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The *in vitro* metabolism of 2-nitroso-1-phenylpropane dimer by fortified 9000*g* supernatants from rabbit liver

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2-Nitroso-1-phenylpropane dimer, a postulated metabolic product of amphetamine, was metabolized by fortified 9000 *g* supernatants from rabbit liver, mainly by oxidation on the carbon *beta* to the nitrogen to form a '*β*-hydroxylated nitroso dimer'. The corresponding nitro compound, ketone and alcohol were also products, the latter two but not the former being cofactor dependant. The nitroso compound was not metabolically reduced under the conditions used.

Beckett & Bélanger (1975) recently proposed a mechanism for the metabolism of primary aliphatic amines via an amine-oxygen-flavoprotein complex. When the amine possessed at least one α-hydrogen atom, the complex gave the hydroxylamine by one route, while a second route gave rise to the corresponding ketone (or aldehyde), oxime and nitroso compound via chemical changes from a common *N*-hydroperoxide; the ketone (or aldehyde) was also derived from *alpha*-carbon oxidation. Postulation of a nitroso compound as a metabolic product of certain aralkylamines prompted the synthesis and characterization of a series of aralkyl nitroso compounds with one or two protons on the carbon atoms *alpha* to the nitrogen. These were isolated in the dimeric form (Beckett, Jones & Coutts, 1977).

Since the nitroso metabolite of phentermine is metabolically reduced extensively to the corresponding hydroxylamine by fortified hepatic fractions (Bélanger, 1975), the *in vitro* metabolism of 'amphetamine nitroso' was investigated to determine if some of the hydroxylamine (Ib) formed during the metabolism of amphetamine *in vitro* (Beckett & Al-Sarraj, 1972; Lindeke, Cho & others, 1973; Parli & McMahon, 1973) might partially arise via the nitroso compound (Id or IIIa). Also, since it might be difficult to detect the nitroso compound (Id or IIIa) directly after the *in vitro* metabolism of amphetamine, the possibility was considered that other products would be formed which might implicate the nitroso compound as an intermediate in their formation. Thus the azoxy- (IIIf), azo-(IIIe) and hydrazo-(IIId) compounds might be obtained by successive metabolic reduction of the nitroso dimer (IIIa) since these reductions have been demonstrated chemically (Beckett & others, 1977).

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MATERIALS AND METHODS

Compounds and reagents

The synthesis of the compounds has already been described: (−)-'amphetamine nitroso' dimer (2-nitroso-1-phenylpropane dimer, IIIa) and (±)-'norephedrine nitroso' dimer (1-hydroxy-2-nitroso-1-phenylpropane dimer, IIIc) (Beckett & others, 1976); 'norephedrine oxime' (1-hydroxy-1-phenyl-2-propanone oxime, IIc) (Beckett, Jones & Al-Sarraj, 1974); (−)-'amphetamine hydroxylamine' (2-hydroxylamino-1-phenylpropane, Ib) (Beckett, Haya & others, 1975). The following compounds were purchased: 1-phenyl-2-propanone (Koch Light), 1-phenyl-2-propanol (Fluka). (See Fig. 1 for the structures.) BSTFA (*N,O*-bis [trimethylsilyl]trifluoroacetamide) (Pierce Chemicals).

Incubations with liver homogenates

Incubations were carried out using 9000 *g* liver homogenates (fortified with NADP, glucose-6-phosphate, nicotinamide and magnesium chloride) from male New Zealand White rabbits (2–3 kg), as described by Beckett & Bélanger (1974). The nitroso dimer substrate (IIIa, (−)-isomer, 5 μmol) was added in 0·1 ml dioxan and the solution incubated for 60 min at 37°; when the subsequent analysis was by mass spectrometry, the incubation time was doubled. Control incubations were carried out in which (a) substrate, (b) cofactor and (c) liver homogenate were separately omitted.

Extraction and analysis

Flasks were placed in ice after the incubation period to halt enzyme activity. The whole incubates were extracted by shaking for 10 min in 2·25 × 7·5 cm

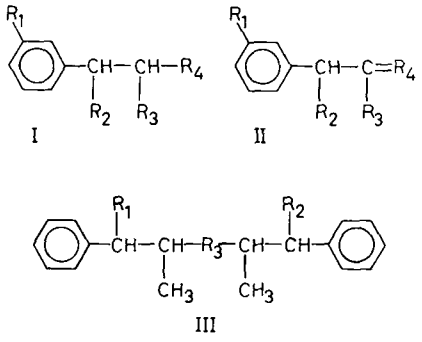

FIG. 1. Structure of amphetamine (Ia) and some oxidation products and analogues.

	R_1	R_2	R_3	R_4
Ia	H	H	CH_3	NH_2
Ib	H	H	CH_3	NHOH
Ic	H	H	CH_3	OH
Id	H	H	CH_3	NO
Ie	H	H	CH_3	NO_2
If	H	OH	CH_3	NHOH
Ig	H	OH	CH_3	OH

	R_1	R_2	R_3	R_4
IIa	H	H	CH_3	NOH
IIb	H	H	CH_3	O
IIc	H	OH	CH_3	NOH
IId	H	OH	CH_3	O

	R_1	R_2	R_3
IIIa	H	H	-N(O) = N(O)-
IIIb	H	OH	-N(O) = N(O)-
IIIc	OH	OH	-N(O) = N(O)-
IIId	H	H	-NHNH-
IIIe	H	H	-N = N-
IIIf	H	H	-N(O) = N-

stoppered centrifuge tubes with *ca* 2 × 10 ml distilled diethyl ether, normally at pH 7·4; however, when required, solutions were made acidic (pH < 1) with 0·5 ml conc. hydrochloric acid, alkaline (pH 9–10) with 50–100 mg anhydrous sodium carbonate and strongly alkaline (pH 13–14) with 0·5 ml 5 N sodium hydroxide. The ethereal extracts were concentrated on a water bath (45°) to about 20 μl and placed on an ice bath pending analysis.

Derivatization. The above extracts were evaporated to dryness under nitrogen, 10 μl of dry acetonitrile (over $CaCl_2$) plus 25 μl BSTFA added and mixed well with the metabolic residue.

Instrumentation

Gas liquid chromatography (g.l.c.)

G.l.c. was on a Perkin Elmer F11 with a flame ionization detector. Glass columns of i.d. 0·40 cm were used; nitrogen was the carrier gas (see below); air and hydrogen pressures were 145 kNm^{-2} each. *System A1*: 1 m column, packed with 2% Carbowax 20M on Chromosorb G 100–120 mesh, acid washed and DMCS treated; nitrogen flow rate was 60 ml min^{-1} (pressure 105 kNm^{-2}) and the oven temperature 130°. *System A2*: as for system A1, but the oven temperature was 160°. *System B*: 2 m column, packed with 2% XE 60 on Chromosorb G 80–100 mesh, acid washed and DMCS treated; nitrogen flow rate was 20 ml min^{-1} (pressure, 75 kNm^{-2}) and the oven temperature 90°.

Thin layer chromatography (t.l.c.)

T.l.c. was carried out on plates spread to a thickness of 0·25 mm with silica gel GF_{254}, dried for 1 h at 110° and developed with benzene–ethyl acetate (3:1 v/v). The plates were viewed under ultraviolet light (λ 254 nm) and either sprayed with 1% copper(II) chloride (oximes appeared as green-spots) or placed in an iodine tank.

G.l.c.-mass spectrometry (g.l.c.-ms)

G.l.c.-m.s. analysis was performed with a Perkin Elmer model 270 g.l.c.-mass spectrometer; column conditions were: *System A*: packed as for g.l.c. system A1; helium (carrier gas) 100–140 kNm^{-2}; oven temperature 120–140°. *System B*: packed as for g.l.c. system B, but a 1 m column; helium, 75–105 kNm^{-2}; oven temperature, 90–120°.

RESULTS AND DISCUSSION

G.l.c. examination of the extracts

Etheral extracts of 'amphetamine nitroso' dimer (IIIa) incubates were made at acidic (pH <1) and alkaline (pH 9–10 and pH 13–14) pH values, but their examination by g.l.c. (system A1, A2 and B) did not reveal the presence of metabolites and metabonates other than those present in the neutral (pH 7·4) extract, which was characterized further.

The ketone (IIb) and alcohol (Ic) were identified as cofactor dependent metabolic products from the nitroso dimer (IIIa) incubates by comparison of their g.l.c. and g.l.c.-ms properties with those of authentic compounds. However, the cofactor dependent disappearance of the nitroso dimer (IIIa) (monitored on g.l.c. as the oxime [IIa, by thermal rearrangement of the nitroso dimer in the injection port—Beckett & others, 1977]) on incubation with fortified rabbit liver homogenates could not be fully accounted for by the formation of the ketone (IIb) and alcohol (Ic), the only obvious metabolites on g.l.c. system A1, or of the nitro compound (Ie) arising from autoxidation of the nitroso compound (Id or IIIa).

BSTFA was used to silylate the nitroso dimer (IIIa) incubate extract; four cofactor dependent peaks (A, B, C and D) were obtained using g.l.c. system B (see Fig. 2). Peak 'A' was due to the TMS (trimethylsilyl) derivative of the alcohol (Ic) and peak 'B' was due to underivatized ketone (2b) and the oxime (2a)-TMS derivative [the nitroso

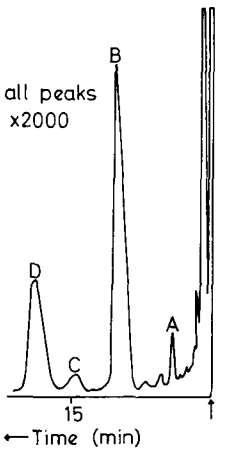

FIG. 2. G.l.c. trace (system B) of the extract (at pH 7·4) of metabolism+ of (−)-'amphetamine nitroso' dimer (IIIa)-derivatized with BSTFA. A = 2-hydroxy-1-phenylpropane-TMS derivative. B = 1-phenyl-2-propanone oxime-TMS derivative and 1-phenyl-2-propanone. C = 1-hydroxyl-1-phenyl-2-propanone oxime-TMS derivative (*syn-* or *anti-*isomer). D = 1-hydroxy-1-phenyl-2-propanone oxime-TMS derivative (*anti-* or *syn-*isomer).
+ Fortified rabbit 9000*g* supernatant (= 1 g orig. liver); 120 min at 37°; 2·5 μmol substrate.

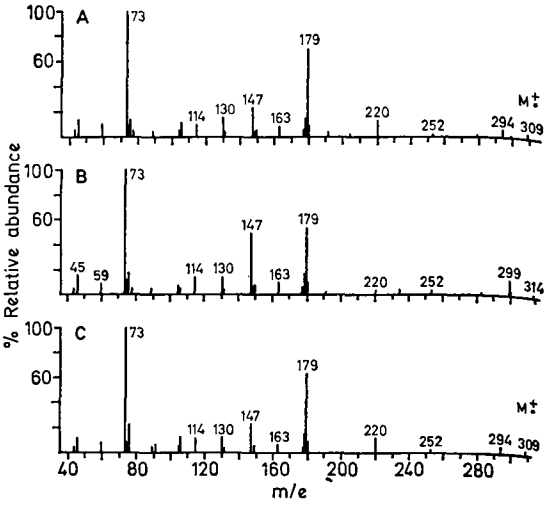

FIG. 3. G.l.c.-mass spectra of: A-peak 'C' and B-peak 'D' (see Fig. 2) the *syn-* and *anti-*'norephedrine oxime' (IIc)-TMS derivative peaks, derived from the metabolism of 'amphetamine nitroso' dimer (IIIa), and C-g.l.c.-ms of the authentic 'norephedrine oxime' (IIc)-TMS derivative.

dimer rearranges to the oxime in the injection port, which is derivatized *in situ* and the product has the same retention time as the ketone (IIb)]. A fairly small peak 'C' and comparatively large peak 'D' both had mass spectra comparable to that of synthetic di(*O*-trimethylsilyl)-'norephedrine oxime' (IIc, 1-hydroxy-1-phenyl-2-propanone oxime) (Fig. 3). The underivatized synthetic 'norephedrine oxime' (IIc), used as a reference compound, was a pure geometrical isomer (nmr evidence—Beckett & others, 1974), which on silylation gave one g.l.c. peak of the same retention time as peak 'D'. However, injection on the same g.l.c. system (B) of a mixture of 'norephedrine nitroso' dimer (IIIc) and BSTFA, gave two g.l.c. peaks of the same retention times as peaks 'C' and 'D' from the derivatized 'amphetamine nitroso' dimer (IIIa) incubate extract, the peak of shorter retention time being considerably smaller. Peaks 'C' and 'D' were therefore concluded to be di-TMS-derivatives of the *syn-* and *anti-*isomers (though not necessarily respectively) of 'norephedrine oxime' (IIc). Resolution of other *syn-* and *anti-*oximes by g.l.c. has been demonstrated previously (Beckett, Van Dyk & others, 1971). The mass spectrum of the smaller peak (C) showed additional ions at *m/e* 299 and *m/e* 314 which were present in the blank spectrum (run after injecting a similarly treated extract, except the substrate was omitted) and probably arose from derivatized impurities extracted from the incubate or the solvent. Extraction of the incubate after adding 50–100 mg sodium carbonate minimized the interfering g.l.c. peak.

T.l.c. examination of the extracts

The nitroso dimer (IIIa) incubate neutral extract was run on t.l.c. and the plate sprayed with 1% copper(II) chloride solution. Two green spots developed with R_F values of 0·18 and 0·45; these were identified as 'norephedrine oxime' (IIc) and 'amphetamine oxime' (IIa) respectively. The plate was then heated to 120° for 30 min, cooled and re-sprayed with 1% copper(II) chloride solution; two additional green spots appeared of R_F values 0·50 and 0·57, due to nitroso compounds, converted to the corresponding oximes, after development, by heat. The spot at R_F 0·57 was recovered substrate, 'amphetamine nitroso' dimer (IIIa) and that at R_F 0·50 due to a more polar metabolite.

The spot of R_F 0·50 (unsprayed) was eluted from the silica gel with ether and run on g.l.c. system A1; two g.l.c. peaks resulted of Rt 2·0 and 28·3 min, corresponding to 'amphetamine oxime' (IIa) and 'norephedrine oxime' (IIc) respectively. Similarly on g.l.c. system B, after the addition of BSTFA,

the t.l.c. extract gave two main peaks corresponding to the TMS derivatives of the oximes IIa and IIc and one minor peak corresponding to the other geometrical isomer of the 'norephedrine oxime (IIc)-TMS derivative. In each case the 'amphetamine oxime' (IIa) peak (or its TMS derivative) was greater in area than the corresponding 'norephedrine oxime' (IIc) peak (or its TMS derivative) [probably due to slight overlap of either the 'amphetamine oxime' (IIa) spot or 'amphetamine nitroso' dimer (IIIa) t.l.c. spot with the metabolite spot at R_F 0·50].

Direct inlet mass spectrometry

Direct inlet mass spectra were run of the t.l.c. eluate of the metabolite at R_F 0·50. Four scans were made; the first, just after insertion of the probe and the remainder at 15–30 s intervals. The first mass spectrum was primarily of 'amphetamine nitroso' monomer (Id), the fourth spectrum primarily of 'norephedrine nitroso' monomer and intermediate spectra were of both. The metabolite was thus identified as the 'mono-β-hydroxy nitroso dimer' (IIIb) which dissociated on the probe and fractionally distilled off under the low pressure giving partially resolved mass spectra of the component monomers.

Possibility of β,β′-hydroxylation

The 'amphetamine nitroso' dimer (IIIa) is metabolically oxidized on at least one side of the alkyl chain, to the 'β-hydroxy nitroso dimer' (IIIb). Whilst there is no apparent evidence to suggest additional oxidation on the second β-carbon (i.e. β′), this possibility cannot be precluded and additional information was sought as follows.

'Norephedrine nitroso' dimer (IIIc), i.e. β,β′-hydroxyl derivative of 'amphetamine nitroso' dimer, obtained from oxidation of (±)-norephedrine base (Beckett & others, 1977) gave a t.l.c. spot of R_F value 0·39 cf 'amphetamine oxime' R_F 0·45; 'β-hydroxy nitroso dimer' metabolite, R_F 0·50; 'amphetamine nitroso' dimer, R_F 0·57); there was no spot corresponding to this value from the metabolic extract. However, other diastereoisomers of the 'norephedrine nitroso' dimer (IIIc) general structure could have slightly different R_F values, possibly overlapping other spots. Since the nitroso dimer derivatives of norephedrine and norpseudoephedrine are difficult to prepare in more than milligram quantities due to considerable oxidation of the amine to benzaldehyde and other products, unequivocal identification of the isomer(s) of the 'mono-β-hydroxy nitroso dimer' (IIIb) found metabolically, or a definite statement that β-hydroxylation does not take place on both sides of the nitroso dimer (IIIa) molecule, is not possible at present.

The 'norephedrine oxime' (IIc) identified on t.l.c. could have arisen from chemical breakdown of the 'β-hydroxylated nitroso dimer' (i.e. IIIb), or metabolic β-oxidation of 'amphetamine oxime' (IIa), which can be produced by chemical breakdown of the nitroso dimer (IIIa) substrate or of the 'mono-β-hydroxylated nitroso dimer' (IIIb).

Other possible metabolic products

The neutral and basic extracts of the 'amphetamine nitroso' dimer (IIIa) incubates were examined for the possible azoxy-(IIIf), azo-(IIIe) and hydrazo-(IIId) metabolites or metabonates on g.l.c. system A2; none were found.

The extracts were also examined for 'amphetamine hydroxylamine' (Ib) by g.l.c. (system A1; also system B after derivatization with BSTFA) and t.l.c.; however, none was detected. This is in contrast to the extensive metabolic reduction of 'phentermine nitroso', to the corresponding hydroxylamine by fortified rabbit liver 9000 *g* supernatant, found by Bélanger (1977). The stability of 'amphetamine nitroso' in the dimeric form (cf monomer) (Beckett & others, 1977) compared with the readily dissociated 'phentermine nitroso' (Bélanger, 1975) could partly explain its lack of metabolic reduction to the hydroxylamine.

The present work demonstrates that β-hydroxylation is a major *in vitro* metabolic pathway of (−)-'amphetamine nitroso' dimer in rabbit liver homogenates. The identity of the enzyme is unknown although it apparently oxidizes the comparable carbon atom on the nitroso dimer, as dopamine β-hydroxylase does on (+)- and (±)-amphetamine [but not the (−)-isomer] to form norephedrine *in vitro* (Goldstein, McKereghan & Lauber, 1964), and *p*-hydroxynorephedrine and norephedrine *in vitro* in rat (Goldstein & Anagnoste, 1965) and man (Caldwell, Dring & Williams, 1972).

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