MICROSOMAL METABOLISM OF N-BENZYL-N-ETHYLANILINE AND N-BENZYL-N-ETHYL-*p*-TOLUIDINE

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SUMMARY

The *in vitro* hepatic microsomal metabolism of two tertiary anilines, N-benzyl-N-ethylaniline (NBNEA) and N-benzyl-N-ethyl-p-toluidine (NBNEPT), was examined in order to determine whether these compounds produce amide derivatives (benzoyl or acetyl) in addition to N-dealkylation and N-oxidation products as metabolites. The preparation of these tertiary anilines and their corresponding potential metabolites was undertaken. The amines and metabolites were separated using TLC and HPLC. Incubations were performed using hamster microsomal preparations fortified with NADPH. The substrates and their potential metabolites were extracted into dichloromethane and examined by TLC and HPLC. The metabolic process of particular interest was the formation of amides from NBNEA and NBNEPT.

The results from these experiments indicated that neither tertiary aniline (NBNEA and NBNEPT) produced amide (acetyl or benzoyl) or N-oxide metabolites. These substrates were dealkylated to the corresponding secondary amines via debenzylation and de-ethylation. Uncharacterised metabolites observed with substrates are proposed to be phenolic (for NBNEA) and hydroxymethyl (for NBNEPT). These findings support the concept that:

- nitrones are essential intermediates for the formation of amides from secondary aromatic amines (chemical rearrangement to amide via an oxaziridine intermediate);

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- carbinolamines produced by NBNEA and NBNEPT are not stable enough to allow further oxidation to amides and therefore these intermediates are broken down to dealkylated products.

The results are discussed in relation to the mechanism of metabolic amide formation from amines.

KEY WORDS

tertiary benzylic amines, *in vitro* metabolism, dealkylation, debenzylation

INTRODUCTION

Studies on the *in vitro* metabolism of substituted N-benzylanilines by hepatic microsomal preparations from various species have previously been performed to establish the formation of the corresponding amides /1-5/. Several mechanisms have been proposed for the metabolic formation of amides /5/; one suggested mechanism is that initial oxygen attack results in hydroxylation of the benzylic α-carbon atom to form a carbinolamine intermediate, which is further oxidized to give the amide (Figure 1, route a). If amide formation utilizes this route, then metabolism of NBNEA or NBNEPT would be expected to give either N-benzoyl or N-acetyl metabolites by further oxidation of an intermediate carbinolamine.

Another route proposed for the formation of an amide from a secondary aniline requires an initial oxygenation step on the constituent nitrogen giving an N-hydroxy compound which is further oxidized to a nitrone, followed by conversion of the nitrone to the corresponding amide *via* an oxaziridine intermediate (Figure 1, route b).

Previous studies on the *in vitro* hepatic metabolism of N-benzylaniline (NBA) and N-benzyl-4-methylaniline (NBPT) showed the formation of the corresponding amide and nitrone /1-5,7/ in a number of species. Therefore, a metabolic study on N-benzyl-N-ethylaniline (NBNEA) and N-benzyl-N-ethyl-p-toluidine (NBNEPT) (Figure 2), the N-ethyl derivatives of NBA and NBPT, which themselves were previously shown to be the best substrates for amide formation /4/, was carried out *in vitro* in order to establish whether the corresponding

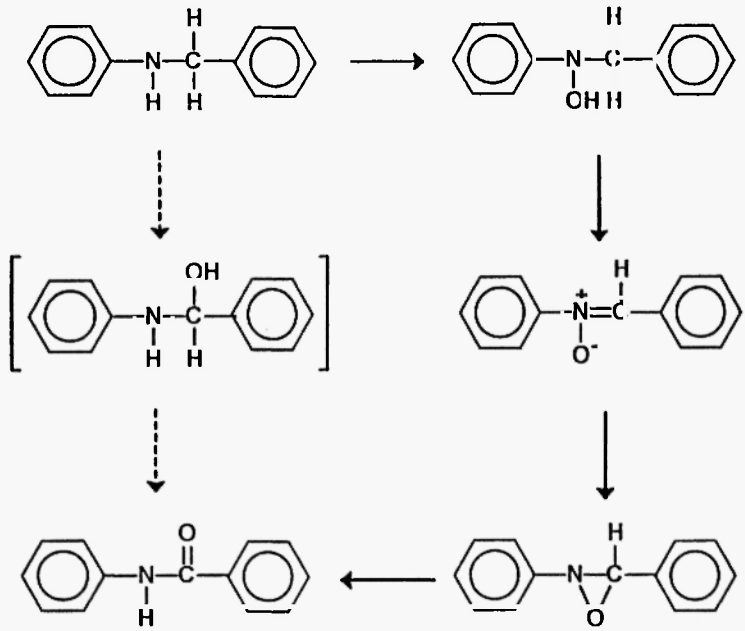
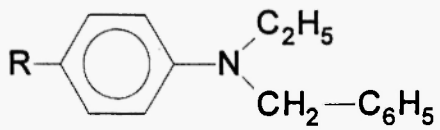


Fig. 1: Proposed mechanisms for the metabolic formation of amides from N-benzylsubstituted anilines. - - - → route a; ⟶ route b.



NBNEA (R= -H), NBNEPT (R= -CH_3)

Fig. 2: Structures of N-benzylic tertiary amines used in the present study.

amides, i.e. the N-benzoyl or N-acetyl derivative, are produced. The aim was to inhibit nitrone formation by introducing the ethyl moiety into the substrate molecule which would prevent oxygenation of the constituent nitrogen to a hydroxylamine and hence nitrone. If the corresponding amides are detected as metabolic products, this would

mean that the N-oxidative route proposed above is probably not involved in the formation of amides, and therefore benzylic carbon oxidation may be involved. In contrast, a negative result for amide formation from NBNEA or NBNEPT would support the concept that metabolic amide formation requires an intermediate nitrone.

From previous experiments on the metabolism of benzylic anilines, it is predicted that a number of other metabolic products, i.e. the phenolic or hydroxymethyl metabolites, debenzylated amines and the corresponding aldehydes, would be produced from these substrates. Additionally tertiary amines would be expected to produce N-oxides as metabolites. The results of the above experiments are the subject of this communication.

MATERIALS AND METHODS

N-Ethylaniline, N-ethyl-p-toluidine, N-benzyl-N-ethylaniline, benzyl, benzoyl and acetyl chlorides were purchased from Aldrich Chemical Company, UK. NBA was obtained from British Drug Houses (BDH), Poole, Dorset, UK. NBPT was synthesized as reported elsewhere /4/. Glacial acetic acid, hydrogen peroxide and all chromatographic solvents were obtained from E. Merck, Darmstadt (Germany). The HPLC column (Spherisorb C18 5 μm (25 cm length x 4.6 mm i.d.) was purchased from Phase Separations Limited, Deeside, UK. The guard column packing material (Whatman Pellicular ODS) was purchased from Whatman International Ltd., Maidstone, Kent, UK. The HPLC system consisted of an isocratic system comprising one LCD analytical constaMetric 3200 solvent delivery system, a Rheodyne syringe loading sample injector valve (model 7125) fitted with a 20 μl sample loop, a Milton ROY spectroMonitor-3100 variable wavelength UV detector, and a Milton ROY integrator. Glucose-6-phosphate dehydrogenase was purchased from the Boehringer Mannheim Corporation (London) Ltd. Nicotinamide adenine dinucleotide phosphate monosodium salt (NADP) and glucose-6-phosphate disodium salt were obtained from Sigma, UK. A rapiscan SA6508 UV detector was connected to the HPLC to directly obtain UV spectra of eluting metabolites from NBNEPT.

Preparation of NBNEPT and potential metabolites

NBNEPT: Equimolar amounts of NEPT and benzyl chloride (0.03 mol) and 10% NaOH solution (30 ml) were refluxed for 3 h /5/. The mixture was cooled and extracted with diethyl ether (3x15 ml). Evaporation of the ether under reduced pressure at 20°C yielded an oil which showed the presence of a secondary amine impurity when analyzed by TLC. The crude oil was heated with benzoyl chloride and 5% NaOH; after cooling the mixture was extracted with ether in the presence of 5% HCl solution leaving the desired compound in the aqueous phase. The aqueous phase was neutralised, and extracted with dichloromethane (DCM) (2x40 ml); the tertiary amine was obtained as an oil in a pure state which was converted to the HCl salt using HCl gas. Finally it was recrystallized from petroleum ether to give yellow crystals. Elemental analysis: Found: C: 71.63; H: 7.61; N: 5.03%. Calculated for $C_{16}H_{19}N.HCl$, NENBPT.HCl (m.w.: 261.84): C: 73.4; H: 7.7; N: 5.3%.

NANBA and NANBPT: In order to prepare NANBA and NANBPT, equimolar secondary amine and acetyl chloride (0.0276 mol) in benzene (40 ml) were refluxed for two hours at 90°C while stirring /5/. The white crystals which formed (NBA.HCl and NBPT. HCl) were collected by filtration and the benzene solution washed with saturated NaCl, 10% $NaHCO_3$ and 12.5% HCl solutions (2x40 ml each). Evaporation of the organic phase gave rise to the desired acetylated amines as oils. The IR spectra of these two amides, NANBA and NANBPT, showed a band at 1655 cm^{-1} which corresponded to the C=O stretching band vibration of tertiary amides. Elemental analysis: Found: C: 79.75; H: 7.25; N: 6.05%. Calculated for $C_{16}H_{17}NO$, NANBPT (m.w.: 239.36): C: 80.28; H: 7.16; N: 5.85%.

NBZNEA and NBZNEPT: For the preparation of NBZNEA and NBZNEPT, benzoyl chloride (0.006 mol) in diethyl ether (20 ml) was added dropwise to a solution of eqimolar secondary amine in diethyl ether (20 ml) while stirring /5/. The product formed in a few minutes (NEA.HCl or NEPT.HCl) were removed and the ethereal phases were evaporated. The crude oil obtained in each case was washed with saturated NaCl solution and 12.5% HCl (2x40 ml each) to remove any secondary amine and benzoic acid impurities. The aqueous phase was extracted with DCM (2x40 ml) and the amides were obtained as oils following evaporation of DCM phases. The IR spectra of these two

TABLE 1

Some physico-chemical properties of NBNEA and NBNEPT and their potential metabolites

Compound	Molecular formula (M.W.)	Melting point (°C) [Physical appearance]	Yield (%)	Mass spectral fragmentation m/e (Relative abundance)	UV λmax. (nm)
NBNEA	$C_{15}H_{17}N$ (211.31)	Oil	C.A.	65(60), 77(61), 92(62), 106(93), 120(45), 134(100), 152(13), 167(11), 180(49), 197(98), 210(76), 211(58), 212(75)	208, 254, 300
NBZNEA	$C_{15}H_{15}NO$ (225.29)	Oil	80	65(3), 77(61), 83(12), 91(6), 105(54), 120(18), 132(13), 197(9), 225(100)	209
NANBA	$C_{15}H_{15}NO$ (225.29)	Oil	65	65(65), 77(67), 91(59), 106(59), 115(10), 128(8), 154(20), 167(15),182(100), 225(14)	206
NBNEAO	$C_{15}H_{17}NO.H_2O$ (245.31)	White crys.	25	77(55), 91(83), 108(57),121(9), 136(100), 149(18), 167(6), 197(5), 211(6), 227(25)	206
NEA	$C_8H_{11}N$ (121.17)	Oil	C.A.	—	205, 245, 293
NBA	$C_{13}H_{13}N$ (183.25)	Pale yellow crys. 38°C	C.A.	—	206, 245, 295
NBNEPT .HCl	$C_{16}H_{19}N.HCl$ (261.84)	Pale yellow crys. 90-94°C	85	65(59), 77(26), 91(66), 92 (55), 105(12), 112(17), 118(53), 134(37), 148(67), 165(6), 180(7), 194(23), 210(100), 211(48), 224(37)	206, 255
NBZNEPT	$C_{16}H_{17}NO$ (239.36)	Oil	84	57(25), 65(99), 77(70), 78(98), 89(57), 91(61), 104(88), 105(52), 120(100), 134(95), 152(21), 165(20), 177(66), 211(55), 238(54), 239(39), 240(45)	206

Table 1 continued

NANBPT	$C_{16}H_{17}NO$ (239.36)	Oil	70	64(53), 76(79), 93(55), 105(43), 121(59), 134(57), 152(63), 167(51), 182(67), 198(42), 238(100)	209
NEPT	$C_9H_{12}N$ (135.21)	Oil	C.A.	—	207, 244, 302
NBPT.HCl	$C_{14}H_{15}N$ HCl (233.28)	White crys.	C.A..	—	206, 255, 306

C.A.: commercially available; m.p.: Buchi apparatus. For abbreviations see Table 2.

amides, NBZNEA and NBZNEPT, showed a band at 1640 cm^{-1} which corresponded to the C=O stretch band vibration of tertiary amides. Elemental analysis: Found: C: 79.43; H: 6.54; N: 6.55%. Calculated for $C_{15}H_{15}NO$, NBZNEA (m.w.: 225.29): C: 80.00; H: 6.7; N: 6.2%.

NBNEAO and NBNEPTO: The method employed for the preparation of NBNEAO and NBNEPTO was oxidation of the corresponding tertiary amine with H_2O_2 /6/. The tertiary amine (0.027 mol) was dissolved in glacial acetic acid (15 ml) and equimolar H_2O_2 (35%) added in three portions over one hour while stirring. The contents were stirred for 5 days at room temperature in the dark. When TLC analysis showed a new compound was produced, the reaction was stopped by adding 40% NaOH solution. The precipitate formed was washed with water (10 ml) and extracted with diethyl ether (2x30 ml). The product which precipitated in the aqueous layer was dissolved in DCM. This solution was evaporated to give a yellow crystalline product and washed with diethyl ether. This procedure gave NBNEAO as a virtually pure compound when examined by TLC. All attempts at complete purification failed in the case of NBNEPTO, which had as impurities the corresponding tertiary amine and trace amounts of several other products. Elemental analysis: Found: C: 72.77; H: 7.88; N: 5.55%. Calculated for $C_{15}H_{17}NO.\ H_2O$, NBNEAO (m.w.: 245.3): C: 73.47; H: 7.75; N: 5.71%.

Table 1 presents some physico-chemical properties of NBNEA and NBNEPT together with their potential metabolites.

Analytical procedures for the detection and identification of NBNEA and NBNEPT and their potential metabolites

The separation techniques used were based on TLC and an isocratic HPLC system. TLC was carried out using prepared silica gel GF254, 0.25 mm on glass plates (E. Merck, Darmstadt, Germany) with suitable solvent systems. Table 2 shows TLC R_f and HPLC R_t values of the two substrates and their potential metabolites, using a variety of solvent systems. The plates were examined after development under UV light (254 nm) and then sprayed with diazotized sulphanilic acid reagent followed by sodium carbonate /7/. The reaction products were eluted with a mobile phase having the composition: acetonitrile:0.2 M phosphate buffer (50:50, v/v) (final

TABLE 2

Chromatographic properties of NBNEA and NBNEPT and some of their potential metabolites

COMPOUND	ABBREVIATION	TLC (R_f x 100)	HPLC (R_t) (mins)
N-Benzyl-N-ethylaniline	NBNEA	77	15.4
N-Benzoyl-N-ethylaniline	NBZNEA	35	5.3
N-Acetyl-N-benzylaniline	NANBA	27	5.3
N-Benzyl-N-ethylaniline-N-oxide	NBNEAO	0	14.6
N-Ethylaniline	NEA	61	4.6
N-Benzylaniline	NBA	53	7.5
Benzaldehyde	B	-	3.4
N-Benzyl-N-ethyl-p-toluidine.HCl	NBNEPT.HCl	68	22.4
N-Benzoyl-N-ethyl-p-toluidine	NBZNEPT	32	6.8
N-Acetyl-N-benzyl-p-toluidine	NANBPT	26	7.0
N-Benzyl-N-ethyl-p-toluidine-N-oxide	NBNEPTO	0	20.0
N-Ethyl-p-toluidine	NEPT	53	6.0
N-Benzyl-p-toluidine	NBPT	48	9.5

HPLC solvent system: Acetonitrile:0.02 M phosphate buffer, 50:50, v/v (pH = 6) [Flow rate: 1.5 ml/min; λmax: 254 nm]
TLC solvent system: Petroleum ether (b.p.40-60°C):acetone, 80:20, v/v

pH=6) at a flow rate of 1.5 ml/min (Table 2). The metabolic products were detected by their absorbance at 254 nm. Retention times of compounds under these conditions are given in Table 2.

Incubation and extraction procedures

The animals used in the experiments were adult Syrian hamsters (80-110 g). Hepatic washed hamster microsomal preparations were prepared at 0°C using the calcium chloride precipitation method of Schenkman and Cinti /8/. Incubations were carried out in a shaking water bath at 37°C using a standard co-factor solution at pH 7.4. Co-factors consisting of NADP (2 μmol), glucose-6-phosphate (10 μmol), glucose-6-phosphate dehydrogenase (1 unit), $MgCl_2$ (20 μmol) prepared in phosphate buffer (2 ml, 0.2 M, pH 7.4) were pre-incubated for 5 minutes before addition of microsomes (5.3 mg protein/ml) equivalent to 0.5 g original liver and substrate (5 μmol in 50 μl methanol per flask). Metabolic reactions were stopped by extraction with DCM (2x5 ml). The DCM extracts were evaporated to dryness using a stream of N_2 at 25°C. Dry organic residues were reconstituted in 200 μl methanol for HPLC and 100 μl methanol for TLC analysis.

RESULTS

Examination of extracts obtained following the *in vitro* metabolism of NBNEA and NBNEPT failed to show the formation of either the N-benzoyl or N-acetyl amines. However, benzaldehyde and the N-ethyl and N-benzyl secondary amines were observed in both cases by HPLC (Figure 3b, 4b). No N-oxides were demonstrated. One unknown metabolite (**X**) of NBNEA (Figure 3b) and two unknown metabolites (**Y1** and **Y2**) of NBNEPT (Figure 4b) were observed. These unknown products, which were also detected by TLC in our system (see Table 2), are thought to be phenolic and hydroxymethyl metabolites because of their chromatographic behaviour /7/. These metabolites also gave a positive response to diazotised sulphanilic acid reagent (with an orange colour) which supports our proposal /7/. Further confirmation of the formation of N-dealkylated metabolites from NBNEPT was achieved using a rapiscan UV detector (Figure 5 a,b). Of the possible N-dealkylated metabolites, only NBA and NBPT were detected by TLC. We propose that the unknown metabolites, **Y1**

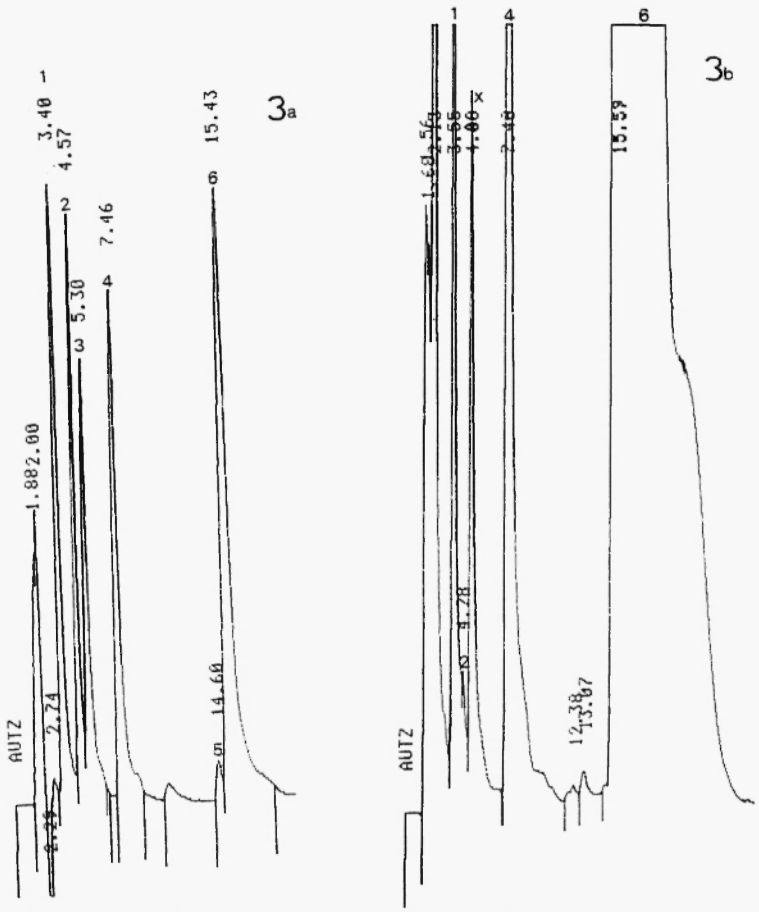


Fig. 3: HPLC chromatogram: (a) from standards; (b) following extraction from male hamster microsomal incubation mixture with NBNEA* as substrate (1 = B, 2 = NEA, 3 = NANBA and NBZNEA, 4 = NBA, 5 = NBNEAO, 6 = NBNEA [substrate], X = unknown metabolite). *For abbreviations see Table 2.

and **Y2,** could be hydroxymethyl metabolites with the structures of N-benzyl-4-hydroxymethylaniline and N-benzyl-N-ethyl-4-hydroxymethylaniline, respectively (Figure 5b). **Y1** was previously reported to be a compound arising during N-benzyl-4-methylaniline metabolism *in vitro* by hamster hepatic microsomes /7/; in the present work the

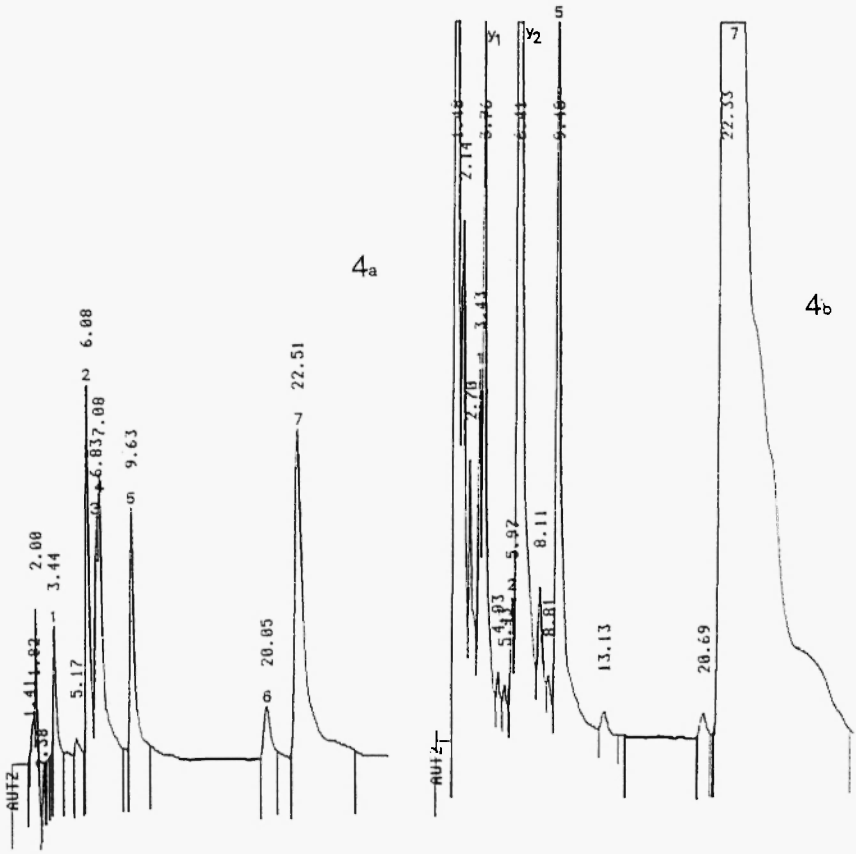


Fig. 4: HPLC chromatogram: (a) from standards; (b) following extraction from male hamster microsomal incubation mixture with NBNEPT* as substrate (1 = B, 2 = NEPT, 3 = NBZNEPT, 4 = NANBPT, 5 = NBPT, 6 = NBNEPTO, 7 = NBNEPT [substrate], Y1 = unknown metabolite 1, Y2 = unknown metabolite 2). *For abbreviations see Table 2.

metabolite possessed exactly the same UV spectrum (λmax = 252 nm) and a similar HPLC retention time as the previously observed metabolite (Figure 5b) /7/. Figure 6 shows established metabolic pathways for NBNEA, one of the two substrates studied in the present work.

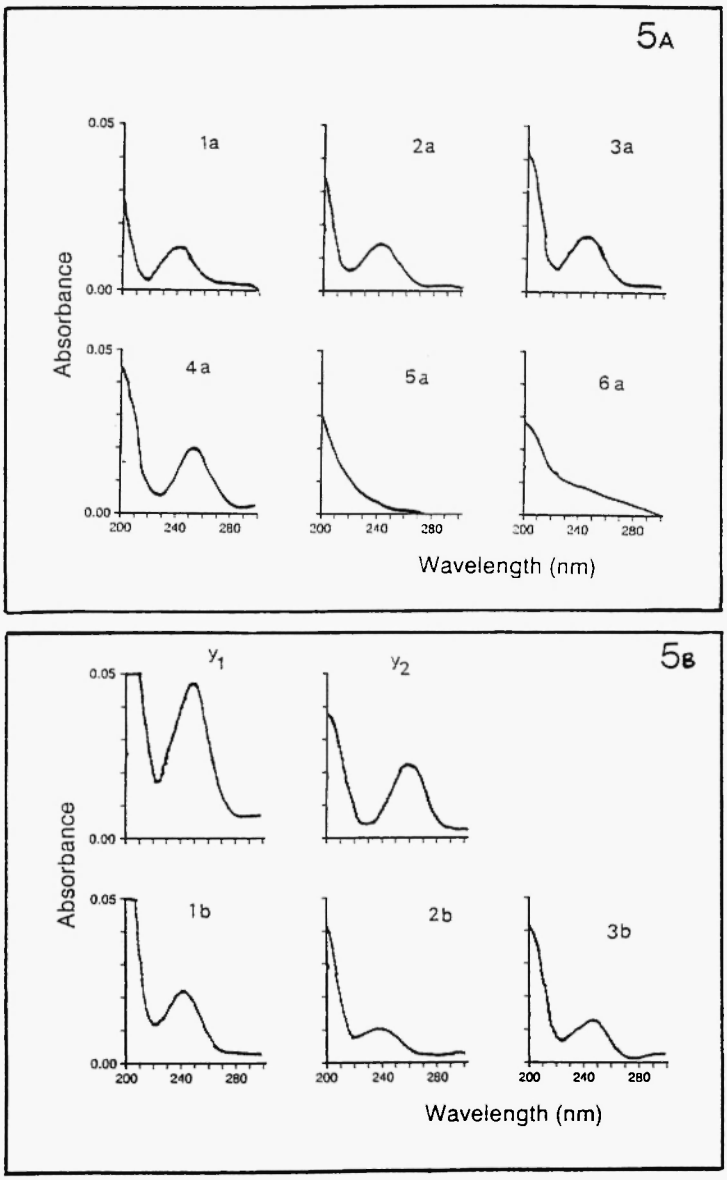


Fig. 5: UV spectra of authentic and metabolic NBNEPT and its potential metabolites. Authentics = (1a = B, 2a = NEPT, 3a = NBPT, 4a= NBNEPT, 5a = NANBPT, 6a = NBZNEPT). Metabolites = (1b = B, 2b = NEPT, 3b = NBPT, Y1 = unknown metabolite 1, Y2 = unknown metabolite 2).

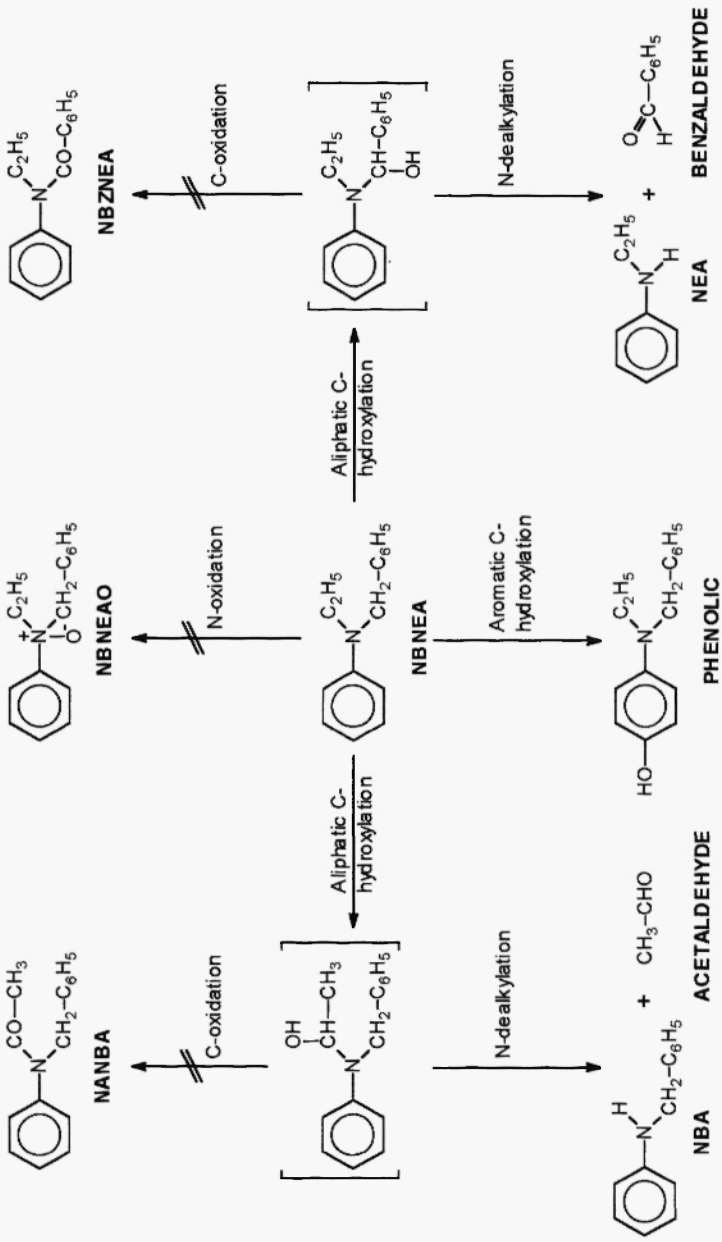


Fig. 6: Possible metabolic intermediates involved in the biotransformation of NBNEA.

DISCUSSION

The *in vitro* microsomal metabolism of both amines, NBNEA and NBNEPT, did not indicate the formation of either corresponding amide. The ethyl moiety introduced into the substrate prevented nitrone formation which clearly supports our previous proposal that α-C benzylic oxidation leads to debenzylation whereas nitrone formation leads to amide formation /4,5,7,9/. The present study clearly supports the proposal that the formation of amides requires initial nitrone formation which can only be formed from secondary amines. We have previously shown that nitrones can chemically rearrange to oxaziridines that yield amides following ring cleavage /9/.

The unknown metabolites observed during the metabolism of NBNEA and NBNEPT could be the phenolic derivative (metabolite **X**), and two 4-hydroxymethyl compounds (metabolites **Y1** and **Y2**), as in addition to studies in our laboratories /2,9/ it was observed that an analogous compound was produced as an *in vitro* metabolite from N-acetyl-4-methylaniline (NAMA) /10/. In a previous study, N,N-dialkyl- and N-alkyl-4-aminophenols derived from N-ethyl-N-methylaniline have also been detected from incubation mixtures of rabbit hepatic microsomes /11/.

In addition, failure to detect the corresponding N-oxide metabolites, which was of secondary importance in this study, may be due to their extreme solubility in the aqueous phase. The possibility exists that they were only produced in low amounts or they were unstable in the biological system which prevented their detection in our experimental conditions. Experiments on the metabolism of these aromatic N-oxides are in progress to examine this proposal further.

ACKNOWLEDGEMENTS

This work was supported by the Research Fund of the University of Marmara (SAGLIK-32, 1995) and the British Council. The authors would also like to thank King's College London Mass Spectrometry Services for EI-Mass Spectral analysis.

REFERENCES

1. Gooderham NJ, Gorrod JW. Microsomal N-oxidation of secondary aromatic amines.In: Gorrod JW, Damani LA, eds, Biological Oxidation of Nitrogen in Organic Molecules. Chichester: Ellis Horwood, 1985; 81-95.
2. Gorrod JW, Gooderham NJ. Microsomal N- and C-oxidation of 4-substituted N-benzylanilines. Xenobiotica 1985; 15: 1021-1031.
3. Gooderham NJ, Gorrod JW. Microsomal formation of amides from secondary aromatic amines. In: Gorrod JW, Gibson GG, Mitchard M, eds, Development of Drugs and Modern Medicines. Chichester: Ellis Horwood, 1986; 318-323.
4. Ulgen M. Metabolic and chemical studies on amide formation from benzylic amines. Ph.D. Thesis, University of London, 1992.
5. Ulgen M, Gorrod JW, Barlow D. Structure-activity relationships in the formation of amides from substituted N-benzylanilines. Xenobiotica 1994; 24: 735-748.
6. Ochiai E. Aromatic Amine Oxides. Amsterdam: Elsevier Publishing Company, 1967.
7. Ulgen M, Gorrod JW. Microsomal formation of N-benzyl-4-hydroxymethyl-aniline from N-benzyl-4-methylaniline. J Pharm Pharmacol 1992; 44: 865-866.
8. Schenkman JB, Cinti DL. Preparation of microsomes with calcium. Meth Enzymol 1978; 52: 83-89.
9. Ulgen M, Gorrod JW. Metabolic and chemical studies on α,*N*-diarylnitrones. Drug Metab Drug Interact 1994; 11: 245-257.
10. Daly JW, Guroff G, Udenfriend S, Witkop B. Hydroxylation of alkyl and halogen substituted anilines and acetanilides by microsomal hydroxylases. Biochem Pharmacol 1967; 17: 31-36.
11. Gorrod JW, Patterson LH. Evidence for p-hydroxylation of N-ethyl-N-methylaniline. Xenobiotica 1980; 10: 603-610.