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Mechanisms of antibacterial formaldehyde delivery from noxythiolin and other 'masked-formaldehyde' compounds

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Formaldehyde release in aqueous solutions of noxythiolin (*N*-methyl-*N'*-hydroxymethyl thiourea) has been monitored by nuclear magnetic resonance (n.m.r.) spectroscopy. The results suggest that antibacterial activity in such solutions resides mainly in the free formaldehyde. N.m.r. spectroscopy also demonstrated slow C–N bond rotation in noxythiolin and *N*-methylthiourea, with $\Delta G^{\ddagger}$ of ca 15 kcal mol^{-1} (63 kJ mol^{-1}). *N*-Hydroxymethyl imidazole is marginally more effective than corresponding hydrated formaldehyde solutions, an effect which is attributed to more rapid turnover of unhydrated formaldehyde as detected by saturation transfer n.m.r. spectroscopy. These observations are combined with the known delivery of lethal iminium ions, $R_2N^+=CH_2$, by compounds of the form R_2NCH_2X (X = OH, NR_2; R is alkyl) to suggest a single consistent explanation of the antibacterial properties of a wide range of masked formaldehyde compounds.

Formaldehyde, I, has long been known as an effective antibacterial agent, but its toxicity and unpleasant gaseous properties have rendered it of little value clinically. In contrast, 'masked' formaldehyde compounds containing a carbon atom at the same oxidation level in the form N–CH_2–N or N–CH_2–O are important both clinically and industrially; for example, noxythiolin, II (*N*-methyl-*N'*-hydroxymethylthiourea) is much used in the topical treatment of bacterial infections (Browne & Stoller 1970; Gilmore et al 1978). Almost nothing is known of the mechanism of antibacterial action of any of this class of compound, although we have recently shown that for R_2N–CH_2–NR_2 derivatives where R is alkyl the 'formaldehyde' is delivered as the iminium ion $R_2N^+=CH_2$ (Gidley et al 1981). We now present results which indicate that noxythiolin exerts its effects by simple release of formaldehyde and we suggest a single consistent explanation for the antibacterial properties of a wide range of masked formaldehyde compounds.

$$\underset{\text{I}}{CH_2{=}O} \equiv CH_2(OH)_2$$

Noxythiolin is known to be unstable in aqueous solution, slowly decomposing to *N*-methylthiourea and formaldehyde [eqn 1]. Several attempts have been made to quantify this formaldehyde release to establish whether it completely accounts for the observed antibacterial activity: Block (1967) used the Nash (1953) assay for formaldehyde, but as the reagents involved may well disrupt the equilibrium,

$$\underset{\text{II}}{Me\overset{H}{N}\overset{S}{\overset{\|}{C}}\overset{H}{N}CH_2OH} + H_2O \rightleftharpoons \underset{\text{III}}{Me\overset{H}{N}\overset{S}{\overset{\|}{C}}NH_2} + \underset{\text{I}}{CH_2(OH)_2} \quad [\text{eq } 1]$$

the possibility of unreliable results cannot be ruled out. Another study (Kingston 1965) attempted the determination of the formaldehyde gas evolved from noxythiolin solutions by placing water and a solution of noxythiolin in separate beakers in a closed container and monitoring the increase in the formaldehyde content of the water by Nash's method. Not surprisingly, this experiment gave inconclusive results.

There was therefore a need for a reliable assay of the formaldehyde produced in solutions of noxythiolin. We have found nuclear magnetic resonance (n.m.r.) spectroscopy to be an ideal way of studying this rection as the relative concentrations of the various species can be easily determined by integration without any disturbance of the equilibrium or reaction rate.

MATERIALS AND METHODS

Noxythiolin (Noxyflex, Geistlich Sons Limited, Wolhusen, Switzerland) and *Escherichia coli* (Manchester University Bacterial Collection, MU352, stored

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on Tryptone Soya Agar slopes) were gifts from Dr M. C. Allwood, Addenbrooke's Hospital, Cambridge.

Commercial aqueous solutions of formaldehyde (formalin) contain methanol as stabilizer, and were therefore unsuitable. Concentrated aqueous formaldehyde solutions were prepared by heating paraformaldehyde and water (or D_2O) at 110 °C in a sealed tube for ca 16 h. Dilute (<5% w/v) solutions were obtained by autoclaving an aqueous suspension of paraformaldehyde at 115 °C, 1 atm for 30 min. Yields were >95% as judged by the sodium sulphite assay (Walker 1964).

Bacterial assay: *E. coli* was grown to stationary phase (18 h, 37 °C) in tryptone soya broth, harvested by centrifugation, washed with distilled water and centrifuged (3 times each), and finally resuspended to 10^8 cells ml^{-1} as judged by optical density at 500 nm. Aliquots of bacterial suspension (0·1 ml) were added to the test solution or water (9·9 ml). After 6 min the suspensions were serially diluted up to 10^7-fold and plated out using the pour-plate technique: 1 ml aliquots of suitable dilution were added to Oxoid Tryptone Soya Agar. Plates were incubated (37 °C, 48 h) and a viable count determined.

The same general procedure was used for the viability assay of challenged bacteria after 3–12 min exposure to the test solution using at least two different dilution factors for each exposure time, and plating out in quadruplicate. The serial dilution ensured that no residual antibacterial activity was present during viability assays. Each assay for bactericidal activity was repeated at least once on separate days using freshly prepared bacterial suspensions.

pD values in D_2O are corrected meter pH readings. The decay of noxythiolin to *N*-methyl thiourea and formaldehyde was followed by ^1H n.m.r. spectroscopy at 80 MHz using a Varian Associates CFT20 instrument operating in the Fourier Transform mode. Relative concentrations of formaldehyde hydrate and noxythiolin were obtained by integration of the relevant signals in the spectrum, care having been taken that the intervals between pulses were greater than 5 times the longest T_1 of interest. Chemical shifts are in δ units (p.p.m.).

Variable temperature n.m.r. experiments were carried out at 100 MHz using a Varian XL100A instrument.

Saturation transfer experiments on *N*-hydroxymethyl imidazole were carried out at 400 MHz on a Bruker WH400 instrument using programs developed by one of us for nuclear Overhauser effect difference spectroscopy (Hall & Sanders 1981): two selective saturation experiments irradiating $N\text{–}C\underline{H}_2OH$ and $C\underline{H}_2(OH)_2$ and a control were carried out automatically under computer control. Subtraction of the control spectrum from the saturation experiments gave difference spectra displaying (apart from positive n.O.e.'s) essentially only negative responses from the irradiated proton and from sites which had exchanged with the irradiated proton. Intensity changes as small as 0·5% are readily detectable by this technique, and were converted to reaction rates by standard methods.

RESULTS

Changes in the concentration of formaldehyde present in noxythiolin solutions as a function of time, determined by ^1H n.m.r., are shown in Fig. 1. A

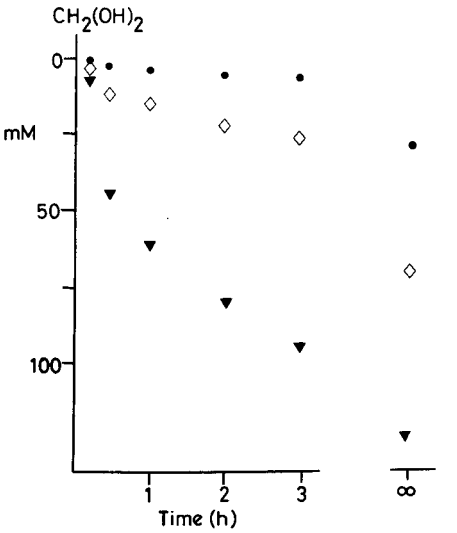


FIG. 1. The release of formaldehyde from various concentrations of aqueous noxythiolin solution as determined by ^1H n.m.r. The equilibrium position (∞) was reached only after several weeks. Initial concentrations of noxythiolin were ●, 33 mM (0·4%); ◇, 83 mM (1·0%); ▼, 207 mM (2·5%).

number of observations can be made about these results:

1. Higher concentrations of noxythiolin show less percentage decomposition at equilibrium. This is to be expected as the decomposition reaction is first order but the re-formation reaction is second order. The equilibrium constant (determined from the equilibrium concentration) for the reaction was found to be constant (7·2 ± 1·0 mol $litre^{-1}$) over the range of concentrations studied.

2. Higher concentrations of noxythiolin equilibrate more quickly than do lower concentrations. This is probably a reflection of the fact that the initial alkalinity of the solutions increases with concentration (initial pD values of 0·4, 1·0 and 2·5% solutions were 7·6, 8·1 and 8·8 respectively). It has previously been found that the rate of decomposition of *N*-hydroxymethylthiourea is proportional to the concentration of hydroxide ions (Dusek 1958) and so, as the equilibrium pD of each solution is nearly the same (6·1–6·4), the initially most alkaline solution (i.e. the highest concentration) might be expected to equilibrate most rapidly.

3. A kinetic analysis in terms of opposing first and second order reactions moving towards equilibrium does not exactly fit the data of Fig. 1. This presumably reflects the varying pD during the reaction.

As noxythiolin is a masked formaldehyde compound, the possibility that its antibacterial activity is due to the presence of iminium ions (Gidley et al 1981) was investigated. Addition of the standard test reagent sodium cyanoborohydride (Gidley et al 1981; Gidley & Sanders 1982) to variously aged solutions of several different concentrations of noxythiolin resulted, in all cases, in the slow reduction of formaldehyde to methanol being the only observed reaction. It would therefore seem that iminium ions are not present in significant concentrations in solutions of noxythiolin.

To determine whether the formaldehyde produced in aqueous solutions of noxythiolin is responsible for the antibacterial activity, the loss of viability of *E. coli* was determined in the presence of solutions of 0·4, 1·0 and 2·5% noxythiolin of various ages. Survival curves are shown in Figs 2 and 3 for the more concentrated solutions. All but the most aged solutions of 0·4% noxythiolin were inactive. These results were then compared with the viability loss caused by exposure to the corresponding concentration of formaldehyde present in each of these solutions (Figs 2 and 3).

It was found that, for all concentrations and ages, the antibacterial activity of noxythiolin solutions was, within experimental error, very similar to that of the free formaldehyde concentration present in the solutions. Thus, for 1% noxythiolin solutions, Fig. 2 shows essentially no difference from the corresponding formaldehyde concentrations. Agreement for 2·5% noxythiolin (Fig. 3) is not quite so ideal. Nevertheless it is clear that the activity of 105 mM formaldehyde is greater than that of noxythiolin solutions containing 95 mM formaldehyde and probably less than those containing 120 mM formaldehyde. Similarly the activity of 70 mM formaldehyde falls between the activities of noxythiolin solutions containing 62 and 80 mM formaldehyde.

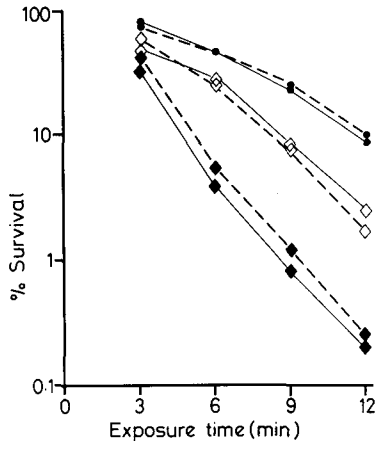


FIG. 2. *E. coli* survival curves following exposure to 1% noxythiolin solutions of various ages and corresponding pure formaldehyde solutions. Period of ageing and equivalent formaldehyde concentrations are —●— 4 h (32 mM), —◇— 24 h (50 mM), and —◆— 8 weeks (72 mM). Pure formaldehyde solutions were - - -● 35 mM, - - -◇ 50 mM, and - - -◆ 70 mM.

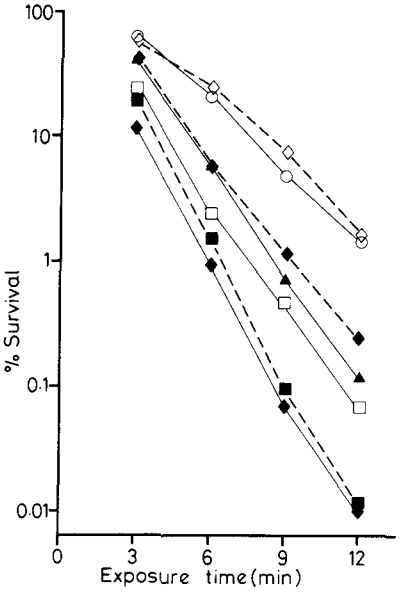


FIG. 3. *E. coli* survival curves following exposure to 2.5% noxythiolin solutions of various ages and corresponding pure formaldehyde solutions. Periods of ageing and equivalent formaldehyde concentrations are —○— 1 h (62 mM), —▲— 2 h (80 mM), —□— 3 h (95 mM) and —◆— 8 weeks (120 mM). Pure formaldehyde solutions were - - -◆ 70 mM and - - -■ 105 mM.

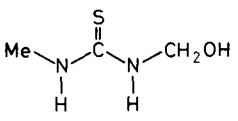
Any differences are of the same order as experimental error. These results indicate that the active species in noxythiolin solutions is probably formaldehyde, particularly when they are compared with the very much greater antibacterial activity of the solutions which can deliver iminium ions (vide infra).

Further evidence supporting formaldehyde as the sole antibacterial agent in these solutions is as follows. First, *N*-methylthiourea, produced along with formaldehyde by the decomposition of noxythiolin, has no antibacterial activity at the concentrations present in noxythiolin solutions. Second, there is no enhancement of the antibacterial activity of formaldehyde by *N*-methylthiourea. Third, the antibacterial activity of noxythiolin solutions increases with time as does their toxicity to animals (Block 1967). As the concentration of noxythiolin decreases with time, this implies that the intact molecule is not the important antibacterial agent, apart from its ability to release formaldehyde.

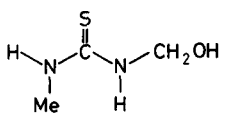
The solution state of noxythiolin

During the course of the n.m.r. studies on noxythiolin it was found that both *N*-methylthiourea III and noxythiolin at or below room temperature show a doubling and broadening of all the expected resonances. This behaviour is due to the partial double bond character of the thiourea C–N bond giving rise to slow interconversion of the possible isomers: the thiocarbonyl group exerts a substantial, geometry-dependent chemical shift effect so the *methyl* chemical shifts in II(a) and II(c) will be essentially the same as each other but different from those in II(b) and II(d). Similarly the methylene CH_2 resonances in II(a) and II(b) will be essentially the same as each other and different from those in II(c) and II(d). Below room temperature the isomerization is slow on the n.m.r. timescale (Jackman & Sternhell 1969) and separate signals are observed; above room temperature it is fast, and single signals are observed. The coalescence temperatures at 100 MHz for the methyl and methylene resonances of noxythiolin were 21 °C and 18 °C respectively. Given shift separations of 20 and 15 Hz these correspond to an activation energy for bond rotation of 15–15.5 kcal mol^{-1} (63 kJ mol^{-1}) in good agreement with published values for *N*-alkyl thioureas (Tompa et al 1969; Sullivan & Price 1975).

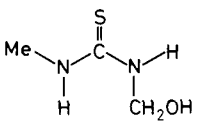
These observations of slow bond rotation are of no biological or clinical significance. They are presented to point out a possible pitfall in the n.m.r. spectroscopy of noxythiolin and related compounds.



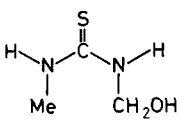
IIa



IIb



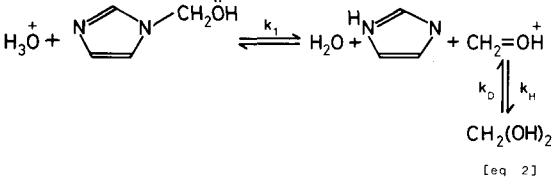
IIc



IId

N-Hydroxymethyl imidazole

We reported elsewhere (Gidley et al 1981) that addition of imidazole to aqueous formaldehyde slightly increased the antibacterial activity: neither 35 mM imidazole nor 17·5 mM formaldehyde alone has any detectable antibacterial effect under the assay conditions but a solution containing both compounds killed about 20% of bacteria in 6 min. ^1H n.m.r. spectroscopy of an aqueous formaldehyde (17·5 mM) and imidazole (35 mM) mixture revealed a singlet at 4·8 δ due to formaldehyde hydrate, $CH_2(OH)_2$, and a singlet at 5·5 δ due to *N*-hydroxymethyl imidazole, IV, in the proportion 45:55 respectively. No iminium ions could be trapped from the solution by sodium cyanoborohydride. We decided therefore to measure the rate of formaldehyde release from IV using the technique of n.m.r. saturation transfer difference spectroscopy (Sanders & Mersh 1983). When the formaldehyde hydrate resonance was irradiated, the hydroxymethyl signal decreased in intensity and vice versa. The overall rate of formaldehyde hydrate formation from IV was found to be 0·2 s^{-1} (eqn 2). As the hydration rate k_H is clearly very rapid, k_1 is also taken to be 0·2 s^{-1}. The possible significance of this result is taken up in the Discussion.



[eq 2]

DISCUSSION

Noxythiolin mechanism

We have shown that the antibacterial activity of noxythiolin is probably due to the formaldehyde which is slowly released in aqueous solution. Differences in activity between noxythiolin solutions and

corresponding formaldehyde solutions are comparable with experimental error. As the rate of production of formaldehyde increases with pH value, the observation that solutions of noxythiolin at pH 11 are more bactericidally effective than similarly aged neutral solutions (Horsfield 1967) is readily explained. When used clinically, noxythiolin solutions are usually prepared immediately before they are required and therefore most of the formaldehyde release takes place at the site of infection. It is intriguing that such slow release of formaldehyde apparently does not damage animal tissue significantly whilst it effectively destroys the bacterial population.

In considering the origin of the antibacterial activity of the formaldehyde/imidazole mixed solution it should be noted (i) that the activity is less than that found in the alkyl-*N*-hydroxymethyl-containing solutions which react via methylene iminium ions and (ii) that iminium ions cannot be trapped from the formaldehyde/imidazole solution. It could still be argued that the biological activity resides in iminium ions if bacteria can trap such a species more efficiently than cyanoborohydride. This seems unlikely because it requires there to be present in the bacterium a better iminium ion trapping agent than the hydride ion produced from sodium cyanoborohydride; furthermore, iminium ions based on imidazole are not produced from the corresponding enamines even under the most forcing conditions (e.g. reflux with 6 M HCl) (Hupe et al 1972). As we pointed out previously, this is only to be expected as the iminium ions (unlike the carbinolamine) would not be stabilized by aromaticity (Gidley et al 1981).

A plausible explanation for the enhancement of the bactericidal effectiveness of formaldehyde solutions by imidazole is however suggested by consideration of the rate of production of free formaldehyde in these solutions. Formaldehyde is predominantly (>99%) hydrated in aqueous solution, but the unhydrated aldehyde (neutral or protonated) is the reactive species and is almost certainly responsible for any antibacterial activity. The rate of dehydration of formaldehyde hydrate (k_D in eqn 2) has been found by Le Hénaff (1963) to be 0·0045 s^{-1} in water at pH 7. In contrast, the rate of production of (unhydrated) formaldehyde from the imidazole carbinolamine is 0·2 s^{-1} under the same conditions. Unhydrated formaldehyde is therefore produced approximately 40 times more often in the presence of imidazole than in its absence.

This could be the basis of the antibacterial activity of imidazole/formaldehyde mixtures especially as the difference between the two rates (once every 5 s vs once every 4 min) is significant when compared with the exposure time necessary for any substantial antibacterial activity to manifest itself (2–3 min), i.e. the turnover of unhydrated formaldehyde at the susceptible biological sites is higher, and so we might expect more biological activity. This admittedly speculative explanation is supported by simple but persuasive logic: if the mixed solution is more effective than formaldehyde alone then the formaldehyde is being delivered to the susceptible biological sites either more efficiently or in a more reactive form. Since there is no detectable transport barrier for small hydrophilic molecules such as $CH_2(OH)_2$ it would not be surprising if greater antibacterial activity reflected delivery of more of the reactive species.

For noxythiolin the most rapid formaldehyde release rate observed (the initial rate at 2·5% concentration) is two orders of magnitude slower than the rate of generation of formaldehyde from its hydrate. This possible mechanism is therefore not applicable to noxythiolin solutions.

A general mechanism

The results presented here on the release of formaldehyde and previously (Gidley et al 1981) on methylene iminium ion delivery can be understood in terms of the chemistry of the N–CH_2–X group. When X is a reasonably good leaving group and the N has a basic lone pair then reaction [3] or [4] can take place to yield a biologically potent iminium ion. For a wide range of alkyl groups, R, these iminium ions would be expected to have essentially identical susceptibilities to nucleophilic attack; this may explain the previously reported insensitivity of biological activity to changes in R for the series of aminals of type $R_2NCH_2NR_2$ (Rehn & Kowatsch 1977a, b). The charged iminium ion is more susceptible to nucleophilic attack than simple carbonyl groups: this would explain the much greater antibacterial activity of compounds which deliver formaldehyde in this way.

$$R_2N-CH_2-X \longrightarrow R_2\overset{+}{N}=CH_2 + X^- \quad \text{where X=OH at pH} \geq 7 \qquad \text{[eq 3]}$$

$$R_2N-CH_2-\overset{+}{X}H \longrightarrow R_2\overset{+}{N}=CH_2 + HX \quad \text{where X=NR}_2 \text{ or OH at pH} < 7 \qquad \text{[eq 4]}$$

$$H^+ \curvearrowright R_2N-CH_2-\ddot{O}H \longrightarrow R_2NH + CH_2=\overset{+}{O}H \underset{k_H}{\overset{k_D}{\rightleftharpoons}} CH_2(OH)_2 \qquad \text{[eq 5]}$$

When X is OH and not easily expelled because the N has a very low pK (i.e. it is not basic), the $N\text{–}CH_2\text{–}OH$ can break down in the opposite sense (eqn 5) to give formaldehyde which, in the absence of other nucleophiles will be rapidly hydrated. In noxythiolin, and possibly in general for this type of system, $k_1 \ll k_D$ (eqn 5) and the turnover of unhydrated formaldehyde is essentially the same as in equivalent solutions of pure formaldehyde. The antibacterial activity of such compounds would therefore be expected to be the same as the equivalent formaldehyde solution.

For the special case of *N*-hydroxymethyl imidazole $k_1 \gg k_D$ presumably because protonation of the unsubstituted nitrogen (pKa ~ 7) makes the imidazole ring a very good leaving group (eqn 2). We speculate that the resultant increase in the turnover of unhydrated formaldehyde compared to simple formaldehyde solutions leads to greater antibacterial activity.

Acknowledgements

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