J. Pharm. Pharmacol. 1994, 46: 585–590
Received September 29, 1993
Accepted January 10, 1994

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The Recognition of a Diarylimine as a Metabonate Produced During Incubation of *N*-Benzyl-4-chloroaniline with Hepatic Microsomal Preparations

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Abstract—Evidence is presented for the formation of *N*-benzylidene-4-chloroaniline as a metabonate during the metabolism of *N*-benzyl-4-chloroaniline. Control studies suggest that the diarylimine is formed as a chemical artifact from the debenzylation products (benzaldehyde and 4-chloroaniline). This novel observation indicates a possible pathway to amide formation from *N*-benzylanilines via diarylimines as intermediates.

The metabolism of *N*-benzyl-4-substituted anilines in-vitro has been reported by Gorrod & Gooderham (1985) to yield a variety of products arising from *N*-dealkylation, ring hydroxylation and *N*-oxidation. They demonstrated that the major route of *N*-benzyl-4-substituted aniline metabolism was *N*-debenzylation in all species studied. Aromatic amides were recognized as minor metabolites.

During the course of investigations into the metabolic reactions involved in amide formation from secondary aromatic amines (Gorrod & Ulgen 1994), studies on possible imine intermediates were carried out and evidence reported which indicated the existence of a new metabolic *N*-oxygenation pathway, which Gorrod & Gooderham (1985) had proposed was the formation of nitrones and amide via oxaziridines. This raises a question as to the possible formation of imines as intermediate metabolites from *N*-benzyl-4-substituted anilines.

The role of imines as intermediates in oxidative aralkylamine metabolism has been reviewed by Gorrod & Raman (1989) who demonstrated for the first time the formation of a stable primary imine metabolite (phenylacetoneimine) from a primary aliphatic amine (*o*-methylbenzhydrylamine). This was shown to be a microsomal process. However, the metabolic formation of secondary imines from *N*-benzyl-4-substituted anilines has not been reported.

The aims of this study were to prepare *N*-benzylaniline (NBA), *N*-benzyl-4-methylaniline (NB4MA) and *N*-benzyl-4-chloroaniline (NB4CA) as substrates, prepare the proposed imine metabolites of the three *N*-benzyl-4-substituted anilines: *N*-benzylideneaniline (NBEA), *N*-benzylidene-4-methylaniline (NBE4MA) and *N*-benzylidene-4-chloroaniline (NBE4CA); and develop sensitive and specific analytical methods to detect and characterize the imines from in-vitro incubation extracts (Fig. 1).

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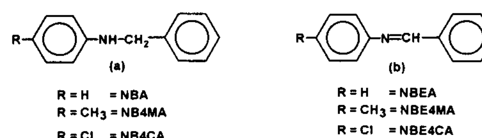

FIG. 1. The structures of substrates (a) and benzilidines (b) searched for as potential metabolites.

Materials and Methods

Chemicals

Acetonitrile, HPLC reagent grade, was obtained from Rathburn Chemicals (Walkerburn, UK); aniline, benzaldehyde, 4-toluidine, nicotinamide adenine dinucleotide phosphate (NADP); glucose-6-phosphate (sodium salt) and all other common chemicals and solvents (analytical reagent grade) were from BDH (Poole, Dorset, UK); 4-chloroaniline was from Aldrich Chemical Co. Ltd; *N*-benzylaniline was from Hopkin and Williams Ltd; glucose-6-phosphate dehydrogenase was from Boehringer Mannheim Corporation Ltd (London, UK); and TLC silica gel $60F_{254}$-coated aluminium sheets were from Merck (Darmstadt, Germany). The corresponding nitrones and amides were prepared as described previously (Vogel 1978; Gorrod & Gooderham 1986).

The preparation of N*-benzyl-4-substituted anilines*

The direct reduction method as described by Vogel (1980) was used to prepare *N*-benzyl-4-substituted anilines; NB4MA and NB4CA. The corresponding *N*-benzylideneaniline (4·0 g) (see preparation of *N*-benzylideneanilines) was dissolved in methanol (50 mL). Upon complete dissolution, sodium borohydride (0·9 g) (Sigma Chemicals Ltd, Poole, UK) was added gradually in small portions over 30 min, with constant stirring. The reaction was monitored by TLC (petroleum spirit: acetone (9:1)). When the addition was complete, the mixture was gently refluxed for 15 min, after which as much methanol as possible was removed by distillation. Distilled water (50 mL) was added to the cooled residue. The emulsion was then extracted with diethyl ether (3 × 10 mL) and the organic extracts bulked and dried using a Büchi RE111 Rotavapour

evaporator with a 35°C water bath. The solids obtained were recrystallized using petroleum spirit (bp 40–60°C). White crystals of NB4CA and a brown oil of NB4MA were obtained. To facilitate subsequent handling during in-vitro incubations, the hydrochloride salt of NB4MA was prepared by passing a steady stream of dry hydrogen chloride gas (BOC Gases Ltd) into a dried ethereal solution of NB4MA. The yellow solid obtained was then recrystallized from benzene.

The preparation of N*-benzylideneanilines*

The *N*-benzylideneanilines were prepared as described by Law (1912). Equal molar amounts of benzaldehyde and the corresponding 4-substituted aniline were heated using a boiling-water bath with constant stirring. The reaction was monitored by TLC and the reaction stopped when the level of the imine appeared maximal. The reaction mixture was cooled and the solids obtained recrystallized from methanol. TLC analyses even after repeated recrystallization showed the presence of traces of benzaldehyde and the corresponding aniline as impurities in all the imines. Since they would only be used as chromatographic references, no further attempts were made to purify them.

Characterization of compounds

All the *N*-benzylanilines and *N*-benzylideneanilines prepared were characterized by IR, UV, elemental analysis, MS and ^{1}H NMR methods. IR for all three imines showed $\nu = 1620\,cm^1$ (Ar-N=CH-Ar stretch); UV (methanol): NB4MA.HCl $\lambda_{max} = 208$ and 247 nm, NB4CA $\lambda_{max} = 209$ and 256 nm, NBEA $\lambda_{max} = 203$ and 262 nm, NBE4MA $\lambda_{max} = 203$ and 262 nm, and NBE4CA $\lambda_{max} = 203$ and 263 nm. Further analytical data are presented in Tables 1 and 2.

TLC separation

TLC systems and chromogenic agents used to separate and identify the substrates and potential metabolites are described in Table 3.

HPLC separations of N*-benzylanilines and their corresponding diarylimines*

An isocratic HPLC system was used, comprising an LCD Analytical ConstaMetric 3000 pump, a model 7125 syringe-loading sample-injector valve fitted with a 20-μL sample loop, an LC3 Phillips Pye Unicam UV detector and a Milton Roy integrator (CI-10B) and plotter. A Rapiscan SA6508 UV detector was connected to obtain UV spectra of eluting metabolites. The analytical column contained Hypersil 5 μm ODS (250 × 4·6 mm i.d.) and the pre-column material was co-pellicular ODS. The mobile phase composition was acetonitrile: 0·015 M phosphate buffer pH 8 (47:53, v/v) at a flow rate of 1 mL min^{-1} for the analyses of NB4CA and NB4MA and their respective putative metabolites. The mobile phase composition for NBA analysis was acetonitrile: 0·015 M phosphate buffer pH 8 (37·5:62·5, v/v) at a flow rate of 1·5 mL min^{-1}. All the column eluates were monitored at 262 nm.

Animals

Male New Zealand White rabbits had free access to water and were fasted overnight and were killed by cervical dislocation. The hepatic microsomes used in the experiments were prepared at 4°C using the calcium chloride precipitation method (Schenkman & Cinti 1978). The cofactor solution (NADPH-generating) consisted of NADP (2 μmol), glucose-6-phosphate (10 μmol), glucose-6-phosphate dehydrogenase (1 unit), magnesium chloride (50% w/v) (20 μmol) in phosphate buffer (0·2 M, pH 7·4; 2 mL).

Incubation and extraction procedures

Incubations were carried out in 25-mL Erhlenmeyer flasks maintained at 37°C in a Grant SS40-D shaking water bath. Cofactor solution (2 mL) was preincubated for 5 min at 37°C and the metabolic reaction was initiated by the addition of substrate (NBA, NB4MA.HCl or NB4CA, 3 μmol in 30 μL methanol) followed by microsomes (1 mL equivalent to 0·5 mg tissue). In control flasks, cofactor solution or microsomal suspension was replaced with phosphate buffer or denatured tissue, respectively. In a third control flask, the substrate was replaced by phosphate buffer. After 20 min, metabolism was terminated by plunging the flasks into ice. The reaction mixture was extracted with dichloromethane (2 × 5 mL). The extracts were bulked

Table 1. Analytical data of *N*-benzylanilines and *N*-benzylideneanilines.

Compound	Mol. wt	Mol. formula	Melting point (°C)		Yield (%)	Description*	Elemental analysis C : H : N : Cl found (required)
			Found	Literature			
NB4MA.HCl	233	$C_{14}H_{16}NCl$	169·3–171·3	181–182[a]	22	White needles	71·93:6·87:6·01:*15·39* (71·94:6·90:5·99:*15·20*)
NB4CA	217	$C_{13}H_{12}NCl$	42·8–43·1	47–48[b]	48	Yellow-white 158D short needles	71·42:5·52:6·40:*16·32* (71·72:5·56:6·43:*16·29*)
NBEA	181	$C_{13}H_{11}N$	49·6–50·2	52[c]	40	Yellow 10D feathery crystals	86·08:6·07:7·74 (86·15:6·12:7·73)
NBE4MA	195	$C_{14}H_{13}N$	29·0–29·5	36[a]	ND	Grey-orange 163D crystals	85·85:6·69:7·15 (86·12:6·71:7·71)
NBE4CA	215	$C_{13}H_{10}NCl$	56·0–59·0	62[d]	60	Yellow 11D crystals	72·44:4·64:6·52: (72·40:4·67:6·49:)

[a] Law 1912, [b] Billman & McDowell 1961, [c] Merck Index 1989, [d] Vogel 1978. ND, not determined. * Royal Horticultural Society colour chart used to record colours.

Table 2. Mass spectra and NMR data of *N*-benzylanilines and *N*-benzylideneanilines.

Compound	Mol. wt	Major mass spectra fragments *m/e* (% relative abundance)	NMR data Chemical shift in ppm[a] (type of signal, number of protons, signal origin)
NB4MA.HCl* (Base)	233 (197)	193(m$^+$ 100) 196(40) 120(25) 106(6) 91(87) 77(6) 65(11)	2·27(*s*,3H,CH_3) 4·36(*s*,2H,CH_2) 7·00–7·15(*m*,2H,C_2C_5) 7·25–7·50(*m*,7H,$C_2C_6C_{10}$–C_{14}) 11·75(*s*,2H,NH_2^+)
NB4CA*	217	219(34) 217(m$^+$ 81) 216(21) 142(3) 140(11) 111(8) 91(100) 77(5) 65(10)	4·24(*s*,2H,CH_2) 4·77(*s*,1H,NH) 6·45–6·65(*m*,2H,C_2C_6) 6·95–7·15(*m*,2H,C_3C_5) 7·15–7·40(*m*,5H,C_{10}–C_{14})
NBEA	181	181(m$^+$ 100) 180(98) 104(12) 77(42)	7·10–7·60(*m*,8H,C_2–C_6C_{11}–C_{13}) 7·80–8·00(*m*,2H,$C_{10}C_{14}$) 8·44(*s*,1H,C_8)
NBE4MA	195	195(m$^+$ 100) 194(79) 118(10) 91(25)	2·33(*s*,3H,CH_3) 7·05–7·30(*m*,4H,$C_2C_3C_5C_6$) 7·40–7·60(*m*,3H,C_{11}–C_{13}) 7·80–8·00(*m*,2H,$C_{10}C_{14}$) 8·47(*s*,1H,C_8)
NBE4CA*	215	217(35) 215(m$^+$ 100) 214(80) 140(4) 138(11) 113(7) 111(21)	7·05–7·25(*m*,2H,C_2C_6) 7·30–7·40(*m*,2H,C_3C_5) 7·40–7·60(*m*,3H,C_{11}–C_{13}) 7·80–8·00(*m*,2H,$C_{10}C_{14}$) 8·42(*s*,1H,C_8)

m$^+$ = molecular ion. * m/e values for both ^{35}Cl and ^{37}Cl given. [a] Downfield from deuterated tetramethylsilane internal standard; $CDCl_3$ as solvent. *s* = singlet, *m* = multiplet.

and evaporated to dryness under a stream of nitrogen in a water bath at 40°C. The residues were reconstituted in 200 μL methanol for HPLC and TLC studies.

Results

In the in-vitro metabolism of NBA and NB4MA by rabbit microsomes, no metabolites having the characteristics of their corresponding imines were detected in the HPLC and TLC systems. However, with NB4CA as substrate, the incubation extracts showed the formation of the proposed imine i.e. NBE4CA using HPLC but not with TLC. A typical HPLC chromatogram of NB4CA and its putative metabolites is shown in Fig. 2a and a chromatogram of actual compounds detected is shown in Fig. 2b. The imine formed had a retention time of 47·6 min, co-chromatographed with authentic *N*-benzylidene-4-chloroaniline (NBE4CA) and had an identical UV spectrum (Fig. 3).

Table 3. TLC systems, R_f (×100) values and chromogenic agents used to separate and identify *N*-benzyl-4-chloroaniline and its metabolites.

Compound	R_f value (×100) insolvent systems		Chromatographic response with detection systems	
	S1	S2	D1	D2
Benzaldehyde	45	81	NR	NR
4-Chloroaniline	12	59	Yellow 12B	NR
α-Phenyl-*N*-(4-chlorophenyl)nitrone	8	64	NR	NR
N-Benzoyl-4-chloroaniline	10	70	NR	NR
N-Benzyl-4-chloroaniline	32	88	Yellow 14A	Grey-orange 165C
N-Benzylidene-4-chloroaniline	51	89	Yellow 12B	Yellow-orange 20B

S1, Petroleum spirit:acetone (9:1). S2, Benzene: ethyl acetate: acetone (8:1·5:0·5). D1, Ehrlichs reagent (*p*-dimethylaminobenzaldehyde 0·1% in HCl). D2, 2,4-Dinitrophenylhydrazine (0·4%) in 2 M HCl. Royal Horticultural colour charts were used to record the colours observed.

No metabolites were observed in control experiments. Other established metabolites, i.e. benzaldehyde, the corresponding anilines, nitrones and amides were observed from all three *N*-benzylanilines studied. From this evidence, it was concluded that the diarylimine of *N*-benzyl-4-chloroaniline was present in the incubation extracts.

To ascertain whether NBE4CA was formed enzymatically or chemically, another control experiment was carried out by incubating benzaldehyde (1 μmol) and 4-chloroaniline (1 μmol) as co-substrates using the same incubation conditions and procedures as for the secondary amine. The HPLC trace of the reconstituted incubation extract is shown in Fig. 4. A prominent peak with a retention time that

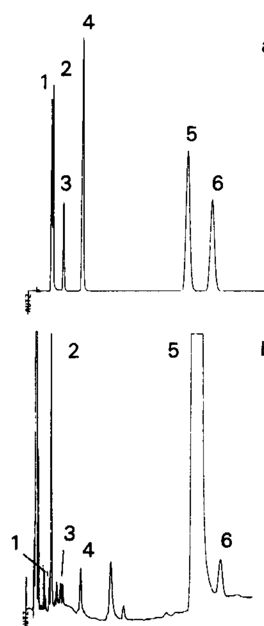

FIG. 2 a. HPLC separation of putative *N*-benzyl-4-chloroaniline metabolites. b. HPLC chromatogram of in-vitro *N*-benzyl-4-chloroaniline metabolites formed by hepatic microsomes of rabbit. 1, Benzaldehyde; 2, 4-chloroaniline; 3, α-phenyl-*N*-(4-chlorophenyl)nitrone; 4, *N*-benzoyl-4-chloroaniline; 5, *N*-benzyl-4-chloroaniline; 6, *N*-benzylidene-4-chloroaniline.

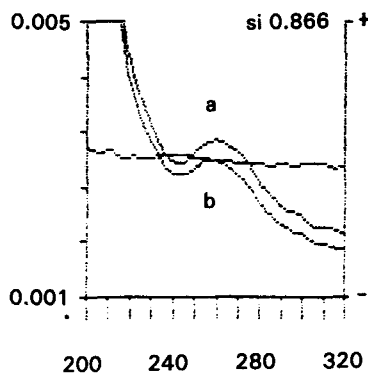

FIG. 3. UV spectral overlay of authentic NBE4CA (a) and metabolite NBE4CA (b), obtained with the Rapiscan detector (peak 6).

corresponded to that of NBE4CA was detected together with a peak with retention time identical to α-phenyl-*N*-(4-chlorophenyl)nitrone.

Discussion

This study has demonstrated for the first time the detection of a diarylimine (NBE4CA) as a product from the metabolism of a secondary aromatic amine (NB4CA). This was shown to be an artifact produced during NB4CA metabolism, and can be explained as follows: NB4CA undergoes debenzylation, giving rise to benzaldehyde and 4-chloroaniline in the incubation mixture (Fig. 5).

Since NBE4CA can be easily prepared chemically by heating benzaldehyde and 4-chloroaniline, it seems these metabolites can react chemically to produce NBE4CA. This explanation also accounts for the fact that no NBE4CA was found in the three control experiments; if NB4CA could not be metabolized, the reactants needed would not be formed.

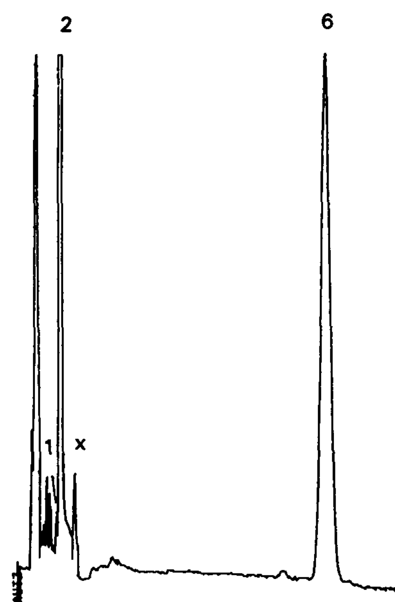

FIG. 4. HPLC chromatogram of metabolic extract, when benzaldehyde and 4-chloroaniline were incubated as co-substrates with rabbit hepatic microsomes. 1, Benzaldehyde; 2, 4-chloroaniline; 6, *N*-benzylidene-4-chloroaniline.

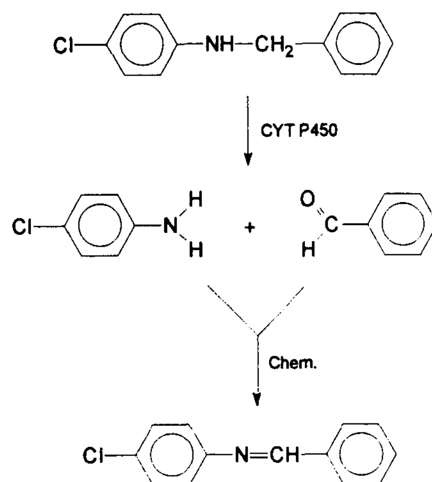

FIG. 5. Formation of *N*-benzylidene-4-chloroaniline from debenzylated metabolites of *N*-benzyl-4-chloroaniline.

One possible explanation for not detecting imines of NBA and NB4MA, even though both substrates underwent debenzylation giving benzaldehyde and their corresponding anilines, could be due to smaller quantities or lower reactivities of the debenzylated products formed, compared with NB4CA. The quantities or rates of formation of debenzylated products are due to differences in metabolism kinetics as a result of differing pK_a values; NB4MA has the highest pK_a while NB4CA has the lowest, among the three *N*-benzylanilines studied. The strong electron withdrawing effect of the chlorine atom on NB4CA will have modified the electron density of the whole molecule and this will have an effect of rendering the N-C bond relatively more susceptible to enzymatic attack.

The novel observations in this study question the proposed route of formation of amides from *N*-benzylanilines via imines as intermediates. They do not support the proposed pathway whereby *N*-benzylaniline undergoes *N*- or *C*-oxidation to the corresponding *N*-hydroxylamine or α-carbinolamine which lose a molecule of water to form the diarylimine. Nor do the results in this study invalidate the suggestion (Gorrod & Ulgen 1994) that oxidation of imines to an oxaziridine, which then isomerizes to either amide or nitrone, is the route to amide formation from *N*-benzylanilines. This study only questions the possibility of imine formation and the mechanism involved.

In the original report on the formation of amides from *N*-benzylanilines it was clear from results using enzyme inhibitors and inducing agents that the overall reaction was enzyme controlled (Gooderham & Gorrod 1986). This study, which shows that imines can be formed chemically from metabolites produced from the oxidative debenzylation of *N*-benzylanilines, indicates that metabolic *N*-dealkylation may be one rate-limiting step. If the levels of aromatic amine and benzaldehyde are modulated by enzyme inhibitors or inducers then these factors would also modulate the levels of imine formed and hence the amount available as substrate leading to amide formation. This leads to a new proposal as to the route of amide formation from *N*-benzylanilines which is summarized in Fig. 6.

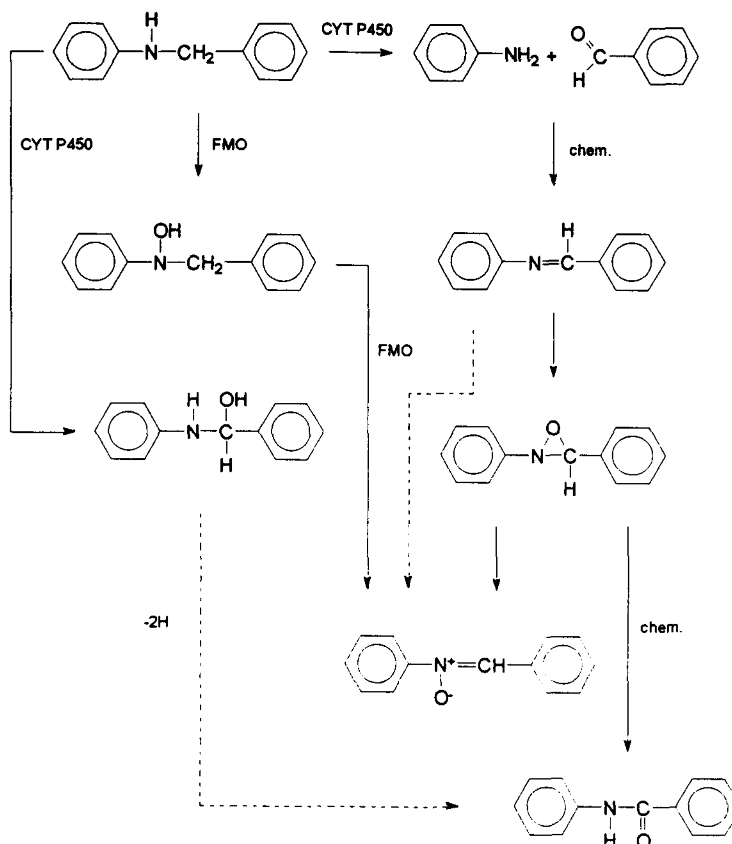

FIG. 6. Proposed route to the formation of amides from N-benzylanilines via diarylimines as intermediates.

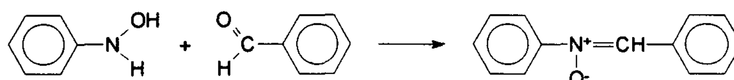

FIG. 7. Formation of α,N-diphenylnitrone from N-hydroxylaniline and benzaldehyde.

During the work on the effects of inducers and inhibitors on N-benzylaniline metabolism, Gooderham & Gorrod (1986) showed that the major route of N-benzyl-4-substituted aniline metabolism was N-debenzylation, a reaction which was highly inhibited by DPEA (2,4-dichloro-6-phenylphenoxyethylamine). They suggested that this inhibitor might have caused a shift in the metabolism of N-benzyl-4-substituted aniline by simply decreasing debenzylation and thereby increasing amide levels. If this were correct, a decrease in the amount of diarylimine formed would be expected. The evidence of Gooderham & Gorrod (1986) suggesting that formation of amides from N-benzyl-4-substituted anilines involved a cytochrome P450 mono-oxygenase system may merely be a reflection of the N-debenzylation activity. Why amide formation should be increased in the presence of DPEA is presently not understood, although it may be of significance that DPEA is known to activate the microsomal flavin-containing amine oxidase (Gorrod & Patterson 1983). It is of interest that during the experiment to ascertain whether the reaction of benzaldehyde and 4-chloroaniline could produce the imine, another product [X] was detected using HPLC (Fig. 4). This compound has a retention time identical to the corresponding nitrone i.e. α-phenyl-N-(4-chloro)nitrone. Normally this type of compound would be formed by reaction of an arylhydroxylamine and benzaldehyde (Fig. 7) (Beckett et al 1979), which suggests that 4-chloroaniline, produced during the metabolism of N-benzyl-4-chloroaniline, undergoes N-hydroxylation.

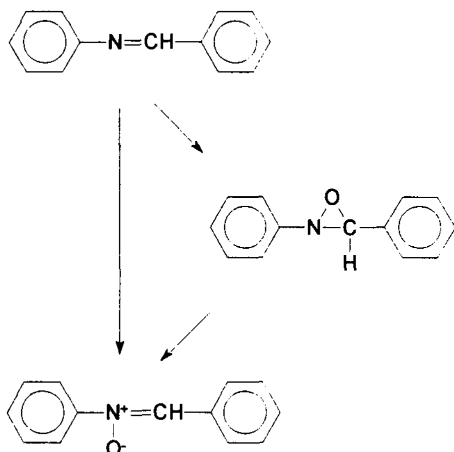

FIG. 8. Proposed routes to formation of nitrone from N-benzylideneaniline.

The *N*-hydroxylation of 4-chloroaniline by hepatic microsomes has been reported previously (Smith & Gorrod 1978) and shown to be a rapid reaction in hamster and guinea-pig preparations. Alternatively the imine formed chemically may be metabolized (Fig. 8) to give the nitrone directly or via an oxaziridine (Gorrod & Ulgen 1994). These possibilities also need further investigation.

If, as is now suggested, imines can be formed through the chemical reaction of metabolically-formed amines and aldehydes, it is possible that other imines will be formed through the reaction of diverse amines and aldehydes produced during metabolic processes. As *N*-dealkylation is a very common metabolic process for drugs and other xenobiotics (McMahon 1966), a wide variety of imines may be formed and reported as metabolites. These metabonates, if formed in-vivo, may account for some iatrogenic effects of drugs, although a systematic study of imine toxicity has not been reported.

The reaction between two metabolites produced from a single substrate to form a new metabonate is rare but not unique. Breck & Trager (1971) showed that an imidazolidinone was formed from the *N*-dealkylation products i.e. *N*-demethyl-lignocaine and acetaldehyde, during the metabolism of lignocaine in man.

If diarylimines can be formed in the body by whatsoever mechanism, as suggested in Fig. 6, then their potential toxicities either directly or via the strained ring oxaziridines, analogous to epoxides and aziridines (Gorrod & Ulgen 1994) should promote further interest in this field of research.

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