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COMMENTARY

THE GENERATION OF POTENTIALLY TOXIC, REACTIVE IMINIUM IONS FROM THE OXIDATIVE METABOLISM OF XENOBIOTIC N-ALKYL COMPOUNDS

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During the past 30 years, a common mechanistic feature has emanated from the many investigations into mechanisms by which chemically innocuous drugs and environmental chemicals exert their toxicity. This is that they undergo metabolic activation to chemically reactive species capable of causing toxicity in specific tissues. Reports that the metabolism of the anaesthetic and psychotropic drug phencyclidine gives rise to an electrophilic iminium species [1, 2] are amongst a number of recent studies which suggest that certain alkylamines and alkylamides may have to be included in the vast array of such chemicals with hazardous potential. In this review, we summarise some of these findings and draw attention to those molecular features of alkylamines and alkylamides which favour the generation of potential toxins. Additionally, some preliminary results are presented which suggest that phase 2 metabolic reactions of metabolites of alkylamines, such as the conjugation of N-(1-hydroxymethyl) compounds, may also give rise to potentially toxic species.

The oxidative metabolism of N-alkylamines (1, Fig. 1) to N-(1-hydroxyalkyl) amines (2) is a ubiquitous metabolic pathway for many classes of xenobiotics bearing an N-alkyl group. This oxidation may be the prelude to the formation of an aldehyde, for example formaldehyde from an N-methyl compound. Alternatively, a relatively stable N-(1-hydroxyalkyl) species may be formed. The fate of this latter moiety is the focus of attention of this review and, in particular, the question is addressed of which molecular feature(s) of specific xenobiotic N-(1-hydroxyalkyl) amines may dispose them to be in equilibrium with the reactive iminium species formed by loss of hydroxide.

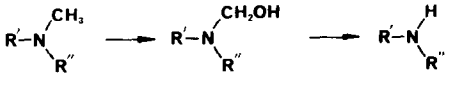

Fig. 1. Metabolism of N-methyl compounds.

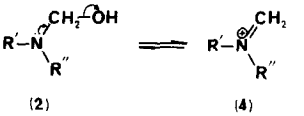

Fig. 2. Equilibrium between a carbinolamine (2) and iminium ions (4).

Several examples of the equilibrium between a carbinolamine (2, Fig. 2) and an iminium species (4) in biological systems have now been reported. The first convincing evidence for the presence of such an equilibrium was presented for a metabolite of nicotine [3]. However, there are other examples of oxidative N-dealkylation reactions where such an equilibrium does not exist, one such being that of N-(hydroxymethyl)carbazole, a metabolite of *N*-methylcarbazole (34, see Fig. 10). Beke [4] has reviewed the chemistry of the heterocyclic pseudo bases, where this equilibrium between carbinolamine and iminium ion exists. The basicity of the nitrogen atom in the carbinolamine (2, Fig. 2) naturally depends upon its structure; the equilibrium is influenced by (a) the electronegativity of substituents attached to the nitrogen (R′ and R″), (b) the situation of the nitrogen in cyclic or acyclic structures, and (c) the aromaticity of the ring. Electron-donating substituents on the nitrogen will tend to facilitate loss of the hydroxide anion. Conversely, electron-withdrawing groups decrease the electron density at the nitrogen atom and do not favour iminium ion production. This latter effect can lead to the cleavage of the N—C bond with concomitant loss of the aldehyde.

The first experiments to provide evidence for the metabolic formation of iminium ions from N-alkylamines were performed by Breck and Trager [5] in studies of the metabolism of lidocaine (Fig. 3). They suggested that the formation of a carbinolamine (6) and consequently an iminium ion (7) may explain the production of a cyclic species (8) from the oxidation of lidocaine (5). These authors also suggested that, if the formation of such a reactive, electrophilic intermediate (7) was a general phenomenon of N-dealkylation, then it might explain the biological responses, whether efficacious or toxic, of various

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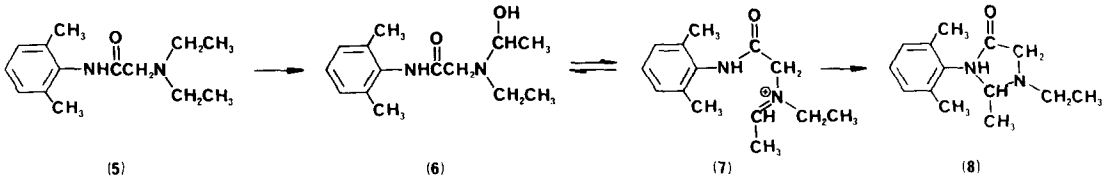
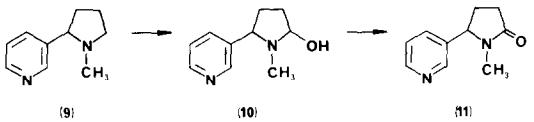


(5) (6) (7) (8)

Fig. 3. Metabolism of lidocaine (5).

amine drugs, as the reaction of such intermediates with nucleophiles at critical enzyme sites [5].

In 1960, Hucker *et al.* [6], in a study of the metabolism of nicotine (9), proposed the pathway illustrated in Fig. 4. The authors studied the effect of metabolic inhibitors upon this pathway. Cyanide inhibited the production of cotinine (11) but this occurred without inhibition of the consumption of nicotine. The authors suggested that the conversion 5′-hydroxynicotine (10) to cotinine (11) was catalysed by aldehyde oxidase which had been shown to be inhibited by cyanide [7]. This suggestion was supported by evidence of the accumulation of an intermediate of metabolism formed in the presence of cyanide, which behaved like the synthetic carbinolamine (10). Murphy [3] reinvestigated these aspects of the metabolism of nicotine with the aim of elucidating the exact enzymatic steps involved in the production of cotinine. When nicotine was metabolised in the presence of cyanide, a novel compound was observed which was characterised as 5′-cyanonicotine (15, Fig. 5). Murphy proposed two pathways to be consistent with the formation of 5′-cyanonicotine (15). Pathway A (Fig. 5) involved the formation of nicotine $\Delta^{1'(5')}$ iminium ion (12) from hydroxynicotine (10), and it was considered that the ion should react readily with cyanide. Pathway B involved the formation of the aminoaldehyde tautomer (13) of hydroxynicotine, followed by reaction with cyanide (14) and ring closure in a manner analgous to the Strecker reaction. To discriminate between these two pathways, the products of the incubation of nicotine with microsomes were treated with sodium borodeuteride. This reduction yielded deuterionicotine, in which the single deuterium was located at the 5′-position (16). This observation could only be consistent with the existence of the iminium ion (12). In addition, the incubation of nicotine with microsomes under an $^{18}O_2$ atmosphere showed that the cotinine (11, Fig. 4) produced did not contain ^{18}O. It was thus demonstrated that the hydroxyl oxygen atom in the 5′-hydroxynicotine molecule was not the same oxygen as was derived from the initial enzymatic hydroxylation of nicotine, which required atmospheric oxygen. This is consistent with a pathway involving the iminium ion (12, Fig. 5).



(9) (10) (11)

Fig. 4. Metabolism of nicotine (9) to cotinine (11).

The iminium salt of nicotine has been synthesised and its structure and stability in aqueous solution have been investigated using NMR spectroscopy [8]. In freshly prepared acid or neutral solution only the iminium form (12) was observed, whereas in strongly alkaline solutions only the carbinolamine (10) was observed. It was estimated that, at physiological pH, 25% or less was present as the carbinolamine but exact determination was not possible as the iminium ion rapidly dimerised. This finding further supports Murphy's conclusions [3] that the 5′-hydroxynicotine can exist in equilibrium with an iminium ion. The authors [8] also demonstrated that the iminium salt can be readily transformed into cotinine by means of an enzyme present in the cytosolic fraction of a liver homogenate.

Another route of nicotine metabolism is demethylation. Nguyen *et al.* [9, 10] suggested that, if nicotine (9) is demethylated via a carbinolamine (17, Fig. 6) intermediate, then an analogous iminium ion (18) might be formed and this may also be trapped by cyanide ions. Metabolic incubations were performed in the presence of cyanide and two metabolites were found; one was 5′-cyanonicotine as shown by Murphy [3], the other was determined to be *N*-(cyanomethyl)nornicotine (19). This was shown to occur, at least in part, without prior nitrogen–carbon bond cleavage, implicating the pathway illustrated in Fig. 6. Some *N*-(cyanomethyl)nornicotine appeared to be generated by an alternative pathway consisting of condensation of nornicotine (the demethylated metabolite), formaldehyde (generated during demethylation), and cyanide. However, this route appeared to be quantitatively less important.

Following the studies on nicotine, cyanide has been used as a trapping agent for iminium ions in metabolic incubations of several other agents. The tertiary amine 1-benzylpyrrolidine (20, Fig. 7) yielded several cyano adducts (21, 22, 23) which the authors proposed to arise from the nucleophilic attack by cyanide ion on a metabolically generated iminium species (24) [11]. Attack can occur at positions 2 and 5 of the pyrrolidine ring, thereby generating the dicyano adduct (22). The pyrrolidinone (23) is formed by oxidation of a hydroxylated intermediate such as (25, 26). The hepatocarcinogenic antihistamine methapyrilene (27, Fig. 8) produced only one identifiable cyano adduct, *N*-(cyanomethyl) normethapyrilene (28) [12]. The experimental evidence did not allow the authors to exclude the formation of another iminium ion (29), however, due to the chemical instability of the corresponding α-cyano amine (30). More recently the formation of an iminium ion from the widely abused drug phencyclidine (31, Fig. 9) has been studied [1, 2]. When

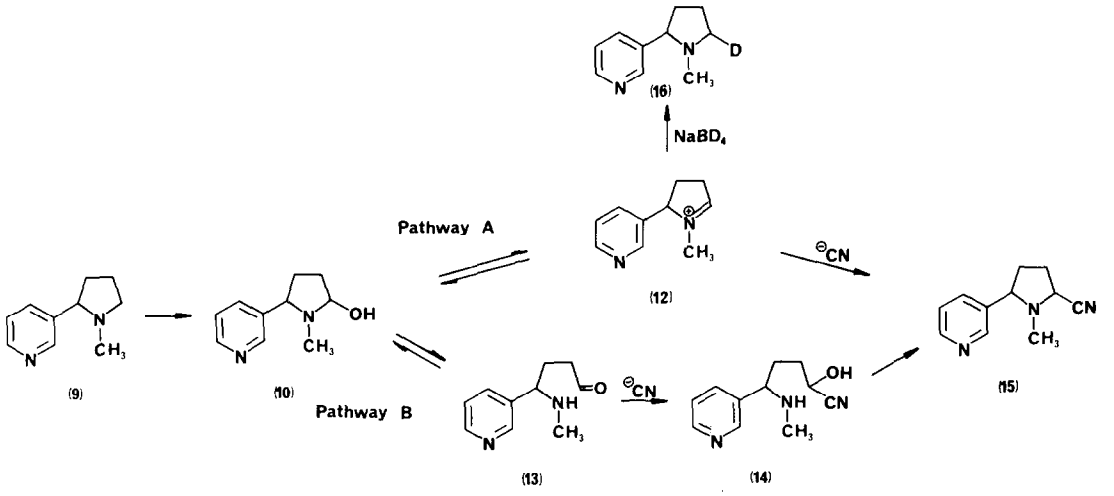


Fig. 5. Proposed pathways for the formation of 5′-cyanonicotine (15) from nicotine (9) in metabolic incubations containing cyanide.

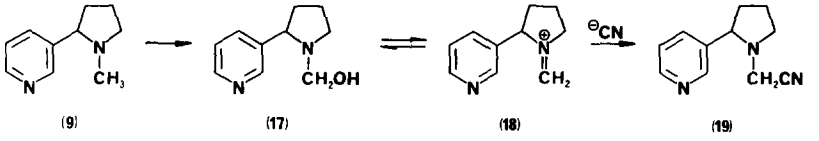
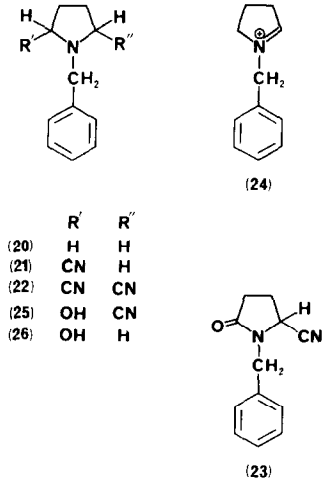
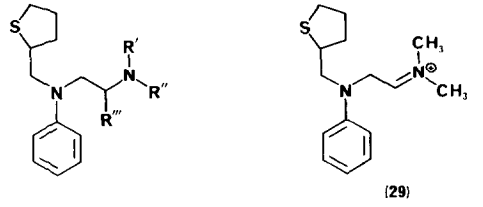


Fig. 6. Pathway for the formation of *N*-(cyanomethyl)nornicotine (19) from nicotine (9) in metabolic incubations containing cyanide.



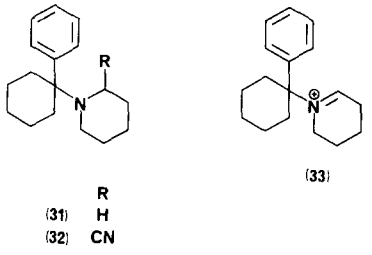
	R′	R″
(20)	H	H
(21)	CN	H
(22)	CN	CN
(25)	OH	CN
(26)	OH	H

Fig. 7. Structures of 1-benzylpyrrolidine (20) and its derivatives.



	R′	R″	R‴
(27)	CH_3	CH_3	H
(28)	CH_3	CH_2CN	H
(30)	CH_3	CH_3	CN

Fig. 8. Structures of methapyrilene (27) and its derivatives.



	R
(31)	H
(32)	CN

Fig. 9. Structures of phencyclidine (31) and its derivatives.

	R
(34)	CH_2OH
(35)	CH_3
(36)	H

Fig. 10. Structures of *N*-methylcarbazole (35), its derivatives and of diphenylamine (37).

cyanide was included in microsomal incubation of this drug, a cyano adduct was observed (32). Again, this was suggested to arise from attack of cyanide ion on an iminium ion (33). The authors also used radiolabeled drug to investigate the metabolism-dependent covalent binding of phencyclidine to microsomal protein. Cyanide, which at the concentrations used here, and in all previous experiments described, does not inhibit significantly microsomal metabolism, was a potent inhibitor of this binding, with an IC_{50} of 57 μM. Glutathione also inhibited the binding to protein. The results support the suggestion that iminium ions may be capable of reactions with nucleophilic groups on microsomal macromolecules and hence other macromolecules. Unequivocal proof of iminium ion production might better be obtained for these compounds by reduction using sodium borodeuteride.

A compound which at first sight appears to be similar to the compounds described above is N-(hydroxymethyl)carbazole (34, Fig. 10), but this compound does not form an iminium ion. Microsomal metabolism of *N*-methylcarbazole (35) under an atmosphere of $^{18}O_2$ produced N-(hydroxymethyl)-carbazole which contained ^{18}O [13]. Conversely, when the metabolism occurred in medium containing $H_2{}^{18}O$, no incorporation of ^{18}O into the N-(hydroxymethyl)carbazole was seen [13, 14]. Thus, no formation of iminium ions appeared to occur, since otherwise an exchange of the hydroxyl oxygen with that of the water would have taken place. Gorrod and Temple [15] suggested the stability of the N-(hydroxymethyl)carbazole to be due to the low basicity of the nitrogen. The nitrogen-protonated conjugate acid of *N*-methylcarbazole has a pK_a estimated to be -8 [16].

The poor basicity of *N*-methylcarbazole at the nitrogen atom and the inability of N-(hydroxymethyl)carbazole to form an iminium ion are presumably due to the involvement of the nitrogen lone pair in the aromaticity of the central ring. Protonation of the nitrogen or formation of an iminium ion would require the involvement of the nitrogen lone pair and would hence destroy this aromaticity. From comparison of the pK_a values of the conjugate acids of carbazole (36, Fig. 10) and diphenylamine (37), an estimate of 42 kJ mole^{-1} has been made for the resonance stabilisation energy derived from the central aromatic ring [16].

The proclivity of certain N-(1-hydroxyalkyl) amines to generate iminium ions appears to be dependent not only on the basicity of the nitrogen,

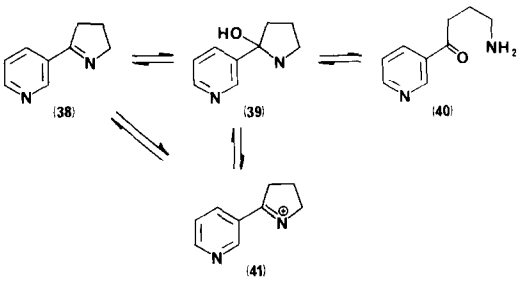
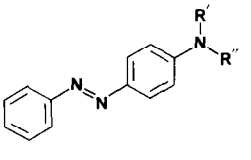


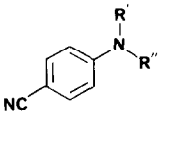
Fig. 11. Proposed equilibria for myosmine (38) in aqueous solution.

as illustrated by N-(hydroxymethyl)carbazole, but also on the extent of alkylation of the nitrogen. A recent communication [17] describes a study by nuclear magnetic resonance spectroscopy of the chemical behaviour in solution of the tobacco alkaloid myosmine (38, Fig. 11), which exists in equilibrium with the α-aminoketone (40). Resonances from myosmine (38) and the γ-aminoketone (40) were seen in the spectra of acidic solutions of myosmine, but there was no NMR evidence for the carbinolamine (39) that should, reasonably, be an intermediate in the reaction myosmine (38) $\rightleftharpoons$ γ-aminoketone (40). Evidently the life time of the carbinolamine (39) is extremely short because of the ease of dehydration to myosmine, and it is unlikely that the iminium ion (41) plays any role in this system. In the nicotine metabolite hydroxynicotine (10, Fig. 5) the presence of the N-methyl substituent prevents dehydration, and iminium ion formation is favoured.

Metabolites of the following three N-methyl containing xenobiotics have recently been suggested to form iminium ions: *N*,*N*-dimethylaminoazobenzene (DAB) (42, Fig. 12) [18], 4-cyano-*N*,*N*-dimethylaniline (44, Fig. 12) [19] and hexamethylmelamine (HMM) (47, Fig. 13) [20]. A major biliary metabolite of the hepatocarcinogen DAB (42) in the rat was identified as *N*-(glutathione-*S*-methylene)-4-aminoazobenzene (43) [18], and a major urinary metabolite of 4-cyano-*N*,*N*-dimethylaniline (44) in the rat and mouse was shown to be *N*-acetyl-*S*-(4-cyano-anilinomethyl)cysteine (45) [19]. In both cases, the thioether metabolites were considered to be the products of the reaction between glutathione and reactive species derived from the N-hydroxymethyl compounds, presumably the methylene iminium ions.

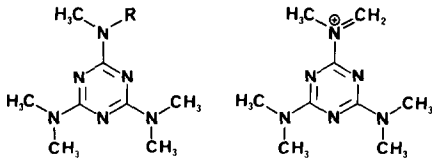


	R'	R''
(42)	CH_3	CH_3
(43)	H	Glutathione



	R'	R''
(44)	CH_3	CH_3
(45)	H	N-Acetylcysteine

Fig. 12. Structures of *N*,*N*-dimethyl-4-aminoazobenzene (42) and 4-cyano-*N*,*N*-dimethylaniline (44) and their metabolites.



	R
(46)	CH_2OH
(47)	CH_3
(49)	H
(50)	N-Acetylcysteine

Fig. 13. Structures of hexamethylmelamine (47) and its derivatives.

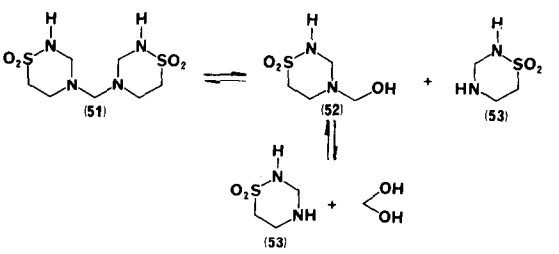


Fig. 14. Equilibria of taurolin (51) in aqueous solution.

The antineoplastic agent HMM (47, Fig. 13) is metabolized *in vitro* to *N*-(hydroxymethyl)pentamethylmelamine (HMPMM) (46) [21]. *In vitro* tests show that HMM has no cytotoxic activity *per se* but instead requires metabolic activation: HMPMM is cytotoxic and it has been suggested that this molecule might be the active cytotoxic species *in vivo* [22, 23]. It is not known, however, if HMPMM is active as an intact moiety or if it is a transport form of cytotoxic formaldehyde. Several studies have shown that the addition of semicarbazide to cell cultures protects them from formaldehyde toxicity [23–25]. The results obtained by treating cells with HMPMM after semicarbazide pretreatment are somewhat equivocal. In some cell lines, cytotoxicity of HMPMM appears to be due to formaldehyde, whereas in others it appears to be directly due to the carbinolamine. Ross *et al.* [24] compared the DNA damage caused by HMM (after metabolic activation) with that caused by formaldehyde and found only low levels of DNA–protein crosslinks with HMM, whereas non-lethal concentrations of formaldehyde caused high levels of DNA–protein crosslinks. They concluded that the cytotoxicity of HMM was unlikely to be due to formaldehyde. Studies involving [^{14}C]ring- and [^{14}C]methyl-labelled HMM *in vivo* have revealed that [^{14}C]ring-HMM binds to a high degree to cellular macromolecules, suggesting that it is the whole molecule that is bound and not only metabolites of oxidised *N*-methyl groups as would be expected if only [^{14}C]methyl-HMM was found to be bound [26]. Ames *et al.* [20] studied the binding of the two differently labelled compounds to calf thymus DNA in a microsomal incubation and found the two labels to be equally bound to the DNA. In contrast, binding of [^{14}C]methyl-HMM to protein was greater than the binding of [^{14}C]ring-HMM which may be explained by assuming that two species are involved in covalent binding: the greater binding of the [^{14}C]methyl-HMM perhaps being due to oxidative metabolism of the N-methyl groups to produce formaldehyde which consequently reacts with proteins. [^{14}C]Ring-HMPMM was shown to bind to DNA and to a lesser extent to protein. However, in this case metabolic activation was not required to obtain binding. On the basis of these results, the formation of an iminium ion (48, Fig. 13) has been proposed by Ames *et al.* [20] to explain the binding of HMPMM to cellular macromolecules, including DNA.

Studies in our own laboratories support this hypothesis. Reduction of HMPMM with cyanoborohydride produced a small quantity of HMM, the major product being pentamethylmelamine (49) from base-catalysed decomposition of HMPMM.* The HMM produced can only result from the reduction of the methylene iminium ion suggested by Ames *et al.* [20]. Furthermore, incubation of pentamethylmelamine, formaldehyde and *N*-acetylcysteine yielded a *N*-acetylcysteine conjugate of HMM (50) [27]. The mechanism of this reaction is not clear, but one possibility is an initial reaction between formaldehyde and PMM to form the carbinolamine HMPMM, which subsequently forms an iminium ion which reacts with *N*-acetylcysteine.

The question as to whether carbinolamines and carbinolamides exert their toxicity via formaldehyde or iminium ions has been investigated recently by Gidley *et al.* [28] with respect to the mode of action of the "masked" formaldehyde antibacterial agent taurolin (51). In aqueous solution the equilibrium is established between (51), the carbinolamine (52), the thiadiazine (53) and formaldehyde as shown in Fig. 14. The amounts of formaldehyde produced in this equilibrium appeared to be insufficient to explain the antibacterial activity of taurolin, and the carbinolamine (52) was implicated as the active moiety. To test this hypothesis, other carbinolamines were generated *in situ* by mixing aqueous solutions of simple amines and formaldehyde [28]. The authors investigated whether it was possible to correlate antibacterial activity with the presence of methylene iminium ions. Iminium ions were detected by adding to the reaction mixture sodium cyanoborohydride which reduces the iminium ion to the corresponding N-methyl compound while only slowly reducing formaldehyde to methanol. The reaction was followed by NMR spectroscopy. Solutions that demonstrated the presence of iminium ions also showed antibacterial activity, whereas those mixtures that did not generate iminium ions, even if they could form carbinolamines, showed only low levels of antibacterial activity. In contrast, the antibacterial agent noxythiolin (54, Fig. 15) decomposes to give only formaldehyde and the amide (55) [29]. Here, treatment with sodium cyanoborohydride yielded methanol with no indication of the presence of an iminium ion. In addition, for different concentrations and ages of the solutions, the antibacterial activity of

* Unpublished observation.

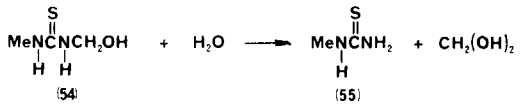


Fig. 15. Equilibrium of noxythiolin (54) in aqeuous solution.

noxythiolin solutions was very similar to that of the amounts of free formadehyde present. These results suggest that formaldehyde is the active antibacterial agent. The authors explained the observations obtained with the different "masked" formaldehyde antibacterials in terms of the chemical properties of the N-CH_2-X group. In cases where X is a reasonably good leaving group and the nitrogen possesses a basic non-bonding electron pair, formation of an iminium ion may occur. However, when X is OH and it is not easily expelled because the nitrogen is not basic, then the N-CH_2-OH moiety may break down differently to give formaldehyde which, in the absence of other nucleophiles, is rapidly hydrated. Investigations in our own laboratories on a number of N-methyl and N-hydroxymethyl compounds are in accordance with this interpretation. We [30, 31] and others [32] found relatively stable N-hydroxymethyl compounds as metabolites of N-methyl compounds when the nitrogen was in the chemical environment causing low basicity, such as in amides (56), triazenes (61) and ureas (60, Fig. 16). Not surprisingly, the loss of hydroxide from these stable N-hydroxymethyl compounds is not favoured, so that, for example, 4-chloro-*N*-(hydroxymethyl)benzamide (57, Fig. 16), a metabolite of 4-chloro-*N*-methylbenzamide (56) [31], did not react with cyanide.* However, the possibility exists that the generation of iminium ions from N-alkyl compounds of low basicity may come not directly from oxidation of the N-alkyl moiety as in the case of, for example, nicotine, but instead after conjugation of the hydroxyalkylamine or hydroxyalkylamide. Indeed, we found that the acetate esters of certain carbinolamides can react to form cyanide adducts. Incubation of *N*-(acetoxymethyl)-benzamide (59) with cyanide produced 4-chloro-*N*-(cyanomethyl)benzamide (58).* An equally striking example of how phase 2 metabolism might activate an otherwise unreactive *N*-hydroxymethyl com-

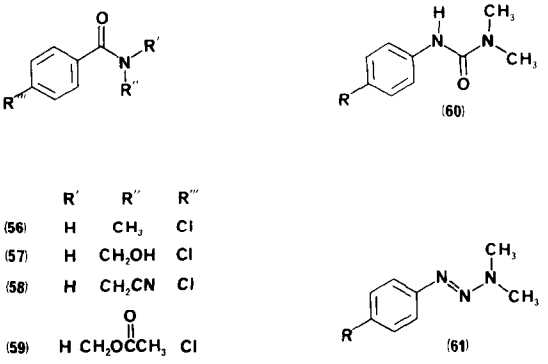


Fig. 16. Structures of 4-chloro-*N*-methylbenzamide (56). its derivatives. *N,N*-dimethylphenylurea (60) and *N,N*-dimethylaryltriazene (61).

pound is found in the solvolysis reactions of some recently reported *N*-(hydroxymethyl)triazene derivatives [33]. The *N*-(acetoxymethyl)triazenes (63, Fig. 17) are readily obtained by base catalysed acetylation of the *N*-(hydroxymethyl)triazenes (62). The acetates (63) undergo solvolysis to the *N*-(methoxymethyl)triazenes (65) perhaps via the intermediate iminium ion (64) in methanol, whereas the underivatised (unconjugated) *N*-(hydroxymethyl)triazene (62) does not generate iminium ions under the same conditions. The only reaction of *N*-(hydroxymethyl)triazenes in methanol is the loss of formaldehyde to produce the monomethyltriazene (Ar-N=N-NHR).

In conclusion, in this review we have summarised the reports which have shown that reactive, electrophilic, iminium ions may be generated by the metabolism of a variety of N-alkylamines and N-alkylamides. The ability of these compounds to form such species is dependent upon their basicity. In the case of hexamethylmelamine (47, Fig. 13) the generation of an iminium ion during its metabolic N-demethylation is suggested to lead to a compound capable of reaction with DNA [20]. This result raises the question of whether this and other xenobiotics which have the potential to form an iminium ion may therefore be genotoxic, a question presently under investigation by us. In addition, such agents may prove to be antitumour agents. The biological activity of these compounds is largely undefined and

* Unpublished observation.

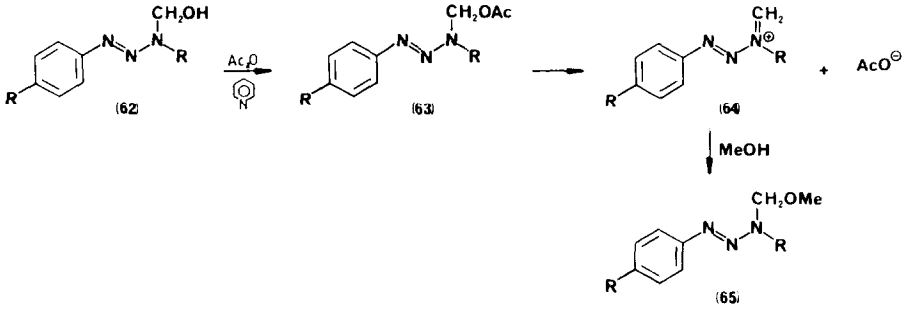


Fig. 17. Chemical formation of an *N*-(acetoxymethyl)triazene (63) and its reaction with methanol.

research on them may usefully provide a wealth of information in the future on both their hazards and utility.

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