INHIBITION OF BACTERIAL GROWTH BY PURE OZONE IN THE PRESENCE OF NUTRIENTS

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(With 5 Figures in the Text)

INTRODUCTION

From time to time the use of ozone has been proposed for the disinfection of air, the sterilization of water, and the inhibition of microbial attack on foodstuffs in storage. There is still, however, doubt as to its efficacy. For instance, Ewell (1938, 1941), has repeatedly stated that concentrations from 0·6 to 3 p.p.m. will inhibit growth of moulds and bacteria on eggs and meat, and similar claims have been made for concentrations as low as 0·1–1 p.p.m. when produced by a special Westinghouse lamp (Anon. 1945); while on the other hand, Cathcart, Wyberg & Merz (1942) found that as much as 4 p.p.m. of ozone failed to reduce appreciably the numbers of Staphylococci and Salmonellae even when suspended *in air* in small numbers, so that they refused to consider its use for foodstuff. Such discrepancies can best be explained by the different circumstances in which results are obtained: for example, by failure to appreciate that, when experiments are made, for example, with ultra-violet lamps, the effects observed may not be due to ozone alone; or that the quantity measured in the atmosphere may not represent that actually applied to the organisms. It is remarkable how little attention seems to have been paid to such points in many statements in the literature.

In these circumstances, it seems desirable to give a complete account of the experiments made here by the late R. B. Haines; for although a summary of his work was published before the war (Haines, 1937), it still remains the most fundamental research in this field.

* The experimental work for this paper was carried out by Dr Haines in 1934–5. Besides a summary which was published, a connected account of all his experiments had been drafted, but its preparation was interrupted by the war and was incomplete when he unfortunately died in December 1943. This paper consists of Haines's description of the experiments (considerably rearranged), with comments on important work which has appeared since. It is a tribute to the memory of one of our foremost food bacteriologists.

The most critical work carried out earlier was that of Heise (1917*a*, *b*), who used a Siemens and Halske ozonizer which gave under certain conditions 0·04–0·06 mg./l. of N_2O_5 together with the ozone; but this he recognized, and the impurity was removed with sodium carbonate solution. Heise sprayed suspensions of organisms on to nutrient gelatine, submitted them to ozonization, and compared the number of colonies developing with controls—in some cases the diameters of the colonies were measured. Heise found that concentrations of ozone varying from approximately 1000 to 2 p.p.m. (by volume) could inhibit development, *provided* that ozonization took place within a short time of inoculation at room temperature, and within 24 hr. of inoculation at 4–5° C. He did not, however, interpret these findings in terms of the growth phases of the organisms.

In Haines's investigation, besides using pure ozone, attention was paid to this last aspect of the matter, and the different effects of admitting the inhibitor during the lag phase, the logarithmic phase, and the maximum stationary phase (Buchanan's nomenclature) were investigated by means of viable counts. Further, Haines recognized that although the process of disinfection by ozone in the absence of nutrients had been exhaustively investigated, little was (and still is) known of the more complex process of inhibition of growth in the presence of nutrients, and he therefore compared the following cases:

(1) The concentration of ozone which destroys bacteria suspended *in water*.

(2) That needed to inhibit growth *in a synthetic medium*.

(3) The concentrations of ozone necessary (*a*) to inhibit growth, (*b*) to destroy the organisms, *in nutrient broth*. (Since the concentration of inhibitor required to arrest growth depends upon the phase in the life history of the culture at which it is admitted, a series of observations was made.)

(4) The concentrations required to inhibit growth of bacteria *on nutrient agar*. (For comparative purposes, a few observations were also made on

moulds.) Comparison of the results for these different media then led to

(5) Investigation of the mechanism of disinfection by ozone.

in a similar flask through which sterile air was passing at a speed comparable with the stream of ozone, were made. The rate of flow of the gases was of the order of 2 to 15 l./hr.

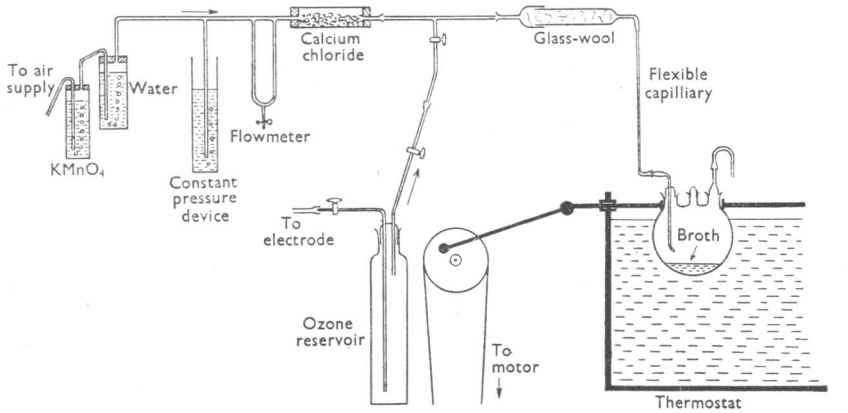

Fig. 1. Diagram of the apparatus.

EXPERIMENTAL METHODS

Pure ozone was prepared by the method of Fischer and Massenez as used by Griffith & Schutt (1921), the procedure of the latter workers being followed exactly in assembling the electrode.* The arrangement of the apparatus is depicted diagrammatically in Fig. 1. Ozone liberated at the anode was collected in a piece of wide glass tubing, the latter being drawn down at one end and sealed to a delivery tube leading to the gas reservoir. All-glass apparatus was used, joints being lubricated with sticky phosphoric acid. Purified dried air from cylinders was admixed with the required proportion of ozone, the mixture passing through a glass-wool filter to the culture-vessel via a flexible capillary allowing mechanical rocking. Any desired concentration of ozone could be obtained by control of the three variables, current density, air pressure, and strength of electrolyte. Analysis of the gas was carried out after the method of Treadwell & Anneler (1905). During a run of a week or more the concentration of ozone fluctuated to some extent, but the limits of variation were estimated by making analyses at intervals of a few hours throughout the run.

In the culture-flasks of about 1 l. capacity, 200 c.c. of nutrient broth pH 7·4 made a shallow layer on the bottom, forming the nutrient fluid in all the experiments except where otherwise stated. It was not possible to bubble the gas through the broth on account of frothing, but by rocking continuously, a steady state could be reached in a few hours. In all cases control experiments, with the organism growing

* Haines was indebted to these workers in the Muspratt Laboratory of Physical Chemistry, University of Liverpool, for advice in assembling the electrode.

In experiments made with the organisms growing on agar, large desiccators containing inverted Petri dishes, lids removed, were placed in the thermostat instead of the culture-flasks, and the gas-mixture containing ozone was passed through them in a similar manner.

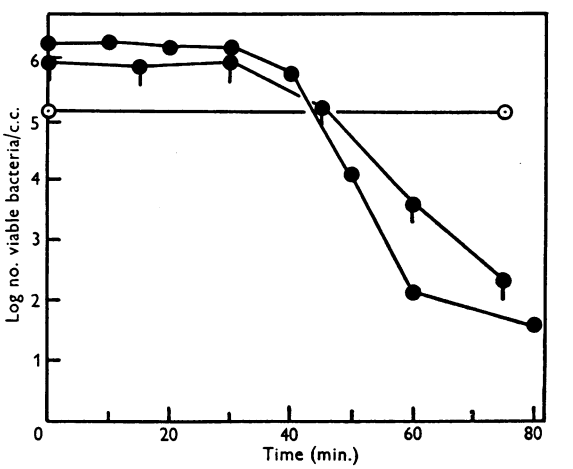

Fig. 2. The death of cells of *Staphylococcus aureus*, suspended in water at 25° C., in the presence of ozone. Ozone and bacteria admitted simultaneously. ⊙, air control; ●, ozone 7 p.p.m. by volume; ●, ozone 21 p.p.m. by volume.

RESULTS

(1) *The disinfection of water*

A suspension of *Staphylococcus aureus* in water was made by transferring two loops of a young culture to 10 c.c. of sterile water, shaking vigorously for some

minutes, and then inoculating 5 drops, from a Pasteur pipette, into 50 c.c. sterile water. Counts were made on this suspension as accurately as possible, the dilutions having been estimated beforehand so that only one was required in the actual experiment. This dilution was made with dropping pipettes individually calibrated by weighing. Ozone in the required concentration was bubbled through the suspension of bacteria, the flask being immersed in a thermostat, and further counts were made at intervals. An example of the figures obtained is shown graphically in Fig. 2. Using a concentration of about 100 p.p.m., a suspension of approximately 10^6 bacteria per c.c. was almost completely sterilized in about 90 min. at 25° C. Various types of curve were obtained for the process of disinfection: in no case was the disinfection strictly logarithmic over the whole of the range from zero time, but often a portion of the curve was logarithmic within the estimated limits of experimental error. With lower concentrations of ozone, rapid sterilization could still be obtained, 21 and 7 parts per million being quite effective. The minimum germicidal concentration for aqueous suspensions of bacteria was not determined.

(2) *Growth in a synthetic medium*

Evidence is given later to show that ozone brings about chemical changes in nutrient broth, rendering it unfit to support bacterial growth. It seemed likely, therefore, that the concentration of ozone required to prevent growth might be smaller in a simple medium containing few or no oxidizable substances. To test this, experiments were made using *Escherischia coli* growing in Nelson's medium (Nelson, 1926), which contains no amino-acids, ammonium succinate being the source of nitrogen. It was found that a concentration of ozone >200 <600 p.p.m., admitted during the logarithmic phase, arrested growth and killed the cells; whereas a concentration as low as 10 p.p.m. was germicidal if admitted simultaneously with the inoculation, even 4 p.p.m. having an inhibitory effect when so applied (Fig. 3).

(3) *Growth in nutrient broth*

A culture of *Staph. aureus*, obtained from the National Collection of Type Cultures, and a culture of *Achromobacter* isolated from slimy beef were selected for the experiments in nutrient media. The former of these organisms belongs to the group rather more sensitive to ozone than the group containing *Achromobacter* and *Pseudomonas*, and the latter had the advantage of growing at low temperatures. A few tests were also carried out with a culture of *Proteus vulgaris* obtained from the Lister Institute.

Inoculation and entry of gas coincident

A selection of the data obtained from the plate counts is shown graphically in Fig. 4. It will be seen at once that much larger concentrations of ozone were needed than in the inorganic media.

Temperature had an important influence. At 20° C., approximately 290 p.p.m. of ozone had a slight inhibitory effect on *Achromobacter*, 350 p.p.m. on the other hand being definitely germicidal, and higher concentrations causing a somewhat more

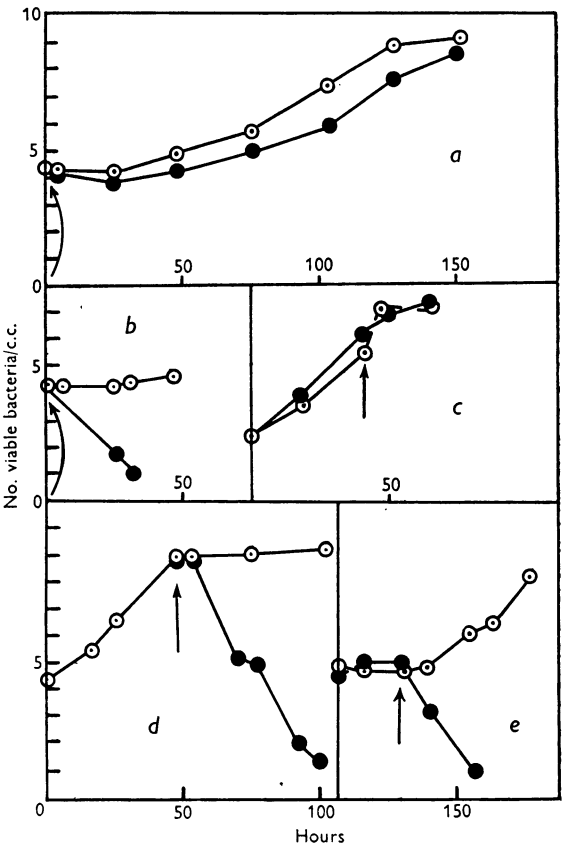

Fig. 3. The effect of ozone on *E. coli* in Nelson's medium at 20° C. (*a*) 4 p.p.m.; (*b*) 10 p.p.m.; (*c*) 190 p.p.m.; (*d*) 680 p.p.m.; (*e*) 715 p.p.m. by volume. The time at which the ozone was first admitted is indicated by the arrows. ⊙, air control; ●, test with ozone.

rapid rate of death. At 0° C., using the same organism, approximately 90 p.p.m. of ozone caused only a slight inhibition of growth, while a concentration of 140 p.p.m. was just germicidal. In other words, a reduction of temperature from 20 to 0° C. allowed the use of about one-third to one-half the concentration of ozone for a similar effect. The solubility of ozone in the broth is unknown, but the difference in solubility of this gas in water between 18 and 0° C. is

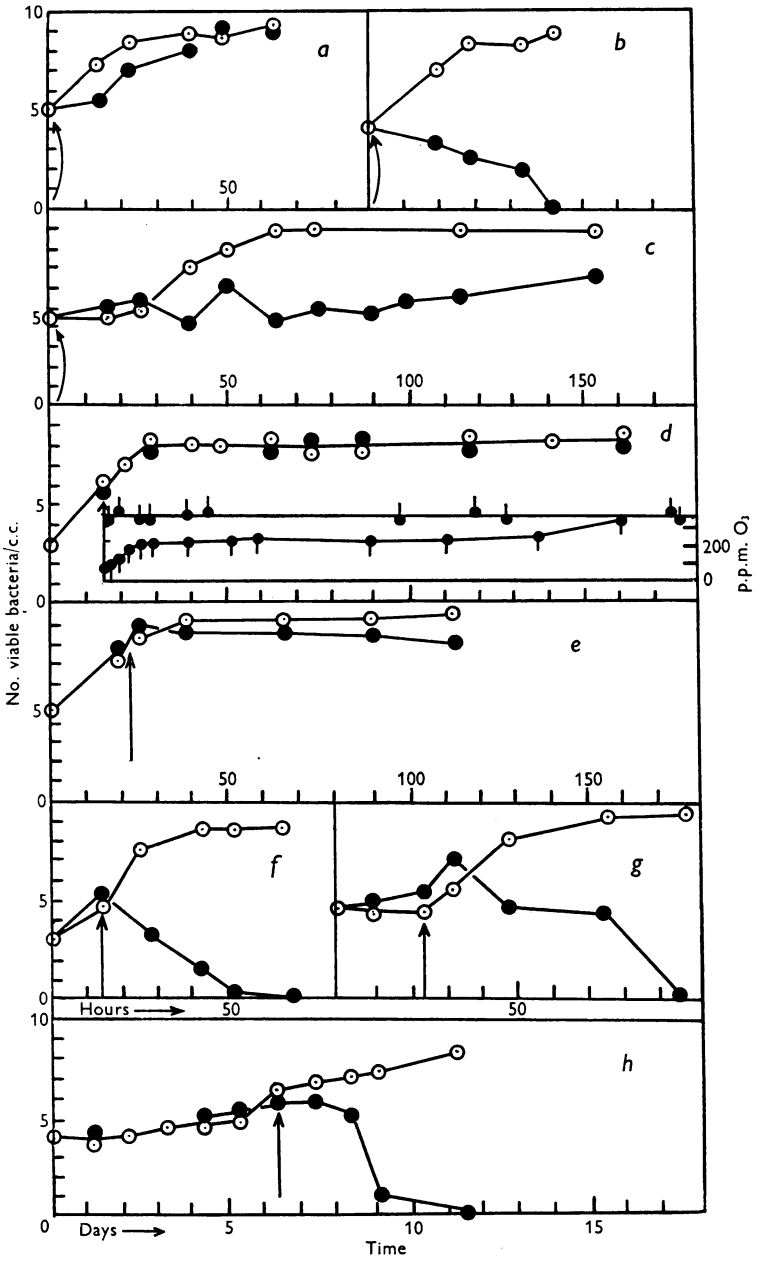

Fig. 4. The effect of ozone on growth of bacteria in nutrient broth. The times at which the ozone was first admitted are indicated by the arrows: ozone and bacteria added together initially (i.e. in lag phase). (*a*) *Achromobacter*, 20° C., ozone 290 p.p.m.; (*b*) *Achromobacter*, 20° C., ozone 350 p.p.m.; (*c*) *Staph. aureus*, 25° C., ozone 280 p.p.m.; ozone applied when growth well established. (*d*) *Achromobacter*, 20° C., ozone 380 p.p.m. (note continued uptake of ozone as shown: ◐, concentration entering; and ●, concentration leaving flask). (*e*) *Achromobacter*, 20° C., ozone 2500 p.p.m.; (*f*) *Achromobacter*, 20° C., ozone 3900 p.p.m.; (*g*) *Staph. aureus*, 25° C., ozone 2600 p.p.m.; (*h*) *Achromobacter*, 0° C., ozone 430 p.p.m. ⊙, air control; ●, air + ozone; ◐, ozone entering flask; ●, ozone leaving flask.

not very marked, so that the effect appears to be more than could be explained on that basis alone.

There was also considerable variation between different organisms. With *Proteus* at 20° C., 240 p.p.m. was almost germicidal, little multiplication of the organisms occurring, while 140 p.p.m. had a definite inhibitory effect. The comparatively greater sensitiveness of *Proteus* is evident. The figures obtained with *Staph. aureus* growing in broth cannot be directly compared with those for *Achromobacter* and *Proteus*, since the former organism was cultured at 25° C. instead of 20° C., in order to facilitate vigorous growth; but even at the higher temperature, a concentration of about 280 p.p.m. greatly retarded (although it did not entirely inhibit) the growth of the *Staphylococcus*, so that this organism, too, appeared to be more sensitive to ozone than the *Achromobacter*.

Ozone admitted subsequent to inoculation

In these experiments both culture flasks were inoculated and the organisms allowed to multiply vigorously before admitting the ozone to one of them. It was first of all shown, by comparative counts made with lots of the same batch of broth, one in a plugged flask and the other with air passing through, that the passage of a stream of sterile air through the shaken culture-vessels had no effect on the rate of growth of the bacteria.

It was then found that a continuous uptake of ozone, apart from that required to saturate the fresh broth, occurred when sublethal concentrations of the gas were passed over growing cultures. In Fig. 5 a typical series of analyses, made both at entry and exit of the flask, is shown. The ozone was admitted 15½ hr. after inoculation, i.e. when the bacteria were in the logarithmic, or most rapidly growing, phase. It will be seen that at 20° C., uptake of ozone continued for at least 100 hr. A similar result was obtained in many other experiments. It seems likely that this decomposition of ozone was due, not to a bacterial enzyme, but to oxidation of reducing substances liberated in the metabolism of the organisms. Two reasons point to this conclusion: first, no marked uptake of ozone was found with water suspensions of the organisms, and secondly little uptake of ozone occurred when the bacteria were growing on agar.

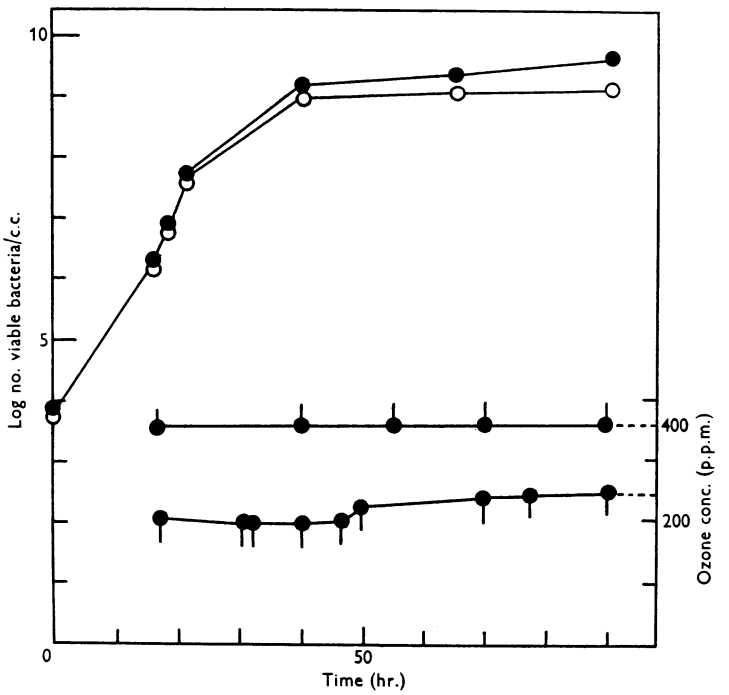

Fig. 5. The continuous consumption of ozone at 20° C., by an active culture of *Achromobacter* (a rate of the order of 10^{-11} g. ozone/hr./cell is suggested). ⊙, air control; ●, air+ozone 380 p.p.m.; ◖, ozone entering flask; ◗, ozone leaving flask.

The concentration of ozone required to affect the organisms was very much greater when they had grown vigorously than when ozone and inoculum entered the broth simultaneously. Reference to Fig. 4*d–f* shows that with increasing concentration of the gas, the first noticeable effect was a shortening

Table 1. *Effect of ozone on bacteria growing on agar at 20° C.*

Organism	Culture*	Ozone			Air			Ozone			Air			Ozone			Air		
		1	2	3	1	2	3	1	2	3	1	2	3	1	2	3	1	2	3
Achromobacter A_1	1	.	.	.	.	.	.	+−−−	+++−	+++−	+++−	++++	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	++++	.	.
A_2	1	.	.	.	.	.	.	+−−−	++++	++++	+++−	++++	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	++++	.	.
A_3	1	−−−−	++−−	+++−	+++−	++++	++++	+−−−	+++−	++++	+++−	++++	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	++++	.	.
A_4	1	−−−−	++−−	++++	+++−	++++	++++	.	.	.	.	.	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	++++	.	.
A_5	1	−−−−	−−−−	−−−−	+−−−	++++	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	+−−−	++++	.	−−−−	−−−−	−−−−	++++	.	.
A_6	1	+−−−	++++	.	++−−	++++	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	+−−−	++++	.	.	.	.	.	.	.
A_7	1	++−−	++++	−−−−	++++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
A_8	1	+−−−	−−−−	+++−	+++−	++++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S_9	1	−−−−	++++	++++	++++	++++	.	.	.	.	.	.	.	+−−−	++++	.	+++−	++++	.	−−−−	−−−−	−−−−	++++	.	.
S_{54}	1	.	.	.	.	.	.	.	.	.	.	.	.	−−−−	++++	.	+++−	++++	.	−−−−	−−−−	−−−−	++++	.	.
S_{56}	1	.	.	.	.	.	.	+−−−	++++	.	++++	++++	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	++++	.	.
A_{17E}	4	.	.	.	.	.	.	+−−−	++++	.	++++	++++	.	++−−	++++	.	+++−	++++	.	.	.	.	.	.	.
A_{645E}	4	.	.	.	.	.	.	.	.	.	.	.	.	+++−	++++	.	++++	++++	.	.	.	.	.	.	.
Bacillus subtilis	2	−−−−	−−−−	−−−−	−−−−	++−−	++++	.	.	.	.	.	.	++++	.	.	++++	.	.	.	.	.	.	.	.
		−−−−	−−−−	−−−−	−−−−	−−−−	++++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
B. mesentericus	3	−−−−	−−−−	−−−−	+−−−	++++	.	−−−−	−−−−	−−−−	+−−−	+++−	++++	.	.	.	.	.	.	.	.	.	.	.	.
		−−−−	−−−−	−−−−	+−−−	++++	.	−−−−	−−−−	−−−−	++−−	++++	++++	.	.	.	.	.	.	.	.	.	.	.	.
B. putreficuscoli Flugge	2	.	.	.	.	.	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	−−−−	−−−−	+++−	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	−−−−	−−−−	++++	.	.	.	.	.	.
Bacterium coli Escherisch	2	−−−−	+−−−	+−−−	+−−−	++++	.	−−−−	++−−	++−−	++−−	++++	.	−−−−	−−−−	−−−−	−−−−	++++	.	.	.	.	.	.	.
		−−−−	++++	++++	+−−−	++++	.	−−−−	−−−−	+−−−	++−−	++++	.	−−−−	−−−−	−−−−	−−−−	++++	.	.	.	.	.	.	.
Bact. coli Connunior	1	++−−	++++	.	++++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		+−−−	++++	.	++++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Micrococcus cinnebareus	1	.	.	.	.	.	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	−−−−	+−−−	++++	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	−−−−	+−−−	++++	.	.	.	.	.	.
Proteus vulgaris	2	−−−−	+−−−	+++−	+++−	++++	.	−−−−	−−−−	−−−−	+++−	++++	.	.	.	.	.	.	.	.	.	.	.	.	.
		−−−−	−−−−	−−−−	++++	++++	.	−−−−	+−−−	+−−−	+++−	++++	.	.	.	.	.	.	.	.	.	.	.	.	.
P. vulgaris	1	.	.	.	.	.	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	−−−−	++−−	++++	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	−−−−	+−−−	++++	.	.	.	.	.	.
Pseudomonas	1	.	.	.	.	.	.	−−−−	++++	++++	++−−	++++	.	.	.	.	.	.	.	−−−−	+−−−	+−−−	++−−	++++	.
		.	.	.	.	.	.	−−−−	+++−	++++	++−−	++++	.	.	.	.	.	.	.	−−−−	++−−	+−−−	++−−	++++	.
Pseudomonas	1	−−−−	−−−−	−−++	.	.	.	.	.	.	.	.	.	+++−	++−+	.	++++	.	.	−−−−	+−−−	++−−	++++	.	.
		++−−	++++	.	−−−−	.	.	.	.	.	.	.	.	++++	++++	.	++++	.	.	+−−−	++−−	+−−−	++++	.	.
Pseudomonas (*B. pyocyaneus*)	2	.	.	.	.	.	.	+−−−	+++−	++++	++−−	++++	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	++−−	++++	.
		.	.	.	.	.	.	+−−−	+++−	+++−	+++−	++++	.	.	.	.	.	.	.	−−−−	−−−−	−−−−	++−−	++++	.
Staphylococcus albus	2	−−−−	−−−−	−−−−	−−−−	++++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		−−−−	−−−−	−−−−	+−−−	++++	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Staph. aureus	2	.	.	.	.	.	.	−−−−	−−−−	−−−−	+−−−	+++−	++++	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	−−−−	−−−−	−−−−	+−−−	+++−	++++	.	.	.	.	.	.	.	.	.	.	.	.
Staph. pyogenes	2	.	.	.	.	.	.	−−−−	−−−−	−−−−	+−−−	+++−	++++	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	−−−−	−−−−	−−−−	+−−−	+++−	++++	.	.	.	.	.	.	.	.	.	.	.	.
		208 ± 70			20·00 ± 0·03° C.			335 ± 60			19·96 ± 0·05° C.			346 ± 40			19·99 ± 0·03° C.			484 ± 45			19·98 ± 0·05° C.	

\+ = Growth along each streak inoculation, as explained in the text.

Numerals at head of columns = time in days (1 day = 24 hr.).

Concentrations of ozone (parts per million by volume) and the exact temperature, given at foot of column.

* 1 = Culture isolated from 'slimy' meat by author. 2 = Culture from Lister Institute. 3 = Culture obtained from Pathological Laboratory, Cambridge. 4 = Culture isolated from 'slimy' meat by W. A. Empey, Australia.

of the maximum stationary phase and an acceleration of the rate of death during the phase of decline, although the gas had been admitted during the logarithmic growth phase. Concentrations of ozone up to about 1000 p.p.m. by volume had no effect on the rate of multiplication, or the rate of death, of the *Achromobacter* at 20° C.; from 1000 to 2000 p.p.m. in general, while having no effect on the rate of growth, caused a shortening of the maximum stationary phase and an acceleration of the rate of death; 2450 p.p.m., while still not affecting the logarithmic growth phase, caused the phase of logarithmic decline to set in almost without any maximum stationary phase; finally, a concentration of about 3890 p.p.m. arrested growth immediately, the culture being almost sterile after about another 30 hr. With *Staph. aureus* (Fig. 4*g*) the smaller concentration of 2560 p.p.m. arrested growth.

At 0° C. a strength of about 430 p.p.m. of ozone was sufficient to arrest the growth of *Achromobacter* (Fig. 4*h*). The interesting point emerges that whereas the rise of temperature 0–20° C. necessitated only a two- or three-fold increase in concentration of inhibitor when admitted simultaneously with the inoculation, roughly a ten-fold increase was needed over the same range of temperature, for arresting established growth.

(4) *Growth on agar*

A single loop of a thick saline suspension of each of a considerable selection of organisms was streaked four times across agar in a Petri dish, thus giving four parallel inoculations of decreasing density, and a + sign in Tables 1 and 2 records the appearance of growth along each streak.

The following results were obtained at 20° C. (Table 1). A concentration of 208 ± 70 p.p.m. by volume of ozone completely prevented the growth of *Staph. albus*, *Bacillus subtilis*, and *B. mesentericus*; had a slight inhibitory effect on *Escherischia coli* (two cultures) and *Proteus vulgaris*; and little effect on four cultures of *Achromobacter* and one of *Pseudomonas*, one culture of *Achromobacter* however being inhibited. Moreover, when the three cultures showing no growth in the atmosphere containing ozone were removed to air and incubated, no further growth resulted, indicating a true germicidal action in these cases. With slightly increased concentration (335–346 p.p.m. approximately) the action was more marked, but most cultures of *Achromobacter* and *Pseudomonas* were still little affected. 485 ± 45 p.p.m. almost completely destroyed all the bacteria tested. At 20° C., therefore, it appears that about double the concentration of ozone germicidal to *Staphylococci*, *Proteus* and *Coli*, and the spore-bearing aerobes *B. subtilis* and *B. mesentericus*, was required to destroy most members of the *Achromobacter* and *Pseudomonas* groups, confirming the difference observed in broth. A possible explanation of the greater susceptibility of the former organisms lies in the fact that their optimum temperature for growth is higher than that of the latter, and their growth at 20° C. was therefore rather less vigorous. This agrees with the fact that the single culture of *Achromobacter* inhibited by the lower concentrations of ozone grew noticeably less vigorously than the other members of this group tested.

At 0° C. (Table 2) a smaller concentration of ozone sufficed to destroy the organisms, 145 ± 10 p.p.m. being germicidal to *Proteus*, two cultures of *Pseudomonas* and nine cultures of *Achromobacter*. 9 p.p.m., on the other hand, while inhibiting the growth of *Proteus*, was germicidal to only one feebly growing *Achromobacter*, and had but slight inhibitory effect on five strains of *Achromobacter* and two cultures of *Torulopsis*.

The growth of mould fungi

Small quantities of growing cultures of various moulds were transferred to the centres of Petri dishes containing malt agar, and the increase in diameter of the colonies was measured when the plates were incubated in various concentrations of ozone, in an atmosphere saturated with water vapour. Controls were simultaneously incubated in air. No attempt was made to distinguish between retardation of germination of spores and inhibition of mycelial growth: the inhibition of fungal growth by volatile substances and gases had already been comprehensively studied by Tomkins (1932), and here it was desired merely to ascertain whether the quantities of ozone required to arrest fungal growth were of the same order as those found with bacteria. The inoculations were therefore made with a mixture of both growing mycelium and spores, the former probably requiring the greater concentration of inhibitor.

While concentrations as low as about 400 p.p.m. exerted at 25° C. a slight inhibitory effect on all the fungi studied, amounts of the order of 1000 or more p.p.m. were required to inhibit growth of all the organisms entirely (Table 3). Differences in resistance among various species were again apparent, *Mucor* and *Botrytis* being the most resistant of those measured. At 0° C., approximately 7 p.p.m. of ozone did not arrest the growth of *Mucor*, *Thamnidium* and *Botrytis*, but caused some retardation (Table 4). A slowly growing *Penicillium* was, however, inhibited.

It thus appears that the concentrations of ozone affecting fungal growth are of a similar order to those found effective against bacteria.

Table 2. *Effect of ozone on bacteria growing on agar at 20° C.*

Organism	Culture*	Ozone 6	Ozone 7	Ozone 8	Ozone 10	Air 5	Air 6	Air 7	Air 8	Air 10
Achromobacter A_1	1	− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	+ + + −	+ + + +	+ + + +
		− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	+ + − −	+ + + −	+ + + +
A_2	1	− − − −	− − − −	− − − −	− − − −	+ − − −	+ + + +	+ + + +	+ + + +	.
		− − − −	− − − −	− − − −	− − − −	+ − − −	+ + − −	+ + − −	+ + + +	.
A_3	1	− − − −	− − − −	− − − −	− − − −	+ + − −	+ + + −	+ + + −	+ + + −	+ + + +
		− − − −	− − − −	− − − −	− − − −	+ + − −	+ + − −	+ + − −	+ + + −	+ + + +
A_4	1	− − − −	− − − −	− − − −	− − − −	+ − − −	+ + + −	+ + + −	+ + + +	.
		− − − −	− − − −	− − − −	− − − −	+ − − −	+ + + −	+ + + −	+ + + +	.
A_8	1	− − − −	− − − −	− − − −	− − − −	+ + − −	+ + + +	+ + + +	+ + + +	+ + + +
		− − − −	− − − −	− − − −	− − − −	+ + − −	+ + + −	+ + + −	+ + + −	+ + + +
S_9	1	.	.	.	.	.	.	.	.	.
S_{54}	1	− − − −	− − − −	− − − −	− − − −	+ + − −	+ + + +	+ + + +	.	.
		− − − −	− − − −	− − − −	− − − −	+ + − −	+ + − −	+ + + +	.	.
A_{17E}	4	− − − −	− − − −	− − − −	− − − −	+ + − −	+ + + −	+ + + +	+ + + +	.
		− − − −	− − − −	− − − −	− − − −	+ + − −	+ + + −	+ + + −	+ + + +	.
S_{645E}	4	− − − −	− − − −	− − − −	− − − −	+ − − −	+ + − −	+ + − −	+ + + +	.
		− − − −	− − − −	− − − −	− − − −	+ − − −	+ + − −	+ + − −	+ + + +	.
Proteus	1	.	.	.	.	.	.	.	.	.
Proteus	1	− − − −	− − − −	− − − −	− − − −	+ − − −	+ + − −	+ + + −	+ + + +	+ + + +
		− − − −	− − − −	− − − −	− − − −	+ − − −	+ + − −	+ + − −	+ + + −	+ + + +
Pseudomonas	1	− − − −	− − − −	− − − −	− − − −	+ + − −	+ + − −	+ + − −	+ + + +	.
		− − − −	− − − −	− − − −	− − − −	+ − − −	− − − −	+ + − −	+ + + +	.
Pseudomonas	4	− − − −	− − − −	− − − −	− − − −	+ + − −	+ + + −	+ + + −	+ + + +	+ + + +
		− − − −	− − − −	− − − −	− − − −	+ + − −	+ + − −	+ + + −	+ + + −	+ + + +
Yeast (*Torulopsis*)	4	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.
Yeast (*Torulopsis*)	4	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	
Concentration of ozone (parts per million by volume) and temperature			145 ± 10					0·7 ± 0·1° C.		

Table 2 (*continued*)

Organism	Culture*	Ozone 6	Ozone 7	Ozone 8	Ozone 10	Ozone 12	Ozone 15	Ozone 17	Air 6	Air 7	Air 8	Air 10	Air 12	Air 15	Air 17
Achromobacter A_1	1	− − − −	+ − − −	+ − − −	+ − − −	+ − − −	+ − − −	+ − − −	+ − − −	+ + − −	+ + − −	+ + + +	+ + + +	.	.
		− − − −	− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	+ − − −	+ + − −	+ + − −	+ + − −	+ + + +	.	.
A_2	1	+ − − −	+ − − −	+ − − −	+ − − −	+ + − −	+ + − −	+ + − −	+ − − −	+ + − −	+ + − −	+ + + +	+ + + +	.	.
		− − − −	+ − − −	+ − − −	+ − − −	+ − − −	+ + − −	+ + − −	+ − − −	+ − − −	+ − − −	+ + + −	+ + + +	.	.
A_3	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	.	.
A_4	1	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	+ − − −	+ − − −	+ − − −
		− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	+ − − −	+ − − −
A_8	1	− − − −	− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	+ − − −	+ + − −	+ + − −	+ + + +	.	.	.
		− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	+ + − −	+ + + +	.	.	.
S_9	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
S_{54}	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	.	.
A_{17E}	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	.	.
S_{645E}	4	− − − −	− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	− − − −	+ − − −	+ + − −	+ + + −	+ + + −	+ + + +	+ + + +
		− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	+ − − −	+ + − −	+ + + −	+ + + −	+ + + −	+ + + −
Proteus	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
Proteus	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	.	.
Pseudomonas	1	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	.	.
Pseudomonas	4	.	.	.	.	.	.	.	.	.	.	.	.	.	.
		.	.	.	.	.	.	.	.	.	.	.	.	.	.
Yeast (*Torulopsis*)	4	+ − − −	+ − − −	+ − − −	+ + − −	+ + − −	+ + + −	+ + + +	+ − − −	+ + − −	+ + − −	+ + + −	+ + + +	+ + + +	+ + + +
		− − − −	− − − −	− − − −	− − − −	− − − −	− − − −	+ − − −	+ − − −	+ − − −	+ + − −	+ + + −	+ + + −	+ + + −	+ + + −
Yeast (*Torulopsis*)	4	− − − −	− − − −	− − − −	− − − −	+ + − −	+ + + +	+ + + +	+ − − −	+ + − −	+ + − −	+ + + +	+ + + +	.	.
		− − − −	− − − −	− − − −	− − − −	+ − − −	+ + + −	+ + + +	+ − − −	+ − − −	+ + − −	+ + + −	+ + + +	.	.
Concentration of ozone (parts per million by volume) and temperature					9·4 ± 1·0							0·5 ± 0·2° C.			

+ = Growth along each streak inoculation as explained in the text.
Numerals at head of columns = time in days (1 day = 24 hr.).
*1 = Isolated by author from 'slimy' meat. 4 = Isolated by W. A. Empey from 'slimy' meat in Australia.

Table 3. *Effect of ozone on fungi growing on malt agar, 25° C.*

Diameter of colony in cm.

Concentration of ozone	Temperature (° C.)	Organism	Air. Time in days						Ozone. Time in days					
			2	3	4	5	6	9	2	3	4	5	6	9
380 ± 40 parts per million by volume	25·00 ± 0·02°	*Thamnidium*	0·8	1·8	2·5	.	.	.	0·3	1·1	1·8	.	.	.
			0·9	1·9	2·5	.	.	.	0·3	0·9	1·4	.	.	.
		Mucor	1·4	4·3	>7	.	.	.	0·5	2·5	4·1	.	.	.
			0·9	3·8	>6	.	.	.	0·7	2·8	4·0	.	.	.
		Penicillium	0·6	1·5	2·5	.	.	.	0·4	0·8	1·8	.	.	.
			0·9	1·8	3·1	.	.	.	0·4	1·1	2·1	.	.	.
		Botrytis	0·6	3·5	>8	.	.	.	0·3	1·8	3·2	.	.	.
			1·2	4·2	>8	.	.	.	0·2	2·1	5·1	.	.	.
		Aspergillus	0·4	1·3	2·5	.	.	.	0·4	1·3	1·7	.	.	.
			0·4	1·4	>5	.	.	.	0·3	1·2	1·8	.	.	.
		Cladosporium	0·7	1·8	2·5	.	.	.	0·2	0·5	1·2	.	.	.
			0·4	1·4	2·4	.	.	.	0·2	0·8	0·8	.	.	.
692 ± 10 parts per million by volume	25·00 ± 0·02°	*Aspergillus*	0·6	2·0	.	3·0	.	>6	0·2	0·2	.	0·2	.	0·2
			0·6	1·6	.	2·5	.	>6	0·2	0·7	.	1·7	.	2·5
		Botrytis	2·0	3·5	.	>6	.	>6	0·3	0·2	.	0·2	.	1·8
			0·8	2·0	.	>6	.	>6	0·5	0·4	.	1·2	.	1·6
		Mucor	2·1	3·5	.	6·1	.	>6	0·9	1·6	.	2·3	.	2·7
			1·5	3·0	.	5·5	.	>6	0·7	1·5	.	2·1	.	2·5
		Thamnidium	0·8	1·2	.	1·8	.	3·5	0·5	0·8	.	0·8	.	0·9
			1·2	1·8	.	2·1	.	3·8	0·3	0·3	.	0·3	.	0·3
		Penicillium	0·5	0·8	.	1·7	.	3·0	0·2	0·4	.	0·6	.	1·1
			0·4	0·8	.	1·8	.	3·0	0·2	0·2	.	0·2	.	0·2
923 ± 20 parts per million by volume	25·00 ± 0·01°	*Aspergillus*	0·3	1·2	.	.	2·2	.	.	0·1	.	.	0·1	.
			0·4	1·8	.	.	2·8	.	.	0·1	.	.	0·1	.
		Penicillium	0·3	0·8	.	.	2·0	.	.	0·2	.	.	0·2	.
			0·2	0·6	.	.	2·0	.	.	0·2	.	.	0·2	.
		Mucor	1·5	3·5	.	.	>5	.	.	1·0	.	.	2·1*	.
			1·5	3·0	.	.	>5	.	.	0·3	.	.	0·3	.
		Botrytis	0·5	2·1	.	.	>4	.	.	0·4	.	.	4·0*	.
			1·0	2·2	.	.	>4	.	.	0·2	.	.	0·2	.
		Thamnidium	0·9	1·2	.	.	3·0	.	.	0·3	.	.	0·4	.
			0·8	1·5	.	.	2·8	.	.	0·2	.	.	0·3	.

* Further growth on incubating in air. None of the others of this group grew.

Table 4. *Effect of ozone on fungi growing on malt agar at 0° C.*

Diameter of colony in cm.
Ozone concentration approximately 7 parts per million

	Air. Time in days					Ozone. Time in days			
	7	14	19	25	27	14	19	25	27
Mucor	0·7	2·1	3·0	3·6	4·0	0·7	1·6	2·2	2·3
	0·8	2·5	3·2	3·8	4·1	1·0	2·0	2·8	2·9
Thamnidium	0·7	1·4	1·7	2·3	2·3	0·6	1·0	1·2	1·3
	0·5	1·2	1·7	2·0	2·1	0·9	1·1	1·5	1·6
Botrytis	0·8	1·1	1·6	2·2	2·3	.	0·3	0·7	0·8
	0·6	0·9	1·5	2·3	2·5	.	0·4	0·7	0·9
Penicillium	.	.	0·4	0·7	0·7	.	.	.	.
	0·3	0·4	0·6	0·8	0·9	.	.	.	.

(5) *The possible mechanism of disinfection by ozone*

In all the cases examined, the inhibitory concentrations of ozone were much less than the germicidal, i.e. an excess was required to destroy the cells (cf. Table 5).

Table 5. *Comparison of the inhibitory and germicidal concentrations of ozone (p.p.m.)*

Organism	Medium	Temperature (° C.)	Inhibitory concentration	Germicidal concentration
E. coli	Synthetic solution	20	4	> 200 < 600
Achromobacter	Nutrient broth	20	290	350
Proteus	Nutrient broth	20	140	240
Fungi	Nutrient agar	25	400	1000+

This suggested that the first action of the ozone might be on the medium. Ozone is chemically very reactive, ammonia and amino-acids being oxidized (Harries & Langheld, 1907; Bergel & Bolz, 1933; Strecker & Thieneman, 1920). It might be expected, therefore, that the ozone would rapidly destroy nutrients available for cellular metabolism.

In order to estimate how far this effect alone would account for the inhibitory action of ozone, both agar and broth were treated with the gas before being sown with bacteria. Agar plates were soaked in ozone for 17 hr. (500 p.p.m. approximately), the ozone was then removed with a current of air, and the agar (which was bleached) inoculated. It was found that simple soaking of the agar in ozone had rendered it incapable of supporting bacterial growth in many cases, while allowing of somewhat retarded growth in others.

The experiment was then repeated with nutrient broth, since in the case of agar there was the possibility that dissolved ozone had remained in it. Ozone (3500 p.p.m.) was passed over the shaken broth for 60 hr., and air then passed for 24 hr. Tests showed that no ozone remained in the broth, which was bleached and had its pH changed from the original 7 to between 4 and 5. On inoculation very limited growth took place. When the experiment was repeated, a portion of the ozone-treated broth having its pH brought back to 7, somewhat slower growth, but eventually as heavy, took place as in the unozonized broth. Evidently therefore one of the major causes of the inhibitory action of ozone is the change of pH brought about by interaction of the ozone with substances in the medium, and not the destruction of nutrients as such. This is confirmed by the fact that smaller concentrations of ozone were effective in a synthetic medium containing less oxidizable matter, and smaller still in the simple disinfection of aqueous suspensions.

A second factor, which was indicated in several ways by Haines's results, is that ozone reacts with the products of bacterial metabolism. First, there is the continual consumption of ozone by a growing culture. Secondly, to produce a given effect much more ozone is needed if the cells have passed into the actively growing phase and have had time to accumulate metabolic products—this is also borne out by the observations of Heise, previously quoted.

Thirdly, the amount of ozone needed was always much greater at higher temperatures (Table 6), which might well be related to the increased rate of

Table 6. *The effect of temperature on the estimated germicidal concentrations of ozone (p.p.m. by volume)*

	° C.	*Achromobacter*	*B. proteus*
Inoculation and admission of ozone simultaneous			
Agar	20	> 340 < 480	210
	0	150	150
Broth	20	350	240
	0	140	—
Ozone admitted when growth was well established			
Broth	20	3900	—
	0	430	—

metabolism. Finally, at a given temperature, a rapidly growing organism required more ozone to prevent its growth than a slowly growing one. The fact that organisms of the *Pseudomonas-Achromobacter* group were particularly resistant to ozone, affords a clue to the kind of reaction that might conceivably be involved. It is generally agreed that the metabolism of such organisms consists chiefly in the degradation of proteins to amino-acids, a minor proportion of which are reutilized for the growth of the cells. Bergel & Bolz (1933) believed that the first stage of the reaction of ozone with amino-acids follows the general scheme:

$$R.CH(NH_2)COOH + O_3 + H_2O \rightarrow R.CHO + CO_2 + NH_3 + H_2O_2.$$

If, therefore, ozone were admitted to an actively metabolizing culture of these organisms, most of the ozone should be consumed in this sort of reaction with the amino-acids, and the growth of the cells would not be seriously hindered until a large part of the amino-acids had been destroyed (as the H_2O_2 could be removed by the catalase which these bacteria possess). Since, however, the aldehydes produced could be easily oxidized to acids, a change

in pH, sufficient to hinder growth, might be produced before this point was reached. Similar considerations would obviously apply to a nutrient medium containing pre-formed amino-acids, in which, in addition, oxidation of carbohydrates might contribute to the acidity.

These complications are absent in the case of the killing of dormant cells suspended in water, which calls for an explanation of the action of ozone on the cell itself. The two modes of germicidal action most generally advanced are either flocculation of the cellular proteins (Bancroft & Richter, 1931) or interference with intracellular enzyme systems. Mellor (1922) states that 'the albumins are destroyed by ozone', but no marked flocculation of a solution of pure egg-albumin could be seen after passage of a stream of ozone through it, so that the other alternative claimed Haines's attention.

A simple system for preliminary investigation was the dehydrogenating enzymes of the cell. Accordingly, washed suspensions of *E. coli* were prepared and examined after the technique of Quastel & Wooldridge (1927), a stream of ozone being bubbled through the suspension for 1 hr., after which it was centrifuged, washed, and made up to bulk again. An example of the type of result obtained is given in Table 7.

Table 7. *Methylene-blue reduction times (minutes) in control and ozone-treated suspensions*

	200 parts per million		800 parts per million	
	Control	O_3	Control	O_3
Formate	2	6	2	31
Lactate	5	13	4	85
Dextrose	5	20	4	> 120
Galactose	7	18	5	> 120
Glycerol	7	> 120	6	> 120
Succinate	12	30	8	> 120
Mannitol	11	> 120	8	> 120
Levulose	—	—	9	> 120

It will be seen that there was a general destruction of the dehydrogenating mechanisms, with some evidence of a selective action, so that ozone may bring about death of the cell by interference with the respiratory mechanism. Even in this case, of course, the effect of ozone may have arisen from specific oxidation of essential components such as coenzymes. For example Keilin (1928) showed that hydrogen peroxide and other oxidizing agents in small amounts completely oxidized all the components of cytochrome, so that ozone might well be expected to have similar effects. Here a wide realm of possibilities was revealed, which Haines did not explore further.

DISCUSSION

Haines's conclusions may be broadly summarized as in Table 8. Despite considerable variations between the organisms tested, differences are plainly distinguishable between the various sets of circumstances represented there.

Table 8. *Approximate concentrations (p.p.m.) of ozone needed to prevent bacterial growth in various media at* 20–25° *C.*

	Applied before growth started	Applied when growth established
In water	< 7	—
In synthetic solution	4–10	200–600
In nutrient broth	150–500	500–4000
In nutrient agar	200–500	—

With quiescent cells, in water or a synthetic solution, quite low concentrations of ozone were bactericidal, which agrees with general experience, e.g. in the sterilization of water (Taylor, 1947). Even these low concentrations, however, are apparently greater than can be tolerated by human subjects. According to the measurements of Hill & Flack (1911) 1 p.p.m. irritates the respiratory tract of man (these authors used a high-tension discharge across sheets of fine gauze to prepare their gas, but they expressly state that no oxides of nitrogen were produced); and Ewell (1938) agrees that distress rapidly follows exposure to 3 p.p.m. Moreover, in synthetic solutions, much more ozone than this is required for inhibition when bacterial growth is established, and still more in organic media, according to Haines's experiments.

The need for more ozone after preliminary growth of the microbes is ascribed to reaction of ozone with products of their metabolism. In practice, this means that more ozone is required to preserve food in which contamination has already begun to develop: which may be illustrated by comparison of eggs and meat. Organisms on the surface of eggs do not begin to multiply seriously until the eggs are collected in a store of high humidity where ozone may be applied from the outset—here Haines found 3 p.p.m. of ozone effective in preventing mould growth, on the outside (but not the inside) of the shells, for 16 weeks (100 % R.H., 0° C.) while controls were mouldy in 4 weeks. With meat, on the other hand, even 10 p.p.m. did not delay bacterial growth appreciably, presumably because it was already under way, and more organic matter was accessible. The careful experiments of Käss (1936) on meat are thus not strictly applicable to normal conditions, because he used specially prepared samples of meat carrying unusually small numbers of bacteria—he found relatively low concentrations of ozone effec-

tive. A second effect of the reaction with products of bacterial metabolism appears to be that much less ozone is required at low temperatures. This was illustrated in Tables 3, 4 and 6; it was observed in all Haines's experiments. General commercial experience confirms this observation, and it is therefore remarkable that Ewell (1941) did not find any difference between 4 and 20° C., which leads one to wonder if the effects he observed were primarily due to ozone at all.

It was noted above that apparently because of diminished production of metabolites reacting with it, ozone is made more effective by anything which reduces growth of the microbes, like low temperature. Dry conditions have a similar effect: Kefford (1948) has recently found that 5 p.p.m. of ozone, shown by measurement to be applied to surface of meat, had no effect on the growth of bacteria there *unless* the ozone was applied during the lag phase of growth, and *unless* growth was greatly hindered by the additional application of a humidity low enough in itself to cause considerable inhibition of growth.

On organic media Haines found that more ozone was required, more with nutrient agar than with broth, which he supposed to be due to waste of ozone by combination with the medium. This is a well-recognized occurrence in commercial conditions, because of the undesirable changes which result from the reaction: as little as 3 p.p.m. of ozone at 7° C. bleach butter and make it rancid in a week, and fat sausage is spoiled in 1–2 days (Ewell, 1938). Even at −10° C., workers in this laboratory have observed marked loss of bloom on meat carcasses after 2–3 weeks in 3–4 p.p.m. ozone. The consumption of ozone by the food creates technical difficulties in large-scale operations, because a rapid atmospheric circulation is essential to ensure that the ozone is not used up before it reaches the remote parts of the system, especially if low concentrations of ozone are in question. This makes it very difficult to assess, in terms of the ozone applied, the results of empirical observations under practical conditions, since the improvement may well be due to other causes like better ventilation, or drying. The point may be emphasized by an observation made by Macara (1947), that oil-taint in frozen meat and rendered fat was not removable with 1 p.p.m. ozone any more quickly than by merely hanging in changing air for the same period. Some additional factors seem necessary, to account for the serious discrepancy between the relatively large quantities of ozone needed to inhibit bacteria in laboratory experiments and the traces which are apparently sometimes effective under practical conditions, and one can only wish that more caution was exercised, in ascribing solely to ozone the effects observed in inadequately controlled commercial-scale experiments.

SUMMARY

Data are given showing the concentration of pure ozone required to inhibit the growth of, and to destroy, various micro-organisms when growing on agar, in nutrient broth, a synthetic medium, or simply suspended in water. The following is shown:

1. Different organisms vary in their susceptibility towards this gas. *Achromobacter* and *Pseudomonas* strains, such as occur on chilled meat, are the most resistant. On the whole, mould fungi are about as susceptible as bacteria.

2. Very much higher concentrations are required to arrest established growth than can be used if inoculation and admission of inhibitor are coincident.

3. Lower concentrations are inhibitory at lower temperatures. These results are ascribed to dissipation of the ozone by combination with products of bacterial metabolism. It seems that any factor which diminishes growth will augment the effectiveness of ozone.

4. Comparatively small concentrations (less than 10 p.p.m.) suffice to destroy bacteria suspended in water. Somewhat larger concentrations inhibit growth in a synthetic medium.

5. Still larger quantities (several hundred p.p.m.) are required in nutrient broth.

6. Nutrient broth treated with ozone will support little or no bacterial growth, due to change of pH, growth taking place slowly if the pH is restored to its original value. Inhibition of growth in such a medium is therefore a complex process depending in part upon the secondary effects of decomposition of the medium.

7. To kill them, bacteria require still more ozone (in some cases several thousand p.p.m.) if growing on agar. These results are ascribed to combination of ozone with the supporting medium.

8. When applied to organisms growing on food, combination with ozone not only spoils the food, but it makes it difficult to interpret the effect on the microbes in terms of the ozone applied.

9. The inhibitory concentrations are higher than humans can tolerate.

10. Ozone destroys the dehydrogenating enzymes of the cell, and it is suggested that its germicidal action may be partly due to interference with cellular respiration.

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