Studies on the Germicidal Action of Ozone

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Ozone is known as the most powerful oxidizing agent practically used in water treatment [7, 3]¹⁾. Its bactericidal properties, however, have hardly been investigated on a quantitative scale, although its action is qualitatively well established. INGRAM and BARNES [5], in a review of the literature, clearly showed the wide divergence of opinion held by various investigators with respect to ozone concentrations necessary for rapid bactericidal action.

In 1955, WUHRMANN and MEYRATH [8] were able to follow quantitatively the action on various organisms including *Escherichia coli* and spores of *Bacillus megatherium*. Survivor curves were obtained with ozone concentrations as low as 0.6 µg/l thus giving clear indications of the extremely high bactericidal properties of aqueous ozone solutions.

In the present paper additional experiments are described. It has been the aim to extend our knowledge of the germicidal power of ozone by testing organisms of rather differing physiological properties. Strains from a variety of taxonomic groups of microorganisms have therefore been selected and investigated with respect to their ozone tolerance.

1. Material and Methods

1.1 Test Organisms

Test organisms were grown under the conditions summarized in Table 1. Prior to testing with ozone, they were centrifuged and washed twice with sterile, distilled water. In the case of *M. tuberculosis*, the surface growth was

¹⁾ Numbers in brackets refer to References, page 122.

washed off, emulsified and filtered through sterile cotton wool before centrifugation. Spores of *Penicillium* were harvested in a similar manner. After re-suspension of the organisms, dilutions were made to bring the number of cells to approximately 10^7 per millilitre (microscopic cell count). From the adjusted cell suspensions 1-ml inocula were used to contaminate 850-ml batches of ozonated water.

Table 1

Growth Conditions for Test Organisms

Organism ¹⁾	Growth medium	Incubation temperature	Incubation time
<i>Escherichia coli</i>	lactose broth tryptone broth	37°	16 hours
<i>Streptococcus faecalis</i>		37°	16 hours
<i>Mycobacterium tuberculosis</i> var. <i>avium</i>	glycerol agar	37°	5 days
Wine yeast	beer wort agar	22°	7 days
Sulphite yeast			
<i>Torula rosea</i>			
<i>Rhodotorula gracilis</i>			
<i>Penicillium notatum</i>			

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1.2 Preparation of Ozonated Water

In accordance with the method used in previous experiments [8], a continuous stream of ozonized air, maintained throughout each experiment, was dispersed in one-litre batches of double-distilled water by means of a vibrator. In all experiments the pH was adjusted to 7.0 by addition of 0.01 *M* sodium bicarbonate to the water and supplementing the ozonized air stream with sufficient carbon dioxide gas. Temperature was kept constant by immersion of the test flask in a thermostat-controlled bath.

The ozone concentration was determined colorimetrically by adding 50 ml of the ozonated water to 2.5 ml o-tolidine reagent, the optical density of the resulting yellow solution being immediately measured with a Beckman-DU-spectrophotometer at a wavelength of 440 μ [6]. The concentration was calculated by means of the following equation:

$$c = \frac{1}{3.7 \times 10^4} \cdot \frac{E}{d}, \quad c' = 1.30 \times 10^3 \frac{E}{d}.$$

c concentration in mol/l; c' concentration in $\mu\text{g/l}$; E optical density; d depth of solution in centimetres.

Concentrations in the range of 0 to 250 $\mu\text{g/l}$ could thus be determined with an accuracy of $\pm 1 \mu\text{g/l}$.

Three ozone samples were taken from each batch of water before contamination with a test suspension. A fourth and occasionally a fifth sample were also withdrawn approximately 3 and 10 minutes after contamination.

1.3 Assessment of Germicidal Action

After contamination of the continuously ozonated water, 1-ml samples were withdrawn at short intervals, immediately transferred to tubes containing 1 ml 10^{-4} N sterile thiosulphate solution and finally poured into plates with the proper medium. In the case of mold spores, serial dilutions of the samples were carried out in order to reduce the number of colonies per plate below 500. Plate counts were made with help of a magnifying lens. Media employed for plating, as well as incubation times and temperatures are stated in Table 2.

The number of viable organisms originally present in the suspensions was determined by pouring two or three plates with samples from duplicate experiments without ozone. Plate counts of the ozone-treated samples were expressed as percentages of the counts obtained from unozonized samples.

Table 2

Culturing Conditions for Ozone-Treated Cell Suspensions

Organism	Culture medium	Incubation temperature	Incubation time
<i>E. coli</i>	nutrient agar	37°	48 hours
<i>Str. faecalis</i>	tryptose agar (Difco)	37°	48 hours
<i>M. tuberc.</i> var. <i>avium</i>	glycerol agar	37°	4 days
Yeasts etc.	beer wort agar	22°	3 days
Fungus spores	beer wort agar	22°	2 days

Figure 1: Survivor curves for *E. coli*. $t = 10^{\circ}$, $\text{pH} = 7.0$. Ozone concentrations: 1, $6 \mu\text{g/l}$; 2, $12 \mu\text{g/l}$.

Figure 2: Survivor curves for *Str. faecalis*. (Experiment a). $t = 10^{\circ}$, $\text{pH} = 7.0$. Ozone concentrations: 1, $4 \mu\text{g/l}$; 2, $10 \mu\text{g/l}$; 3, $18 \mu\text{g/l}$.

Figure 3: Survivor curves for *M. tuberc.* var. *avium*. $t = 20^{\circ}$, $\text{pH} = 7.0$. Ozone concentrations: 1, $8 \mu\text{g/l}$; 2, $16 \mu\text{g/l}$; 3, $27 \mu\text{g/l}$.
 Figure 4: Survivor curves for wine yeast 'Champagne'. $t = 20^{\circ}$, $\text{pH} = 7.0$. Ozone concentrations: 1, $2 \mu\text{g/l}$; 2, $10 \mu\text{g/l}$; 3, $16 \mu\text{g/l}$; 4, $47 \mu\text{g/l}$.

Figure 5

Figure 6

Figure 5: Survivor curves for *Str. faecalis*. (Experiment b). $t = 10^{\circ}$, pH = 7.0. Ozone concentrations: 1, 5 $\mu\text{g/l}$; 2, 13 $\mu\text{g/l}$.Figure 6: Survivor curves for *Penicillium notatum* (spores). $t = 20^{\circ}$, pH = 7.0. Ozone concentrations: 1, 55 $\mu\text{g/l}$; 2, 90 $\mu\text{g/l}$; 3, 200 $\mu\text{g/l}$; 4, 340 $\mu\text{g/l}$.

Figure 7

Concentration/time relation for 99.99% kill. 1, *E. coli* at 10°; 2, *Str. faecalis* at 10°; 3, *M. tuberc. var. avium* at 20°; 4, wine yeast 'Champagne' at 20°; 5, *Penicillium notatum* (spores) at 20°.

2. Results

2.1 Fluctuations in Ozone Concentration During Tests

Since steadiness of the ozone concentration during each experiment was a prerequisite for the quantitative evaluation of the germicidal action, ozone levels were carefully checked during the tests. Analytical figures from some of our experiments (i.e. ozone concentrations before and after contamination of the continuously ozonated batches) are given in Table 3, together with the presumed average concentrations deduced from the analytical data.

As shown by the figures in Table 3, it is possible to obtain ozone levels of sufficient stability within a wide range of ozone concentrations. It is necessary, however, to use double-distilled water and well-washed cell suspensions with little dead material in order to avoid pronounced fluctuations.

Table 3

Ozone Concentrations in Some Experiments with Continuous Ozonation

Experiment	Ozone concentration at various sampling times (o-tolidine method)				Presumed average concentration $\mu\text{g/l}$
	5 minutes before contamination	3 minutes before contamination	3 minutes after contamination	10 minutes after contamination	
	$\mu\text{g/l}$	$\mu\text{g/l}$	$\mu\text{g/l}$	$\mu\text{g/l}$	
A	2.2	1.9	2.2	2.3	2
B	9.8	9.8	9.1	8.8	9
C	11	11.5	12.2	12.0	12
D	15	16.2	16.5	—	16
E	390	402	390	396	395

2.2 Survivor Curves

In plotting the logarithms of survival percentages against ozone contact time, linear relations are revealed for all *vegetative cell* suspensions (see Figures 1 to 4). Deviations from linearity, as noted in some experiments with *Str. faecalis* (Figures 2 and 5 resp.) are probably attributable to secondary factors or experimental errors, such as irregular dispersion of cells (partial clumping) in the test suspension.

Spores of Penicillium notatum displayed a marked 'short-time' resistance towards ozone (Figure 6). After a lag phase—depending on ozone concentration—the usual linear relationship was exhibited.

Preliminary tests were also performed with various yeast strains (sulphite and wine yeasts, *Torula*, *Rhodotorula*) and suggested a more or less similar sensitivity of these organisms to that displayed by the wine yeast '*Champagne*' (Figure 3).

2.3 The Concentration versus Contact Time Relation

The available data do not contradict the assumption that there is a linear dependence between the logarithm of contact time (for a specified percentage of kill, e.g. 99.99%) and the logarithm of ozone concentration (Figure 7). It can therefore be assumed that

$$c^n t_k = \text{const.}$$

c ozone concentration; n concentration exponent; t_k contact time for specified percentage of kill.

The present findings indicate a n -value of approximately 1.0 for all organisms tested with the exception of *Str. faecalis* where n equals 0.45.

In comparing ozone with other disinfectants, as well as in estimating the ozone tolerance of various organisms, death rate constants and contact times for specified killing rates are of interest. It should be pointed out, however, that differences in order of magnitude of such figures rather than small differences in absolute values are significant. In Table 4 contact times for 99.99% kill at three arbitrarily chosen ozone concentrations are given, together with some death rate constants K , calculated from the linear parts of the survivor curves according to the equation

$$K = \frac{\log z_1 - \log z_2}{t_2 - t_1}.$$

K death rate constant (decimal logarithms); z percentage of survivors; t contact time

Table 4

Estimated Contact Times for 99.99% Kill
at Selected Ozone Concentrations and Death Rate Constants

Organism	Temperature °C	Ozone concentration					
		10 µg/l		30 µg/l		100 µg/l	
		t min	K min ⁻¹	t min	K min ⁻¹	t min	K min ⁻¹
<i>E. coli</i>	10	1.5	2.2	0.5 ¹⁾	—	0.2 ¹⁾	—
<i>Str. faecalis</i>	10	3	1.4	1.7	2.4	1 ¹⁾	—
Wine yeast ' <i>Champagne</i> '	20	2.5	1.6	0.8	5	0.2 ¹⁾	—
<i>M. tuberc. var. avium</i>	20	10	0.4	3	1.4	0.7 ¹⁾	—
<i>Pen. notatum</i> (spores)	20	150 ¹⁾	—	50 ¹⁾	—	10	0.3

¹⁾ Extrapolated values (outside experimental range).

In comparing the ozone sensitivity shown by various organisms (the temperature effect being taken into account), the following order with respect to increasing resistance may be established:

E. coli < *Str. faecalis* < wine yeast '*Champagne*' < *M. tuberc. var. avium* < *Penicill. notatum* (spores).

3. Discussion

The present findings agree with earlier results from our laboratory [8] as to the extremely high bactericidal activity of ozone solutions. They give further evidence of the rate of kill which can be expected for a number of

organisms. The control experiments with *E. coli* at 10° showed some minor deviations from earlier results. For instance, this investigation indicated a K -value of 2.2 min^{-1} at $10 \mu\text{g/l}$ ozone, while data from WUHRMANN and MEYRATH [8] lead to $K = 4.4 \text{ min}^{-1}$ at $10 \mu\text{g/l}$ and 12°. Such differences do not seem to be significant, as they can easily be attributed to experimental variations, such as difference of strain, age of inoculum, temperature and ozone analytics. Furthermore, in the previous investigation a nonlinear relation between the logarithm of survivals and the contact time was observed at very low ozone concentrations (upward convex at $2.3 \mu\text{g/l}$ or less ozone). We tend to explain this deviation from linearity as due to a probable, short-time drop in ozone level at the moment of contamination of the ozonated water.

The *Penicillium* spores behaved in a similar manner to the *B. megatherium* spores referred to in the earlier paper, although no initial stimulation of germination could be detected with fungus spores. Quite generally, an initial lag in ozone action seems to be characteristic for spores.

Results published by other investigators vary quite markedly from the present findings. HOLLUTA and UNGER [4] obtained with *E. coli* less than 60% kill within 5 minutes at $100 \mu\text{g/l}$ ozone (in tap water). A complete kill within 1 minute was observed, however, at $120 \mu\text{g/l}$. Such findings seem to be due rather to errors in ozone estimation than to some peculiar bactericidal action. The iodometric method used by these authors is subject to interference by various compounds present in ozonated water. FETNER and INGOLS [1] experimenting with *E. coli* at 1° reported that an initial ozone concentration of 0.53 mg/l would be required to produce a 99% kill in 1 minute. Irregular action with lower ozone concentrations led them to believe that small deviations from a certain critical concentration would give an 'all-or-none-type effect' of ozone within the first minute of contact. Such a claim does not find support by our observations.

Several factors have to be considered in basic investigations on the germicidal properties of ozone. If reproducible results are to be obtained ozone concentrations should be maintained at *steady levels* during experiments and should be determined by reliable analytics. The use of specially distilled water, carefully washed cell suspensions, as well as continuous ozonation seem to be essential.

The relative sensitivity of the tested bacteria, as shown by the distribution of their contact-time/concentration curves, corresponds with their order of tolerance towards several other chemicals. Yeasts do not seem to possess a resistance markedly higher than bacteria. *Penicillium* spores, however,

exhibit a fairly high resistance, similar to that of bacterial spores. Some reports on ozone resistance of yeasts and fungus spores [2,5] suggest that such organisms might exhibit an *extremely* high tolerance of ozone. Such an assumption does *not* seem to be justified in view of the present findings.

Summary

The germicidal action of highly dilute, aqueous ozone solutions has been investigated. Tests were performed with various bacteria and yeasts at ozone concentrations between 2 and 50 $\mu\text{g/l}$, and with fungus spores at concentrations between 50 and 350 $\mu\text{g/l}$. Survivor curves and the respective contact-time/concentration relations suggested the following sequence of test organisms with respect to increasing ozone tolerance: *Escherichia coli* $\ll$ *Streptococcus faecalis* $\ll$ wine yeast ('*Champagne*') $<$ *Mycobacterium tuberculosis* var. *avium* $\ll$ *Penicillium notatum* (spores).

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