transferred to a finely-tapered test-tube and concentrated to about 40–50 μl in a water bath at 42°. The amount of DBA (Ia) present was determined on g.l.c. system A with reference to a standard curve. To determine the amount of the *N*-dealkylated metabolite present as benzylamine, the procedure was identical to that of the DBA (Ia) analysis, except that 2 ml aliquots, to which had been added 100 μg (+)-amphetamine sulphate in water (1 ml) as internal marker, were extracted and that 5 μl n-butanol were added to the first ethereal extract to minimize loss of benzylamine due to volatilization and "creep" up the test tube walls. The amount of benzylamine in the incubation extract was determined on g.l.c. system B with reference to a standard curve.

T.l.c. analysis of incubation extracts

After extraction and subsequent concentration of the ether extract to 40 μl, aliquots were applied to glass plates (20 × 20 cm) spread to 0·25 mm with silica gel G (1:2, silica to water mixture), which had been dried at room temperature and activated at 110° for 60 min before use. Authentic samples of the metabolite were co-chromatographed along with the incubation extracts. The chromatograms were run in one dimension by using four different solvent systems (Table 1). The metabolic products were visualized with either Dragendorff's reagent, ammoniacal silver nitrate, triphenyltetrazolium chloride or Ninhydrin reagent. In certain cases, after initial visualization of metabolic products on t.l.c., equivalent undeveloped areas of the chromatogram were scraped off, extracted with ether and the ether extracts were analysed by g.l.c. system C. Blank areas of the chromatogram served as controls in this instance.

Table 1. R_F *values of DBA and* NN-*dibenzylhydroxylamine, and their response to various locating agents.*

Compound	Solvent System	R_F Values: Authentic material	R_F Values: Metabolic product	Reaction with locating agents: Dragendorff's reagent	Reaction with locating agents: Ammoniacal silver nitrate	Reaction with locating agents: Triphenyltetrazolium chloride	Reaction with locating agents: Ninhydrin reagent
Dibenzylamine	1	0·41	0·44	Immediate brown spot	No reaction	No reaction	No reaction
	2	ND*	ND*				
	3	0·83	0·80				
	4	0·85	0·85				
NN-Dibenzylhydroxylamine	1	0·83	0·83	Immediate orange spot	Black spot quickly develops	Red spot quickly develops	Purple spot slowly develops
	2	0·41	0·44				
	3	0·93	0·92				
	4	0·93	0·91				

* ND not determined. Solvent systems: 1. Benzene–ethyl acetate–water (36:12:1); 2. Methanol-cyclohexane (1:19); 3. Methanol–benzene (1:1); 4. Chloroform-methanol (4:1).

Combined gas-liquid chromatography-mass spectrometry

All mass spectra were obtained using a Perkin Elmer model 270 gas chromatograph-mass spectrometer system at an electron energy of 70 eV fitted with a 1 metre glass column, ¼″ outside diameter, containing 10% UC-W98 on Chromosorb W (high

performance), 80 to 100 mesh. Carrier gas (helium) was 15 p.s.i., the column temperature at 200° and the injection block temperature at 250°. Neutral ethereal extracts from the equivalent of four standard incubations were pooled and concentrated to a small volume (approximately 20 μl), and analysed on the combined g.l.c.–mass spectrometer system.

Gas-liquid chromatography

The instrument used was a Perkin-Elmer F11 model fitted with a flame ionization detector and the conditons used were as follows. *System A*—glass column, 1m, $\frac{1}{4}''$ outside diameter, 5% Carbowax 20M containing 10% KOH on dimethylchlorosilane (DMCS) treated chromosorb G, 80 to 100 mesh, oven temperature 180°, N_2 25, H_2 30, air 20 p.s.i., flow rate 240 ml min^{-1}. *System B*—glass column, 2m, $\frac{1}{4}''$ outside diameter, 10% Apiezon L containing 10% KOH on DMCS treated chromosorb W, 80 to 100 mesh, oven temperature 120°, $N_2$20, $H_2$22·5, air 20 p.s.i., flow rate 80 ml min^{-1}. *System C*—glass column, 2m, $\frac{1}{4}''$ outside diameter, packed with 2% XE 60 on Chromosorb G, 80–100 mesh, oven temperature 150°, $N_2$20, $H_2$22·5, air 20 p.s.i., flow rate 80 ml min^{-1}. Injection port temperatures were operative at 200–220° in all three systems. All columns were conditioned by injecting 3 × 5 μl aliquots of hexamethyldisilazane onto the column and left overnight at 30° higher than the operating temperature before use.

RESULTS AND DISCUSSION

Specificity of silylation

Preliminary experiments with *NN*-dibenzylhydroxylamine (Ib) suggested that the compound broke down on g.l.c. columns in a manner comparable to that reported for *N*-hydroxyamphetamine (Beckett, 1971; Beckett & Al-Sarraj, 1972). Initial attempts to stabilize *NN*-dibenzylhydroxylamine (Ib) as its TMS derivative using *N, O*-bis (trimethylsilyl)-trifluoroacetamide as silylating reagent, were only partially successful because the reagent also formed a TMS derivative with DBA (Ia) which could not be resolved from the TMS ether of *NN*-dibenzylhydroxylamine (Ib) on various g.l.c. columns. *N*-Trimethylsilylimidazole is a more specific reagent and only silylates hydroxyl groups (Horning, Moss & Horning, 1967; Horning, Moss & others, 1968). This was verified using DBA (Ia) and *NN*-dibenzylhydroxylamine (Ib); analysis on g.l.c. system C showed that only the latter formed a TMS derivative. The internal marker used for quantitative analysis (a ketone), did not silylate. Silylation of *NN*-dibenzylhydroxylamine (Ib) by this procedure is quantitative and instantaneous. With synthetic or metabolically produced *NN*-dibenzylhydroxylamine, equivalent peaks appeared after silylation with identical retention times of 10·4 min on g.l.c. system C.

Extractability and calibration curves

Repeated extractions of DBA (Ib) and its *N*-dealkylated metabolite benzylamine, from aqueous alkaline solution showed that three extractions were sufficient to partition the compounds completely into ether. Under these conditions, no decomposition of DBA (Ia) or benzylamine occurred, and although there was decomposition of *NN*-dibenzylhydroxylamine (Ib), the decomposition products did not interefere with the analysis of the former compounds to any significant extent. In contrast, *NN*-dibenzylhydroxylamine (Ib) decomposed in basic and acidic media, but was stable at

neutral pH; two ether extracts were sufficient to extract all the hydroxylamine from neutral solution without decomposition. Calibration curves were constructed using liver homogenates to which had been added known quantities of DBA (Ia) and its metabolites, and the curves were linear (mean of triplicates) over the ranges investigated (DBA [Ia] 1.25–10·00 μmol/assay; benzylamine, 0·25–2·00 μmol/assay and *NN*-dibenzylhydroxylamine [Ib]0·1 to 1·0 μmol/assay). Regression analysis of these lines yielded correlation coefficients of not less than 0·995.

Thin-layer chromatography

The t.l.c. behaviour of DBA (Ia) and its *N*-oxidized metabolite in various solvent systems, and their responses to locating agents are depicted in Table 1, which shows an excellent correlation between synthetically and metabolically produced *NN*-dibenzylhydroxylamine (Ib). The *N*-hydroxy metabolite (Ib) was further characterized. Metabolic extracts were run without decomposition in solvent system 1, the hydroxylamine was localized and an equivalent area from an undeveloped chromatogram was scraped off and extracted with ether. When the ether layer was treated with *N*-trimethylsilylimidazole as described above, then chromatographed, it was found to contain a product which had a retention time (10·4 min) identical to that of the authentic *NN*-dibenzylhydroxylamine-TMS derivative. The thin-layer chromatographic characterization of benzylamine is not reported, because it is metabolically formed in small amounts and is unusually resistant to visualization by the locating reagents used.

Mass spectral characteristics of synthetic and metabolic NN-*dibenzylhydroxylamine* (*Ib*)

Under the conditions used, *NN*-dibenzylhydroxylamine (Ib) broke down to the parent amine (Ia) and the nitrone (II) on g.l.c.-mass spectrometry. This is substantiated by the fact that g.l.c. chromatograms of the hydroxylamine (Ib) showed three distinct peaks with retention times of 10·3, 13·3 and 33 min and when these peaks were scanned, they exhibited molecular ions of *m*/*e* at 197, 213 and 211 corresponding to the amine, hydroxylamine and nitrone respectively; base peaks occurred at *m*/*e* 91 in all three spectra (fragment c, Fig. 2). The spectra of authentic and metabolically

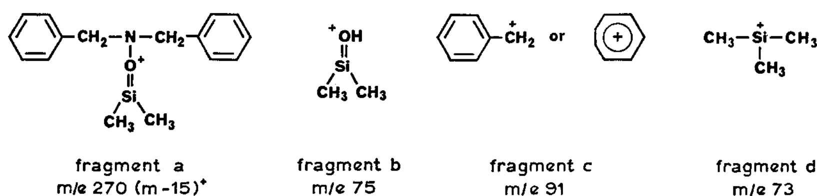

FIG. 2. Diagnostic fragments in the mass spectrum of the TMS derivative of *NN*-dibenzylhydroxylamine from incubation extracts.

produced *NN*-dibenzylhydroxylamine (Ib) were identical. The spectrum of the nitrone (II) formed on g.l.c. treatment was also identical to that of an authentic sample (Beckett & others, 1973). Fragment C was again the base peak in the spectrum of the TMS-ether of Ib. Other diagnostic ions in the spectrum of Ib-TMS ether are identified in Fig. 2 (Budzikiewicz, Djerassi & Williams, 1967; Lindeke, Cho & others, 1973). The spectra of the metabolically produced hydroxylamine (Ib) and its TMS

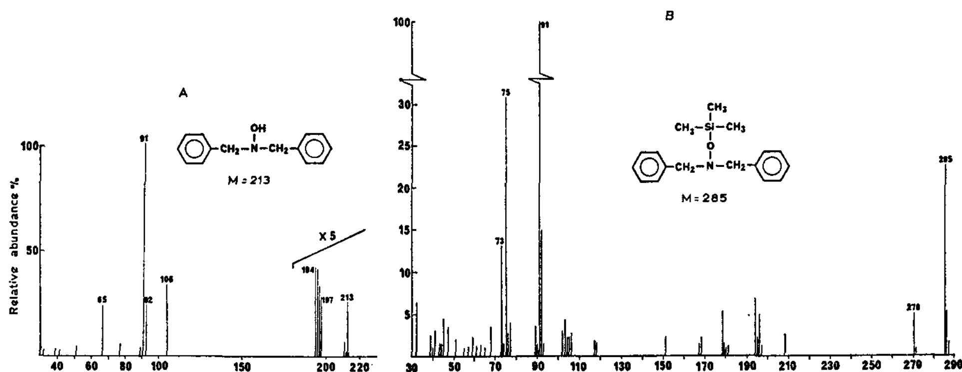

FIG. 3. Mass spectra of *NN*-dibenzylhydroxylamine (A) and its TMS derivative (B) from incubation extracts.

derivative are shown in Fig. 3. When the hydroxylamine (Ib) was silylated, thus imparting more stability to the molecule (on g.l.c.), neither the nitrone (II) breakdown peak nor the amine degradation product could be detected, substantiating the fact that the nitrone (II) and amine (Ia) are decomposition products of the hydroxylamine (Ib). The mass spectrum of the parent amine Ia (not shown) had an abundant ion at *m/e* 106 (75%) corresponding to the fragment $PhCH_2NH^+$. The origin of this ion is not clear. It is less abundant in the spectrum of *NN*-dibenzylhydroxylamine (Ib), Fig. 3, and is of very low abundance in the mass spectrum of the TMS derivative of the latter. The mass spectral evidence presented, verifies the existence of the hydroxylamine as a metabolite of dibenzylamine.

G.l.c. analysis of incubation extracts

The hydroxylamine of dibenzylamine from standard incubation extracts was identified as its TMS derivative on g.l.c. system C, and had a retention time (10·4 min) identical to that of the synthetic compound under the same conditions. When the enzyme was denatured by heat treatment (90°, 15 min) or omitted, of if the substrate or cofactor cocktail was omitted, then no hydroxylamine (Ib) could be detected on either g.l.c. or t.l.c. A more extensive study of the cofactor requirements for the metabolic *N*-oxidation of Ia to the hydroxylamine (Ib), and characterization or the enzyme system will be published elsewhere (Beckett & Gibson, 1975). When DBA (10 μmol) was incubated with the fortified 9000*g* hepatic homogenate from the rabbit, an analysis of unchanged DBA (Ia) and its metabolites showed that 3·72 μmol of DBA were metabolized, 3·37 μmol of the hydroxylamine (Ib) were formed together with 0·23 μmol of benzylamine; an overall recovery of 97%. Consistent results were obtained with different rabbit liver 9000*g* homogenates. *NN*-Dibenzylhydroxylamine (Ib) was also incubated at 37° for 60 min with a fortified 9000*g* supernatant fraction, but recovery was quantitative, showing that at least under these conditions, the hydroxylamine was not further metabolized by Phase 1 oxidative mechanisms. The analytical data presented above indicates that metabolic ring hydroxylase activity contributes little, if any, to the overall, *in vitro* metabolism of DBA (Ia) and that *N*-oxidation is the major metabolic pathway, occurring to the extent of 91% of the total amount of DBA metabolized, compared to a 6% production of benzylamine; even this product of *N*-dealkylation could arise from metabolic oxidation of N_2 rather than the α-carbon

atom (Beckett & Bélanger, 1975). To our knowledge there is no other drug, analogue or substrate that exhibits this specificity with respect to *N*-oxidation over *C*-oxidation to such an extent, and thus DBA constitues a useful substrate for the study of the *N*-oxidation metabolic pathway. Additional preliminary studies suggest that a wide range of basic amine drugs can compete with DBA for the *N*-oxidation system in the rabbit (Beckett & Gibson, 1975).

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REFERENCES

BECKETT, A. H. & ROWLAND, M. (1965). *J. Pharm. Pharmac.*, **17**, 59–60.

BECKETT, A. H. (1971). *Xenobiotica*, **1**, 365–383.

BECKETT, A. H. & AL-SARRAJ, S. (1972). *Biochem. J.*, **130**, No. 1., 14P.

BECKETT, A. H. (1973). In, *Frontiers in Catecholamine Research.* Editors, Usidin, E. & Snyders, S. pps. 139–143, Oxford: Pergamon.

BECKETT, A. H., COUTTS, R. T. & OGUNBONA, F. A. (1973). *Tetrahedron*, **29**, 4189–4193.

BECKETT, A. H. (1974). In, *Ciba Foundation Symposium.* No. 26. Amsterdam: Associated Sci. Pub.

BECKETT, A. H. & BÉLANGER, P. M. (1974). *Xenobiotica*, **4**, 509–519.

BECKETT, A. H. & BÉLANGER, P. M. (1975). *J. Pharm. Pharmac.*, **27**, 547–552.

BECKETT, A. H. & GIBSON, G. G. (1975). *Xenobiotica*, in the press.

BICKEL, M. H. (1969). *Pharmac. Rev.*, **21**, 325–355.

BUDZIKIEWICZ, H., DJERASSI, C. & WILLIAMS, D. H. (1967). *Mass Spectrometry of Organic Compounds*, Holden-Day Inc., San Francisco, pps. 471–477.

CUCINELL, S. A., ISRAILI, Z. H. & DAITON, P. G. (1972). *Am. J. trop. Med. Hyg.*, **21**, 322–331.

HORNING, M. G., MOSS, A. M. & HORNING, E. C. (1967). *Biochim. biophys. Acta*, **148**, 597–600.

HORNING, M. G., MOSS, A. M., BOUCHER, E. A. & HORNING, E. C. (1968). *Analyt. Letters*, **1**, 311–321.

LINDEKE, B., CHO, A. K., THOMAS, T. L. & MICHELSON, L. (1973). *Acta pharm. suecica*, **10**, 493–506.

WEISBERGER, J. H. & WEISBERGER, E. K. (1973). *Pharmac. Rev.*, **25**, 1–66.