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Comparative toxicity of various ozonized olefins to bacteria suspended in air

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SUMMARY

Air containing olefin vapour was treated with known amounts of ozone simulating natural concentrations. The bactericidal effect of the mixture was tested using microthreads sprayed with washed cultures of *Escherichia coli* var. *communis* or *Micrococcus albus*, aerosol strain. With 20 different olefins a wide range of activity was found, those in which the double bond formed part of a ring being the most bactericidal; petrol vapour was about as active as the average open-chain olefin. The two organisms behaved similarly at the experimental relative humidity of 80 %. The estimated amount of bactericidal substance present was only about one hundredth of that required to give the same kill with a 'conventional' air disinfectant; a simple physical explanation is proposed for this enhanced effect.

INTRODUCTION

Rural air often contains a bactericidal component, present in such low concentration that direct chemical identification has not yet been possible (Druett & May, 1968). For convenience it has been called the Open Air Factor (OAF) and indirect evidence suggests that it arises from chemical reaction between ozone and olefins (Druett & Packman, 1968; Druett & May, 1968). Olefins are present in the open air because of the widespread dissemination of petroleum products, while ozone is a natural constituent of clean air. OAF is presumed to form whenever such clean upper air is brought down by turbulence and mixes with polluted lower air. This paper describes laboratory experiments on the bactericidal properties of air initially containing about 1 part per million of olefin vapour and about 1 part per hundred million of ozone, the latter concentration being comparable to that found naturally.

EXPERIMENTAL

OAF was discovered using the microthread technique, in which bacteria are held on spider escape line wound across a metal frame (May & Druett, 1968). In this way organisms are recoverable after exposure to open air for any required length of time. The same technique was used in the present work, the bacteria being exposed to the atmosphere inside a closed box. Bacteria on threads do not behave exactly as though they were free-floating, but the differences are not important in the present investigation of the comparative bactericidal activity of different atmospheres.

Organisms

Two test organisms were used. The first (EC) was *Escherichia coli* commune (MRE strain 162), a robust organism used in most of the open-air tests already mentioned. The second (MA) was *Micrococcus albus*, aerosol strain (N.C.T.C. 7944), which does not form large clumps and had been used before in tests of air disinfectants (Nash, 1962). The marker organism (BG) was *Bacillus subtilis* var. *niger* spores, also as used in the earlier Porton tests. The use of BG as a marker has been thoroughly investigated (Anderson, 1966; May & Druett, 1968).

Preparation and assay of microthreads

Microthreads on frames were infected by loading into a tubular brass manifold down which could be passed a concentrated bacterial cloud from a Collison spray. The spray pot contained a distilled water suspension of 2–3 $\times 10^8$ EC or MA and 1–2 $\times 10^8$ BG per ml. The cloud was somewhat diluted by bleeding in dry air until the relative humidity (R.H.) was near that of the experiment, generally 80%. For assay, groups of three frames were dropped into 12 ml. of phosphate buffered sucrose/alginate solution, and after dilution this was plated on agar containing hydrolysed casein as nutrient.

Exposure chamber

Bacteria on microthreads were exposed in a rectangular welded aluminium box 4 ft. high, 3 ft. wide and 2 ft. deep, of volume 670 l. A removable panel occupied part of the front face, leaving a rectangular hole 29 in. wide by 13 in. high. The box was stiffened by angle aluminium around this opening in order that the panel should fit well. Below it there was a row of regularly spaced circular holes 1 in. in diameter, each closed by a cork. A narrow metal tube projected centrally through each cork to a depth of 4 in. inside the box, for holding the frames. In this way any number of frames up to twelve could be exposed to the box atmosphere at the same time, as long as required, and then withdrawn and tested for viable bacteria.

At the side of the box there was a small ozone generator consisting of a cylindrical 2-l. tin containing five Philips OZ4 lamps connected in series with each other and with a 100 W. filament bulb outside as a ballast resistor. An electronic timer allowed mains voltage to be placed across bulb and lamps for accurate periods of 1, 2, 3, 4 or 5 sec. Oxygen from a cylinder could be passed through the tin and into the box, at such a rate that 95% of the ozone was swept through in 25 sec., after the lamps had been switched off.

Olefin vapour was introduced through one of the 1 in. holes by means of a calibrated syringe. The air inside the box was stirred by a large slow-speed fan at the top; using a Kata thermometer it was found that the air velocity past the frames was 10 to 15 cm./sec. The humidity inside the box could be increased by hanging up a damp cloth, and removing it when experience indicated. Relative humidity inside the box was measured by a polymer-film resistor on a separate small removable panel at the side. After an experiment the box was ventilated by removing

the front panel and passing in a length of 6 in. trunking connected to the laboratory air-conditioning system.

Air quality

The concentration of ozone used in the experiments was no greater than is often found naturally in rural air. Such ozone was not removed by the laboratory air-conditioning system, to any great extent, so that it was essential to remove it in some way from the box air before an experiment. On the other hand, when the outside air was polluted, the ozone might well have been used up leaving an excess of pollutants capable of reacting with more ozone if it was added. In order to deal with both of these contingencies, every experiment was preceded by a clean-up, as follows.

Test procedure

The air in the box was humidified as described above, and the box closed. Ozone was then injected, using one or two 5 sec. bursts from the lamps, and allowed 20 min. to burn up any reactive hydrocarbons or nitric oxide present. The particular olefin under test was then added, and allowed to stand for another hour. This destroyed residual ozone and also allowed time for the decay of any bactericidal product. The experiment proper was then started by exposing a control set of three frames for 10 min., to the olefin vapour only. After this frames were exposed, also for 10 min. at a time, to the olefin vapour and also to ozone which was injected with mixing at the beginning of the exposure. Successive sets of frames were exposed to increasing amounts of ozone, by switching on the lamps for 2, 4 or twice 5 sec.; the olefin was however nearly always in vast excess and its concentration could be assumed constant. Ten minutes was allowed between exposures in order that the bactericidal effect of the previous 'shot' of ozone should have decayed. The procedure of roughly trebling concentrations of ozone in successive tests also ensured that carrying-over of this kind would be negligible.

Choice of ozone and olefin concentrations

The initial ozone concentration in the box during an experiment was estimated by two methods which gave good agreement. In the first, the whole output of the generator was taken through a solution of colorimetric reagent (Nash, 1967) giving the total dose. In the second, the ozone concentration in the box was estimated (in the absence of olefin) by using a Brewer bubbler and cell (Brewer & Milford, 1960). The actual concentrations of ozone used were known from earlier work to give reasonable kills. The olefin concentration was more or less dictated by the conditions of the experiment. In comparing the effects of exposure to different substances, it was desirable to keep the time of exposure constant. If the various olefins had all been used at some fixed concentration, the rate of generation of bactericidal product would have varied in a gross and arbitrary manner from substance to substance, because olefins differ widely in the rate at which they react with ozone. It was therefore decided to use each olefin at such a concentration that

the ozone half-life in it was always 5 min. In all the experiments, therefore, the concentration of bactericidal substance presumably rose to a maximum during the 10 min. exposure, and then fell away, in a similar manner for all the olefins.

Measurement of ozone–olefin reaction rates

Varying the olefin concentration to suit its reactivity seems a good way of obtaining meaningful comparative results, but it has to be established first that with all of the olefins the required concentration is sufficiently high for the reaction to be pseudounimolecular, and also that the olefin itself is not bactericidal at this concentration. The latter condition was established in the course of the work, through the control exposures, while the former was put on a sound basis by a long series of preliminary experiment on the decay of ozone in the presence of various concentrations of each olefin. A box atmosphere containing 3–5 parts per hundred million (pphm) of ozone was prepared, and a known volume of olefin vapour injected. The decay of the ozone was then followed on the above-mentioned Brewer instrument, and plotted logarithmically. A half-life was then calculated and the experiment repeated once or twice until the 5 min. half-life was bracketed.

With ethylene, propylene and the butenes, gas from a small cylinder was used. With the other olefins, which are liquids at room temperature, a known volume of saturated vapour was injected into the box from a graduated syringe holding excess liquid at the plunger end. Data on vapour pressures were obtained from the *Handbook of Chemistry and Physics* (40th ed.) and when these were not given the vapour pressure of the corresponding saturated hydrocarbon was used, with a small correction for the double bond. With anethole considerable extrapolation was required, owing to its exceptionally low vapour pressure.

Any errors regarding vapour pressure can always be corrected later, as the actual volume of vapour injected is known. As a precaution against the presence of more volatile and reactive impurities, the air in the syringe was blown out a few times before injection. With only one substance was there definite evidence of a change in the composition of the vapour during volatilization. This was cyclopentadiene (not reported in the next section), which gave a spuriously high kill from the first vapour fraction, probably cyclopentene. When this was blown off the kill was quite low.

RESULTS

When the ozone concentration was plotted against time it was found that the die-away in the absence of added olefin was quite slow, with a half-life of 4 hr. As it was intended to add sufficient olefin to reduce the half-life to 5 min., such background die-away could be ignored. In the presence of olefin, the die-away followed the unimolecular law quite well in most cases, but with the conjugated ethers (ethyl vinyl ether, butyl vinyl ether and dihydropyran) there was considerable 'tailing'. It was always possible to define a half-life for the purpose of the bactericidal experiments, but only when the die-away showed a good straight logarithmic plot was it possible to calculate reaction velocity constants. When this was done they were found to be in broad agreement with those available in the literature;

different workers by no means agreed among themselves, however (Leighton, 1961; Bufalini & Altshuller, 1965).

Olefin concentrations required to give an ozone half-life of 5 min. are listed in Table 1, and were calculated as described above; the actual volumes of vapour injected into the box are also given.

Table 1. *Concentrations in parts per million of olefins required to give an ozone half-life of 5 min., at 19–22° C.*

(Figures in parentheses are ml. of saturated vapour added to 670 litres reaction volume.)

Terminal olefins			Internal olefins		
Ethylene	70	(45)	*Cis* 2-butene	0·7	(0·45)
Propylene	5	(3·5)	*Trans* 2-butene	0·3	(0·20)
1-butene	7	(4·5)	*2-pentene	0·8	(1·0)
1-pentene	10	(13)	*2-hexene	1·2	(4)
1-hexene	14	(45)	*3-heptene	2·0	(20)
Oxygen compounds			**Miscellaneous**		
Ethyl vinyl ether	0·3	(0·5)	Cyclopentene	0·15	(0·3)
Butyl vinyl ether	0·3	(3)	Cyclohexene	2·0	(12)
1,2 dihydro-pyran	0·25	(2)	Cycloheptene	0·8	(10)
Anethole	0·25	(2400)	2-Me-2-butene	0·5	(0·45)
Crotyl alcohol	0·7	(70)	2,4,4 trimethyl 2-pentene	2·0	(25)

* Commercial mixtures of *cis* and *trans*.

Main series of olefins

The results of the bactericidal tests are given in Table 2. The EC/BG or MA/BG ratio after exposure to olefin alone was taken as the baseline throughout, varying from experiment to experiment between 1 and 4. The ratio in the spray solution was also found, by plating out a sample before the experiment, and it was found that never less than 50% of either MA or EC survived the process of spraying, collection on microthreads, exposure to olefin and rehydration in phosphate buffered sucrose/alginate solution. At lower relative humidities EC does not survive so well (see below), but on the whole it is clear that frames prepared in this way make a satisfactory test vehicle for the population being tested.

Effect of relative humidity

The main series of experiments was done at a high R.H. because most air disinfectants show maximum activity in this region (Nash, 1962). Some experiments were also done however with one olefin, cyclohexene, over a range of humidities. As expected, there was good survival in olefin vapour alone throughout the R.H. range for MA, but poor survival at the low end for EC, down to 20% of the spray solution ratio at R.H. 40%. As in the main series, however, survival after exposure to olefin vapour alone, whatever the R.H., was taken as the baseline, and the results are plotted in Fig. 1.

Table 2. *Survival of microorganisms on microthreads after exposure to ozone–olefin mixtures.*

(Olefin concentrations are as in Table 1. Initial ozone concentrations are in parts per hundred million (p.p.h.m.). Ten minute exposures at 19–22° C., relative humidity 76–79 %. Figures are percentage of the survival when exposed for the same time to olefin alone; the first figure is for *Micrococcus albus*, the second for *Escherichia coli*.)

Olefin	Initial ozone concentration, p.p.h.m. 0·4	1·1	3·3
Cycloheptene	10, 10	4, 6	—
Methyl cyclopentene	15, 10	7, 4	5, 3
Cyclopentene	25, 15	6, 6	3, 2
Dihydropyran	25, 35	2, 6	3, 3
Anethole	45, 55	4, 7	3, 2
Cyclohexene	45, 35	15, 7	2, 2
Crotyl alcohol	65, 55	15, 10	2, 5
2-hexene	65, 70	15, 20	5, 5
2-pentene	45, 80	10, 50	5, 10
Petrol, top	95, 90	30, 35	10, 10
1-pentene	80, 65	50, 20	10, 5
Trans 2-butene	70, 85	45, 30	10, 10
3-heptene	80, 90	25, 45	10, 35
Petrol, bottom	100, 70	50, 30	15, 20
Propylene	80, 70	55, 70	10 ,20
1-hexene	85, 75	50, 70	15, 20
1-butene	90, 90	90, 35	50, 35
cis-2 butene	*	75, 75	40, 20
2-methyl 2-butene	*	80, 75	20, 55
Vinyl butyl ether	*	100, 70	50, 45
Vinyl ethyl ether	*	*	25, 30
2,4,4 trimethyl pentene	*	*	*

* Not significantly different from 100.

DISCUSSION

The olefins listed in Table 2 are placed roughly in order of activity, averaging figures for the two organisms. A limitation of the microthread technique as used here is that about 2 % of the organisms are deposited on the frames, where they are to a large extent protected against toxic vapours. Survivals of 2 or 3 % may therefore indicate considerably greater kill on the microthreads themselves.

It can be seen first of all that there is perhaps a rather poor correlation between reactivity with ozone (Table 1; least p.p.m. denotes greatest reactivity) and bactericidal effect. The most significant correlation is probably that between structure and activity, in that all the ring olefins are at the top of the table with anethole, which is also a ring compound although the olefinic portion in this case is outside. This correlation fits in with what is known about the ozone–olefin reaction, together with the well-established principle that a good air disinfectant must have a very low vapour pressure (Nash, 1951).

Attack by ozone splits the double bond, one end becoming ketone or aldehyde

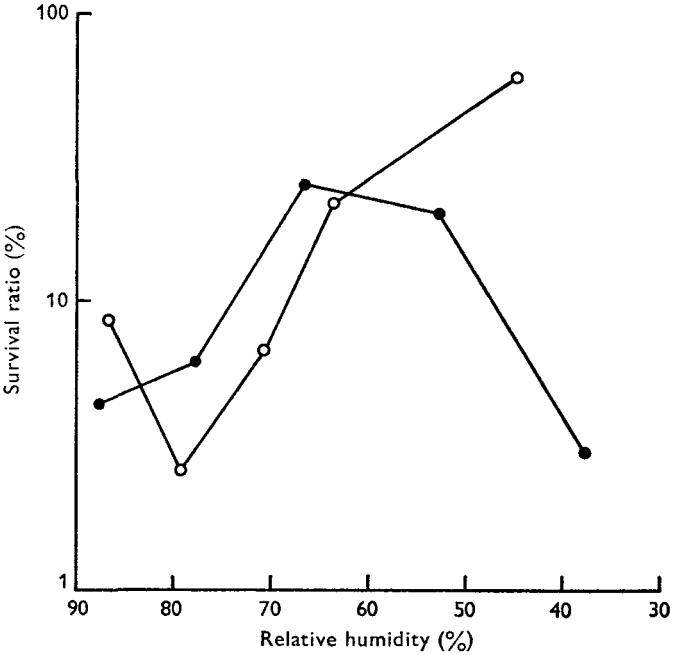

Fig. 1. Survival on microthreads exposed to mixtures of ozone (1·1 parts per hundred million) and cyclohexene (2 parts per million) mixtures for 10 min. at different relative humidities. ●, *Escherichia coli* strain 162; ○, *Micrococcus albus*, N.C.T.C. 7944. Both organisms sprayed in distilled water. Survivals are expressed as percentages of the survivals of the organisms exposed to cyclohexene vapour alone for the same length of time.

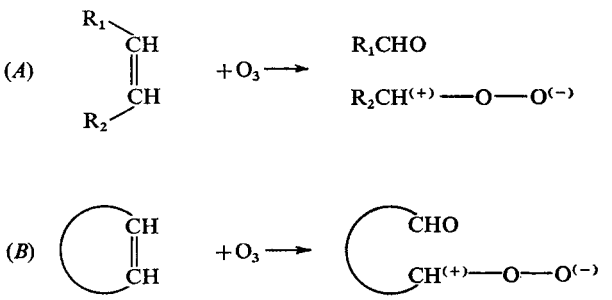

Fig. 2

and the other a peroxide 'zwitterion', this being probably the active fragment (Fig. 2). Comparing two olefins of the same molecular size, one a ring and the other an open chain, it is clear that the active fragment from the open chain olefin will be smaller and have a higher vapour pressure than the product from the ring olefin, where the molecule cannot be split into two separate portions.

Chemical constitution must not be neglected, but there is no point in drawing any further inferences at this stage without knowing the exact course of the ozone reaction in each case. For instance, it is difficult to see why the two butenes should differ so much in activity, when the products of ozonation should be the same.

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It is possible that recombination of the fragments to form true ozonides, which occurs at high concentration, also occurs at low concentration and is critically dependent on constitution. There is scope here for a more detailed investigation of selected olefins.

There is one further aspect of the results which deserves some discussion, that is the concentration at which the ozone–olefin reaction products are bactericidal in comparison with the concentrations at which known air disinfectants are bactericidal. Taking resorcinol as an example, the amount required for a good kill is about 100 times the amount of ozone, if a suitable olefin is present. There is a simple physical explanation of this, going back to the known behaviour of triethylene glycol (Nash, 1951). When this substance is vaporized from a hot plate, for air disinfection tests, it condenses again to a cloud of droplets, because the vapour pressure is exceptionally low. Its (rather poor) bactericidal effect is mainly due to vapour slowly distilling from these droplets onto the bacteria-carrying particles. It is easy to imagine that with compounds of still lower vapour pressure bactericidal action will get less and less, from the same quantity of material, because the rate of production of vapour from the condensed aerosol will get less and less and finally be so small that aerial disinfectant activity will be reduced to zero. A substance whose vapour pressure is a hundred times less than that of resorcinol may well be active at a 100-fold less concentration of vapour, but this concentration would never be approached in practice because of aerosol formation immediately after vaporization. The ozone–olefin reaction products, on the other hand, are produced initially as single molecules in the very dilute gas phase, with the maximum chance of condensation on to bacteria-carrying particles, or any other surface in the neighbourhood.

There is no need, therefore, to postulate exceptional bactericidal activity on the part of these products, but merely exceptionally favourable physical circumstances.

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