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The bactericidal properties of compounds which protect living cells against freezing damage

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INTRODUCTION

The kind of bactericidal action considered in this paper is that which occurs during air disinfection, but this is no different in principle from disinfection *in vitro*. The bacteria-carrying particles in contaminated air can be regarded as tiny 'test tubes' in which the mixture need not be homogeneous, and where the disinfectant is probably present in high concentration. Its action should stop immediately the particle impinges on to a wet surface, owing to rapid diffusive removal of the substance from around the bacteria.

There has been little progress in the field of chemical air disinfection since the publication of the *M.R.C. Report* (Bourdillon, Lidwell & Lovelock, 1948*a*). It is known that transfer of disinfectant to the bacteria-carrying particle occurs exclusively through the vapour phase (Puck, 1947; Nash, 1951), and that it is easy enough to kill the bacteria in aerosols produced by spraying cultures, at either high or low relative humidity. It is also possible to deal with naturally contaminated air at high relative humidity. No substance has yet been found, however, which achieves a satisfactory rate of kill in the most important practical application, that of air disinfection under indoor winter conditions when the relative humidity is low and the bacterial pollution high because of poor ventilation. The purpose of this communication is to report a new approach to the latter problem, suggested by work in another field.

KNOWN AIR DISINFECTANTS

Exceptional behaviour of the glycols

Most air disinfectants require a fairly high relative humidity (R.H.) to be effective (Lidwell & Lovelock, 1948), presumably because the bacteria-carrying particles must be moist before they can dissolve and exert their bactericidal action. The simple glycols (mono-, di- and tri-ethylene and -propylene) on the other hand are most effective at low R.H. They fail at high R.H. because too much water then condenses on to the particle at the same time, so that the glycol concentration is not high enough to be bactericidal (Lidwell, Lovelock & Raymond, 1948). The importance of high particle concentration was established by detailed *in vitro* studies (Robertson, Appel, Puck, Lemon & Ritter, 1948). For triethylene glycol

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vapour used against *Streptococcus haemolyticus*, the lethal effect is a maximum at R.H. 25 %, and falls almost to zero at R.H. 65–70 % (Lester, Robertson, Puck, Wise & Smith, 1949).

Experimental demonstration of the effect of relative humidity

Some work is now reported which was carried out in the M.R.C. Air Hygiene Laboratory using a test chamber constructed for such experiments after experience with the Salisbury chamber (Bourdillon, Lidwell & Lovelock, 1948*a*). Sixty-five compounds were tested against the standard organism, a micrococcus, aerosol strain, N.C.T.C. 7944. Compounds were selected according to criteria of volatility and water solubility which were considered at the time to be most relevant (Nash, 1951). The general procedure was as follows: after various checks, a 5:1 dilution of an overnight broth culture in distilled water was sprayed from a fine baffled spray and allowed to equilibrate for one minute in the air of the chamber. A half-minute control plate was then taken on a slit sampler, and the compound under test rapidly vaporized from an electrically heated stainless steel boat. Test plates were taken at suitable intervals subsequently. If the killing rate was very high, only one plate was used and the compound was vaporized during sampling.

Table 1. *Air disinfection at high and at low relative humidity*

The indicated amounts of material were vaporized into a space of 8400 l. uniformly infected by micrococci. The air was sampled with a slit sampler at 28 l./min. and the number of colonies per sector counted after incubation. Killing rates are reckoned in equivalent air changes per hour: −, < 30; +, 30–100; ++, 100–300; +++, 300–1000; ++++, > 1000. S indicates the hydrophilic character of each compound (see text). * Known air disinfectants.

	R.H. 40 %		R.H. 65–70 %				
Substance	30 mg.	100 mg.	3 mg.	10 mg.	30 mg.	100 mg.	S
Diethylene glycol*	−	+++	.	.	−	++	+5/7
Monoacetin	−	++	.	.	−	+	+3/9
Monobutyrin	−	+	.	−	+	+++	+1/11
Hexa-hydro resorcinol	.	−	.	−	+	++	0/8
2-Hydroxymethyl cyclo-hexanol	.	−	.	−	++	++	−1/9
3-Hydroxymethyl cyclo-hexanol	.	−	.	−	+	+	−1/9
Tropine	.	−	.	.	−	+	−1/9
Ephedrine	.	−	−	+	++	++++	−1/9
α-Phenyl glycol	.	−	.	−	+	++++	−1/9
α-Hydroxy-α-methyl butyric acid*	.	−	.	−	+	++++	−1/8
Resorcinol*	−	+	+	+++	+++	.	−1/8
Succinimide	.	−	.	.	−	+	−1/7
1,2-Octane diol	.	−	.	.	−	+++	−2/10
Terpin hydrate	.	−	.	.	−	+	−4/12

Results for fourteen of the more active compounds are summarized in Table 1, which also includes a parameter S as a rough measure of the hydrophilic character of each compound. The parameter is in the form of a fraction whose denominator is

the total number of non-hydrogen atoms in the molecule, and whose numerator is the number of methylene groups which have to be added or removed in order that the compound should be only just miscible with water. The fraction is negative if hypothetical methylene groups have to be removed. S is a simpler version of a 'solubility index' calculated from the parachor and based on a survey of published solubility data and when necessary the synthesis of model compounds (Nash, 1962*a*).

Apart from succinimide, which is almost inactive, all the compounds listed in the table are hydroxylic, while eight of them are diols. Only two of the latter, however, retain their effectiveness at low R.H., and these have the highest values of S. The exceptional behaviour of the simple glycols is therefore confirmed, and one might suspect that the mechanism of their bactericidal action is different from that of other compounds.

PROTECTION OF LIVING CELLS AGAINST FREEZING DAMAGE

In the ordinary way the glycols are among the least toxic of compounds, and in particular can protect red blood cells against lysis when they are frozen to a low temperature and then thawed. Such lysis is almost certainly due to the concentration of salt inside the cells, because of the separation of pure water as ice (Lovelock, 1953). In the presence of glycol, glycerol or related compounds, cells can be frozen without lysis because the compound lowers the concentration of salt in equilibrium with ice at any temperature below freezing. Thus it is possible, by adding enough solute (about 3 M) to maintain the salt concentration below the critically damaging level (Lovelock, 1954). It follows that the glycol must remain in the liquid phase at low temperatures, and must also retain dissolved salts even when most of the water has been removed.

Connexion with air disinfection

Of the compounds listed in Table 1 only the first two, which are also the best air disinfectants at low R.H., are known to be good protectors against freezing damage (Lovelock, 1954). The others are either too surface active, or not soluble enough, or obviously unsuitable for other reasons. It seemed possible that the solvent properties of the glycols, as well as allowing them to function in protection against freezing damage, might also be responsible for their air disinfectant activity at low R.H. Under these conditions the normally stronger disinfectants such as the hydroxy acids or resorcinol fail because they require a certain amount of water in which to dissolve before any bactericidal action can take place. When dry particles, coated with such inactive disinfectant, are collected on to agar or in liquid medium, the potentially lethal compound rapidly diffuses away leaving the bacteria unharmed.

As regards the possible solvent properties of the simple glycols, it is known that ethylene glycol will dissolve some of the macromolecular components of bacterial cell walls (Morgan, 1937). It is also known that *in vitro* concentrations of glycol, only a little less than critical, are comparatively harmless to bacteria (Robertson *et al.* 1948). This would be difficult to understand if the glycol were acting as

individual toxic molecules. On the other hand, a small change in properties, such as would result from a change in the amount of water present, could easily, by Bronsted's principle (Bronsted, 1938) change the mixture from a non-solvent to a solvent as regards a particular macromolecule. It is therefore tentatively proposed that the lethal action of the glycols in air disinfection at low R.H. is due to some kind of massive physical attack on the cell wall, possibly followed by leakage of essential constituents.

Unlike bacteria, red cells are freely permeable to glycol, as indeed they must be to all such protective agents (Lovelock, 1954). Their membranes would therefore not be exposed to a high concentration gradient of glycol, which is perhaps a contributory cause of damage in bacteria.

OTHER KNOWN PROTECTIVE AGENTS AS AIR DISINFECTANTS

As long as glycerol and the simple glycols were the only compounds known to protect against freezing damage, their action as air disinfectants could be dismissed as a coincidence. Recently, however, three quite different kinds of compound have been shown to protect: dimethyl sulphoxide (Lovelock & Bishop, 1959), pyridine *N*-oxide (Nash, 1961) and four mono- and di-*N* substituted amides (Nash, 1962*b*). The last-named compounds are rather too volatile for testing as air disinfectants, but dimethyl sulphoxide, tetramethylene sulphoxide and pyridine *N*-oxide were tested and found to give high rates of kill against the micrococcus at R.H. 40 % and also to a somewhat lesser extent at R.H. 28 %. The experiments were carried out in a smaller chamber, of only 1400 l. volume, and the quantities of disinfectant used are not directly comparable to those used in the main series because of the different surface to volume ratio of the chamber. However, the slit sampler plates showed the typical cut-off of bacterial growth with a slightly swept-back radius which is given by very fast air disinfectant action (Bourdillon, Lidwell & Lovelock, 1948*b*). This is unprecedented behaviour for non-hydroxylic compounds, and is strong evidence in favour of the foregoing arguments. Not all amides are protective agents, and a useful check on the correlation between protection against freezing damage and air disinfectant action was provided by 2-pyrrolidone, which is not a protector although it penetrates red cells (Nash, 1962*b*). The compound is undoubtedly of the right volatility, but was not effective in air against the micrococcus, up to concentrations at which it became inhibitory on the plate. For 2-pyrrolidone, S is 1/6; for dimethyl sulphoxide, tetramethylene sulphoxide and pyridine *N*-oxide it is 4/4, 3/6 and 6+/6 respectively.

Formamide, another non-protector (Lovelock, 1954), was also found inactive as an air disinfectant, but this result is not of great significance since the compound is rather too volatile.

Changes in the chemical constitution of active compounds

A compound which is both a protective agent against freezing damage and an air disinfectant at low R.H. may be altered in various ways so as to lose its activity. For ethylene glycol, either polymerization or the introduction of an alkyl chain or other fairly large substituent is sufficient. As regards polymerization, diethylene

glycol is still a good protective agent (Lovelock, 1954) and a good air disinfectant at low R.H. (Table 1), but triethylene glycol is only partly protective (Lovelock, 1954) and is also much less bactericidal *in vitro* than, for example, propylene glycol (Robertson *et al.* 1948). Its behaviour as an air disinfectant is complicated by its persistence in air as droplets as well as vapour (Nash, 1951), but there are indications that its maximum rate of kill is less than that of the simpler glycols (Lidwell & Lovelock, 1948).

The effect of the introduction of a substituent into the molecule of ethylene glycol is clear from Table 1. Monobutyrin, 1,2 octane diol and α-phenyl glycol have all reverted to the 'ordinary' type of air disinfectant, almost inactive at low R.H. At high R.H., on the other hand, they are more bactericidal than the simple glycols, probably because they are surface-active; the latter property would also make them unsuitable as protective agents.

Dibutyl sulphoxide and di-*N*-propyl acetamide were also tested as air disinfectants and found inactive at low R.H. These two negative results are considered significant since the vapour pressure of each compound should be in a much more favourable range for air disinfection than that of the parent compounds, dimethyl sulphoxide and *N*-dimethyl acetamide.

CHEMICAL CONSTITUTION

The dative oxides and the dialkylamides are Lewis bases, i.e. strong electron-pair donors which can co-ordinate active hydrogen strongly to form hydrogen bonds (Coulson, 1957). It is suggested that activity in both the fields under discussion is due to these lone-pairs of electrons and the strong attraction they have for the hydrogen of water. Other Lewis bases of suitably low vapour pressure for air disinfection are *N*-methyl pyrrolidone (note failure of parent compound, above), *N*-dimethyl glycolamide and trimethyl phosphine oxide. These three compounds should also be protective agents against freezing damage, as long as they remain in the liquid state. It must be remembered, however, that otherwise irrelevant geometric factors may so stabilize the crystal of any of these compounds (e.g. trimethyl phosphine oxide, m.p. 137° C.) that the full hydrophilic properties of its polar group may not be realized. These properties are however revealed in the higher homologues with lower melting-points (Nash, 1962*a*).

SUMMARY

Most air disinfectants fail at low relative humidity. Those which remain active appear to be the same compounds which, in other circumstances, can protect living cells against the lethal damage caused by freezing to low temperatures and then thawing. The compounds are either simple glycols, whose respective activities in the two fields are already known, or the weaker Lewis bases, whose air disinfectant properties have not hitherto been investigated. It is suggested that both freezing protection and bactericidal action are due to the special solvent properties of these compounds, which come into play when most of the water usually present has been removed, by freezing or by drying respectively.

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REFERENCES

BOURDILLON, R. B., LIDWELL, O. M. & LOVELOCK, J. E. (1948*a*). Studies in air hygiene. *Spec. Rep. Ser., med. Res. Coun., Lond.*, no. 262. H.M.S.O.

BOURDILLON, R. B., LIDWELL, O. M. & LOVELOCK, J. E. (1948*b*). Technique of bacterial killing tests. Studies in air hygiene. *Spec. Rep. Ser., med. Res. Coun., Lond.* no. 262, p. 54, H.M.S.O.

BRONSTED, J. N. (1938). Solubility relations of high molecular weight substances. *C.R. Lab. Carlsberg* (Ser. chim.), **22**, 99.

COULSON, C. A. (1957). The hydrogen bond—a review of the present position. *Research, Lond.*, **10**, 149.

LESTER, W., ROBERTSON, O. H., PUCK, T. T., WISE, H. & SMITH, M. (1949). The rate of bactericidal action of triethylene glycol vapor on micro-organisms dispersed into the air in small droplets. *Amer. J. Hyg.* **50**, 175.

LIDWELL, O. M. & LOVELOCK, J. E. (1948). Certain general features in air disinfection by chemicals. Studies in air hygiene. *Spec. Rep. Ser., med. Res. Coun., Lond.*, no. 262, p. 147. H.M.S.O.

LIDWELL, O. M., LOVELOCK, J. E. & RAYMOND, W. F. (1948). Studies in air hygiene. *Spec. Rep. Ser., med. Res. Coun., Lond.* no. 262, p. 75. H.M.S.O.

LOVELOCK, J. E. (1953). The haemolysis of human red-blood cells by freezing and thawing. *Biochim. biophys. Acta*, **10**, 414.

LOVELOCK, J. E. (1954). The protective action of neutral solutes against haemolysis by freezing and thawing. *Biochem. J.* **56**, 265.

LOVELOCK, J. E. & BISHOP, M. W. H. (1959). Prevention of freezing damage to living cells by dimethyl sulphoxide. *Nature, Lond.*, **183**, 1394.

MORGAN, W. T. J. (1937). The isolation and properties of a specific antigenic substance from *B. dysenteriae* (Shiga). *Biochem. J.* **34**, 2003.

NASH, T. (1951). Physical aspects of air disinfection. *J. Hyg., Camb.*, **49**, 382.

NASH, T. (1961). Prevention of freezing damage to living cells by pyridine *N*-oxide. *Nature, Lond.*, **192**, 360.

NASH, T. (1962*a*). The solubility of organic non-electrolytes in water. (To be published.)

NASH, T. (1962*b*). The chemical constitution of compounds which protect living cells against freezing damage. (Submitted for Publication.)

PUCK, T. T. (1947). The mechanism of aerial disinfection by glycols and other chemical agents. *J. exp. Med.*, **85**, 729.

ROBERTSON, O. H., APPEL, E. M., PUCK, T. T., LEMON, H. M. & RITTER, M. H. (1948). A study of the bactericidal activity *in vitro* of certain glycols and closely related compounds. *J. infect. Dis.* **83**, 125.