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Metabolic *N*-oxidation of atropine, hyoscine and the corresponding nor-alkaloids by guinea-pig liver microsomal preparations

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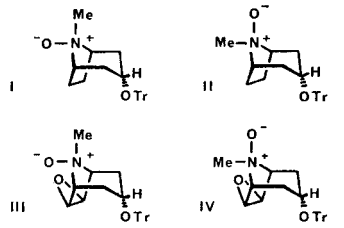
Incubation of guinea-pig liver microsomal preparations with atropine or hyoscine resulted in the formation of the corresponding nor-alkaloids and both isomers of atropine *N*-oxide from atropine and one isomer of hyoscine *N*-oxide from hyoscine. Separate incubations of guinea-pig liver microsomal preparations with nor-atropine and nor-hyoscine yielded the corresponding hydroxylamines. The *N*-oxide and hydroxylamine metabolites were identified by comparison of their t.l.c. behaviour and m.s. with prepared compounds and also by their reduction to the corresponding tertiary or secondary amines.

Tertiary amines yield *N*-oxides and secondary amines produce hydroxylamines during mammalian metabolism (Bickel, 1969; Beckett, 1971). *N*-oxidation of some alkaloids occurs in plants (Culvenor, 1953; Phillipson, 1971) and *N*-oxides of hyoscyamine and hyoscine have been isolated as natural products from several genera of the Solanaceae (Phillipson & Handa, 1975). We have investigated the *N*-oxidation of these alkaloids and their corresponding nor-compounds in a mammalian system. For this purpose (−)-hyoscyamine and (−)-hyoscine were converted to their *N*-oxides and also atropine [(±)-hyoscyamine] and (−)-hyoscine were converted to the corresponding nor-alkaloids which were further oxidized to hydroxylamines. Hyoscyamine forms two isomeric *N*-oxides which have been termed isomer 1 (I, equatorial N^+–O^-) and



I–IV Tr = $-CO.CH(CH_2OH).C_6H_5$

isomer 2 (II, axial N^+–O^-); similarly, hyoscine also forms two *N*-oxides, isomer 1 (III, equatorial N^+–O^-) and isomer 2 (IV, axial N^+–O^-) (Huber, Fodor & Mandava, 1971).

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MATERIALS AND METHODS

Preparation of compounds

N-oxides. (−)-Hyoscyamine and (−)-hyoscine *N*-oxides were prepared by oxidation of the corresponding tertiary bases (BDH Chemicals Ltd) with *m*-chloroperbenzoic acid and each pair of isomers were separated and characterized as previously described (Phillipson & Handa, 1975). T.l.c. R_F values (system A) are given in Table 1.

Nor-atropine (Carr & Reynolds, 1912). Atropine [(±)-hyoscyamine] (1 g) was dissolved in water (30 ml) and potassium permanganate (1·5 g) added over 1 h (Benholzer, Schutz & Zeil, 1912), during which the pH was adjusted to between 7·5 and 8·0 with $NaHCO_3$ and maintained at 30°. The suspension was filtered and the filtrate made alkaline with sodium carbonate and extracted with chloroform (5 × 50 ml). The combined chloroform extracts were washed, dried with anhydrous sodium sulphate, filtered and concentrated to dryness under reduced pressure to yield nor-atropine (350 mg, 48%).

The R_F values (systems A and B) are given in Table 1; nmr, δ 7·33 (5H, *s*; aromatics), 5·10 (1H, *t*; H-3), 4·20 (1H, *t*; benzylic), 3·80–4·00 (2H, *m*; CH_2OH), 2·73–3·40 (2H, *m*; H-1 and -5), 1·95 (2H, *m*; H-2 and -4, *axial*), 1·70 (2H, *m*; H-2 and -4, *equatorial*), 1·15–1·50 (2H, *q*; H-6 and -7). *Oxalate*, the base was dissolved in absolute ethanol (2 ml) and 10% aqueous oxalic acid solution added dropwise until just acidic. On cooling, colourless prismatic crystals of oxalate salt, m.p. 260–262° were obtained. M.s. of the oxalate, *m/e* 275 (parent ion of base, 5%), 136 (5·3), 121 (5·3), 118 (2·3),

111 (17), 110 (100), 109 (8·4), 108 (4·6), 104 (3·8), 103 (12·3), 91 (13), 90 (5·3), 83 (5·3), 82 (16·9), 81 (20), 80 (23), 79 (6·1), 78 (3·8), 77 (13), 69 (10·7), 68 (24), 67 (23).

Nor-hyoscine (Gmelin, 1941). Hyoscine hydrobromide (384 mg) was treated, as described above, with potassium permanganate (379 mg), to yield nor-hyoscine (280 mg, 97%), hydrochloride m.p. 238–240°.

The R_F values (systems A–E) are given in Table 1 and are identical with those of an authentic sample; nmr, δ 7·23 (5H, *s*, aromatics), 5·00 (1H, *t*; H-3), 4·20 (1H, *t*; benzylic), 3·68–4·00 (2H, *m*; CH_2OH), 2·93–3·13 (2H, *m*; H-1 and -5), 2·91 (2H, *m*; H-6 and -7), 1·20–2·03 (4H, *m*; H-2 and -4 *axial* and *equatorial*). *Hydrochloride.* The base was dissolved in absolute ethanol (5 ml) and 5% HCl in absolute

Table 1. *Thin-layer chromatography of atropine and hyoscine, their nor-derivatives and* N-*oxidation products.*

Compound	*RF* values in solvent systems A	B	C	D	E
Atropine	0·73	0·24	0·07	0·05	0·06
Hyoscine	0·80	0·70	0·42	0·34	0·25
Atropine *N*-oxide (isomer 1)	0·30	0·06	0	0·03	0
Atropine *N*-oxide (isomer 2)	0·35	0·08	0	0·03	0
Hyoscine *N*-oxide (isomer 1)	0·20	0·01	0	0	0
Hyoscine *N*-oxide (isomer 2)	0·45	0·01	0	0·08	0
Noratropine	0·53	0·23	0	0·05	0
Norhyoscine	0·72	0·62	0·30	0·31	0·14
N-Hydroxynoratropine	0·74	0·75	—	—	0·25
N-Hydroxynorhyoscine	0·76	0·82	0·60	0·42	0·37

Silica gel G (Merck)
Solvent systems: A. ethylacetate–isopropanol–20% ammonium hydroxide (45:35:15); B. acetone–water–conc. ammonium hydroxide (90:7:3); C. acetone–conc. ammonium hydroxide (99:1); D, chloroform–methanol (90:10); E. chloroform–acetone–conc. ammonium hydroxide (50:50:1).

ethanol added dropwise until the solution was just acidic. Dry diethyl ether was added slowly until slightly turbid and on cooling colourless needle crystals of the hydrochloride, m.p. 238–240° were obtained. M.s. of the hydrochloride, *m/e* 289 (parent ion of the base, 66%), 141 (5·7), 140 (14), 124 (23), 123 (40), 122 (51), 121 (29), 118 (13), 112 (9), 110 (10), 107 (13), 106 (77), 105 (14), 104 (17), 103 (74), 97 (11), 96 (17), 95 (20), 94 (100), 93 (14), 91 (34), 90 (14), 89 (9), 83 (22), 82 (11), 81 (14), 80 (94), 79 (21), 78 (11), 77 (21), 68 (85), 67 (18).

N-*Hydroxynoratropine and* N-*hydroxynorhyoscine.* The nor-alkaloid (200 mg) was liberated from the salt and dissolved in chloroform (15 ml) and *m*-chloroperbenzoic acid (200 mg) added gradually while stirring. After 8 h the solvent was removed with a stream of nitrogen. The residue was dissolved in diethyl ether, washed with 10% aqueous potassium carbonate (2 × 10 ml) and distilled water (10 ml). The ethereal solution was dried over anhydrous sodium sulphate, filtered and evaporated to dryness under reduced pressure. Nor-hyoscyamine yielded 160 mg (76%) of a pale yellow solid which was crystallized as the oxalate, while nor-hyoscine yielded 192 mg (91%) of an amorphous solid which was crystallized as the hydrochloride.

N-*Hydroxyatropine* was obtained as an amorphous solid; R_F values (systems A, B and E) are given in Table 1, an immediate black spot was produced by spraying with 7% ammoniacal silver nitrate; nmr, δ 7·30 (5H, *s*; aromatics), 4·90 (1H, *t*; H-3), 4·21 (1H, *m*; benzylic), 3·65–4·05 (2H, *m*; CH_2OH), 3·20–3·60 (2H, *m*; H-1 and -5), 1·55–2·10 (8H, *m*; H-2, -4, -5 and -6). Accurate mass measurements, found M^+, 291·1481, $C_{16}H_{21}NO_4$ requires M^+, 291·1471; found 110·0968, $C_7H_{12}N$ requires 110·0969. *Oxalate*, m.p. 246–248° was prepared as described above. M.s. *m/e* 291 (parent ion of base, 0·7%), 27 6(1·2), 275 (5), 126 (7·4), 125 (2), 121 (4), 111 (14), 110 (100), 109 (8), 108 (4), 103 (8), 92 (2), 93 (3), 91 (7), 83 (4), 82 (12), 81 (15), 80 (16), 79 (4), 77 (8), 69 (9), 68 (18), 67 (15).

N-*Hydroxynorhyoscine* was obtained as an amorphous solid; R_F values (systems A–E) are given in Table 1, an immediate black spot was produced by spraying with 7% ammoniacal silver nitrate. *Hydrochloride*, m.p. 196–198°, was prepared as described above; found C, 55·84%; H, 5·69%, N, 4·14%; $C_{16}H_{19}NO_5$, HCl requires C, 56·10%; H, 5·86%; N, 4·11%. Nmr, δ 7·40 (5H, *s*; aromatics), 5·10 (1H, *t*; H-3), 4·25 (1H, *t*; benzylic), 3·80–4·10; (4H, *m*; overlapping $-CH_2OH$ and H-1 and -5), 2·82 (2H, *q*; H-6 and -7), 1·60–2·20 (4H, *m*; H-2 and -4). M.s. *m/e* 305 (parent ion of base, 2·6), 304 (0·9), 303 (0·5), 290 (0·7), 289 (2·3), 276 (0·5), 275 (1), 158 (6), 157 (66), 149 (3), 140 (17), 139 (12), 131 (4), 129 (3), 128 (4), 124 (7), 123 (12), 122 (26), 121 (100), 118 (11), 110 (6), 109 (24), 107 (3), 105 (6), 104 (8), 103 (43), 98 (12), 97 (8), 96 (11), 94 (13), 91 (18), 81 (13), 80 (13), 79 (23), 78 (5).

Reduction of hydroxylamines. The hydroxylamine (2 mg) was allowed to stand overnight with titanous chloride (2 ml, 15% $TiCl_3$, BDH and 1 ml 5N HCl). The solution was made alkaline with 2N, ammonium

hydroxide and extracted with chloroform. T.l.c. (systems A–D) indicated that each hydroxylamine reduced to only one alkaloid which had identical R_F values (Table 1) with those of the corresponding nor-alkaloids.

Thin-layer chromatography (*t.l.c.*). 20 × 30 cm glass plates were coated with 0·25 mm layers of silica gel G (Merck) and activated by heating to 110° for 1 h. The solvent systems A–E and the R_F values of the compounds are given in Table 1. The tertiary alkaloids, their *N*-oxides and the nor-alkaloids were detected with Dragendorff's reagent and the hydroxylamines with 7% ammoniacal silver nitrate.

Mass spectroscopy. Mass spectra were determined with an AEI MS-902 high resolution mass spectrometer using an ionization potential of 70 eV and inlet temperatures between 185 and 195°.

Nmr spectroscopy. 60 MHz nmr spectra were determined in $CDCl_3$ solution using TMS as internal reference.

Preparation of liver microsome fraction. Liver homogenates were prepared from male Dunkin-Hartley guinea-pigs using the method previously described (Gorrod, Temple & Beckett, 1975). The homogenate was centrifuged at 10 000 *g* for 20 min, then 140 000 *g* for 80 min. The pellet was resuspended in isotonic tris/KCl buffer pH 7·4 and recentrifuged at 140 000 *g* for a further 1 h. The pellet resuspended in isotonic tris/KCl buffer is referred to as the washed microsomal preparation.

Incubation of alkaloids and guinea-pig liver microsomal preparations

Substrate solution. Each substrate (25 μM) in distilled water (0·1 ml) and phosphate buffer (pH 7·4, 0·2M, 0·4 ml) was divided into five equal volumes, transferred to 5 separate 25-ml incubation flasks and placed on ice. The following substrates were used: atropine [(±)-hyoscyamine] sulphate, hyoscine hydrobromide, nor-atropine oxalate, nor-hyoscine hydrochloride.

Co-factor solution. Freshly prepared solutions of the following co-factors were used per flask: NADPH tetrasodium salt, type I (Sigma) 3 μM, glucose-6-phosphate (Boehringer Corporation Ltd) 15 μM, glucose-6-phosphate dehydrogenase (Boehringer Corporation Ltd.) 1 unit, magnesium chloride (0·1 M) 0·6 ml, phosphate buffer (0·2 M, pH 7·4) up to 2·5 ml.

Incubation. Substrate (5 μM in 0·1 ml), co-factor solution (2·5 ml) and washed liver microsomal preparation (1 ml ≡ 0·5 g liver) were incubated for 1 h at 37° with constant shaking. Three controls were run simultaneously as follows: (*a*) substrate, washed microsomal preparation but no co-factor solution; (*b*) substrate, co-factor solution and inactivated microsomal preparation (previously heated in boiling water for 15 min and cooled) and (*c*) washed microsomal preparation, co-factor solution and no substrate.

Isolation and identification of metabolites

After incubation, either methanol (20 ml, used for tertiary alkaloids) or ethanol (20 ml, used for secondary alkaloids) was added to each flask. The precipitate was removed by centrifuging at 10 000 *g* for 30 min and the separated supernatant evaporated to dryness under reduced pressure. The residue was extracted with 2% sulphuric acid (3 × 5 ml) and the combined filtered acid extracts made alkaline (pH 10) with ammonium hydroxide and extracted with chloroform (3 × 15 ml) and subsequently with chloroform-methanol (9:1) (2 × 15 ml). The organic extracts were combined, dried with anhydrous sodium sulphate, filtered and evaporated to 0·1 ml. The following basic metabolites were separated and identified:

1. *From atropine and from hyoscine*. Preparative t.l.c. (system A) was used to separate the following metabolites: (a) *Nor-alkaloids*. Atropine and hyoscine yielded the corresponding nor-alkaloids which were identified by their R_F values (system A) and mass spectra which were identical with those of the prepared compounds. (b) N-*oxides*. Atropine yielded both isomers 1 and 2 of atropine *N*-oxide (Phillipson & Handa, 1975) while hyoscine yielded only isomer 1 of hyoscine *N*-oxide (Phillipson & Handa, 1975). The *N*-oxides were identified by their R_F values (system A) and mass spectra, which were identical with those of the prepared compounds. The reduction of each isolated *N*-oxide with titanous chloride resulted in only one alkaloidal spot on t.l.c. (systems A and B) and this had identical R_F values in each case with the corresponding tertiary alkaloid.

2. *From nor-atropine and nor-hyoscine*. *N*-hydroxynoratropine and *N*-hydroxynorhyoscine were obtained by preparative t.l.c. (system E) from incubates of the corresponding nor-alkaloids. Each hydroxylamine was identified by its R_F value (system E), immediate black colour on spraying

with 7% ammoniacal silver nitrate and by its mass spectrum. These properties were identical with those of the synthetic compounds. Reduction of the hydroxylamine metabolites with titanous chloride (see above) resulted in the formation of only one alkaloidal spot which had R_F values (systems A and B) which were identical with those of the corresponding nor-alkaloid.

Control experiments

Each control incubation was processed, as described above, and examined by t.l.c. for the presence of oxidation products. All control incubations were negative for the presence of *N*-oxides, hydroxylamines and nor-compounds.

Estimation of basic metabolites of atropine. Atropine (17·5 mg) was incubated with the washed liver microsomal preparation and the basic metabolites extracted and separated by preparative t.l.c. as described above. Unchanged atropine, nor-atropine and total *N*-oxides were assayed colorimetrically using the Vitali-Morin reaction (Freeman, 1955). Bands corresponding to these compounds were scraped off t.l.c. plates (system A) and extracted with chloroform–methanol (1 : 1), filtered and made up to 5 ml. Aliquots were evaporated to dryness and the residue treated with fuming nitric acid (1 ml) which was subsequently removed by evaporation. On cooling, the residue was dissolved in dimethyl formamide, 25% tetraethylammonium hydroxide (0·5 ml) added and the volume made up to 10 ml with dimethylformamide. After 5 min the colour density was measured at 540 nm and the alkaloid content calculated as atropine from a calibration curve. The results showed that the total basic metabolites extracted (11 mg, 62·8%) contained unchanged atropine (6 mg, 34%), nor-atropine (0·75 mg, 4·3%) and atropine *N*-oxides (0·80 mg, 4·6%).

RESULTS AND DISCUSSION

Although purified microsomal liver preparations have been used in the study of atropine and hyoscine metabolism, no *N*-oxidation products have been isolated and characterized (Ziegler, Mitchell & Jollow, 1969). Rat and guinea-pig liver preparations contain enzyme systems which oxidize atropine (Truhaut & Yonger, 1967a, b) but *N*-oxidation was not observed. It has been proposed that *N*-oxidation is a major pathway for the metabolism of some basic drugs such as phenothiazines (Beckett & Hewick, 1967; Gorrod, Lazarus & Beckett, 1974), tobacco alkaloids (Jenner, Gorrod & Beckett, 1973) and some antihistamines (Kuntzman, Phillips & others, 1967). Similarly, some secondary amines have been shown to undergo *N*-oxidation during metabolism and with amphetamines, hydroxylamine formation appears to be one of the major pathways (Beckett & Al Sarraj, 1972; Cho, Lindeke & Hodshon, 1972; Parli & McMahon, 1972). Hence we considered that *N*-oxidation could be involved in the metabolism of atropine, hyoscine and their *N*-demethylated derivatives. Atropine, hyoscine and their nor-derivatives were therefore separately incubated with guinea-pig liver microsomal preparations and the basic metabolites were extracted and separated by preparative t.l.c. Each metabolite was identified by chromatography on t.l.c. with the prepared compound and by mass spectrometry. The *N*-oxides and hydroxylamines were also reduced to their corresponding tertiary bases (from *N*-oxides) and secondary bases (from hydroxylamines) which were identified chromatographically. Atropine on incubation with guinea-pig liver microsomal preparation yielded nor-atropine and isomers 1 and 2 of atropine *N*-oxide (I, II) whereas hyoscine yielded nor-hyoscine and isomer 1 of hyoscine *N*-oxide (III). *N*-Dealkylation of tertiary amines to secondary amines and further oxidation to hydroxylamines is known for a number of basic drugs (Beckett & Salami, 1972; Essien, Cowan & Beckett, 1975) but hydroxylamines were not detected when atropine and hyoscine were incubated with guinea-pig liver microsomal preparations in the present study although they could possibly have been formed from nor-atropine and nor-hyoscine. To examine whether this was so, the nor-alkaloids were separately incubated with a microsomal preparation and the corresponding hydroxylamines were obtained. Thus atropine and hyoscine are metabolized by guinea-pig liver microsomes to the corresponding nor-alkaloids which are further oxidized to the corresponding hydroxylamines.

Preliminary quantitative estimation with atropine indicates that on incubation with the guinea-pig liver microsomal preparation used, the total basic metabolites extracted accounted for 63% of the atropine incubated, and that unchanged atropine (34%), nor-atropine (4·3%) and atropine *N*-oxides (4·6%) were present. This indicates that for this preparation the *N*-oxidation route for atropine is as significant as the *N*-demethylation route. Whether *N*-dealkylation proceeds via *N*-oxidation for atropine and hyoscine or via direct attack on the *N*-methyl groups as demonstrated recently for *N*-methyl-

carbazole (Gorrod & Temple, 1976), is not known. It has been predicted that amino compounds having pKa values greater than 7·0 should be oxidized by microsomal NADPH dependent flavoprotein known to be present in the guinea-pig (Gorrod, 1973). The results presented herein are consistent with this concept.

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