Factors Affecting the Toxicity of Oxygen Towards Airborne Coliform Bacteria

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SUMMARY

The maximum bactericidal effect of oxygen on airborne coliform bacteria occurred at the lowest relative humidity tested. *Escherichia coli* COMMUNE and *Klebsiella aerogenes* were very much less sensitive than *E. coli* B and *E. coli* JEPP. Only a small partial pressure of oxygen in a nitrogen-oxygen mixture at atmospheric pressure was required to produce the maximum effect. The oxygen toxicity for each bacterial strain was altered by changing the growth conditions or by including certain chemicals in the spray fluid. These chemicals are protective to freeze-dried bacteria and therefore inactivation of oxidative enzymes in freeze dried and airborne bacteria probably occur by similar mechanisms. The same oxidative enzymes in bacterial colonies were slightly inactivated by hyperbaric oxygen pressures.

INTRODUCTION

Cox (1966) and Cox & Baldwin (1967) showed that *Escherichia coli* sprayed from distilled water died rapidly in air or in oxygen and that death was very marked at low relative humidity (RH) values. At RH values above 70% other death mechanisms prevail (Cox & Baldwin, 1966; Benbough, 1967) which may be associated with re-hydration on collecting the partially dried bacteria.

Past investigations into metabolic disturbances caused by oxygen have been hampered by difficulty in recovering adequate quantities of bacteria from laboratory aerosols. However, comparison of the oxygen effects on freeze-dried bacteria (Lion & Bergmann, 1961; Lion & Avi-Dor, 1963) with those on airborne bacteria and simulation of the aerosolized state by drying bacterial suspensions on filter membranes (Benbough, 1967) have been successful in attempts to elucidate the death mechanisms of airborne bacteria. In dried bacteria enzymes and/or coenzymes concerned with pyruvate degradation and with the reduction of tetrazolium salts were affected. The effect on pyruvate degradation recalled the findings of Thomas, Neptune & Sudduth (1963) who attributed the toxicity of hyperbaric oxygen on pyruvic oxidase activity to oxidation of the dithiol moiety of α -lipoic acid, a cofactor of pyruvic oxidase.

Polyhydroxycompounds have been the most widely used protective agents of aerosolized bacteria (Webb, 1960); according to Cox (1966) they protect because they form supersaturated viscous layers around bacteria and thus limit loss of water. An increase in the bacterial polysaccharide content ought then to be beneficial towards aerosol stability by a similar mechanism to the added polyhydroxycompounds; this is reported in this paper.

METHODS

Escherichia coli (strains B, JEPP and COMMUNE) and *Klebsiella aerogenes* (H.8) were either grown in a chemically defined medium (Benbough, 1967) or in a complex tryptone medium (Anderson, 1966).

Aerosol survival estimation. This was described by Benbough (1967). In tests involving the effect of oxygen concentration the quantities of oxygen were admitted to the drum previously containing pure nitrogen. The bacteria were sprayed with pure nitrogen so the quoted oxygen concentrations in the drum were corrected for the volume displaced by that nitrogen.

Chemicals whose protective ability were being tested were added to the spray fluid before aerosolization.

Polysaccharide content of bacteria. This was expressed as a percentage of the dry weight and was measured as follows. To 1.0 ml. suspension containing 2×10^{11} bacteria/ml. 1.0 ml. 5% redistilled Analar grade phenol was added followed by 5.0 ml. concentrated sulphuric acid. These were thoroughly mixed and the red colour compared at 545 m μ with standard glucose solutions. The dry weight was measured as described by Anderson & Dark (1966).

Drying and storage of dried bacteria in a controlled RH. A bacterial suspension was spread evenly over Oxoid membrane filter (diam. 6 cm.). The culture fluid was drained by suction before placing the membrane filter in a desiccator containing 100 ml. of saturated K₂CO₃ solution which kept the RH constant at 44%.

The effect of oxygen on the enzymic activity of dried bacteria. The bacteria-laden membrane filters were stored in either nitrogen, air or oxygen for 1 hr. Then a small volume of phosphate buffer was used to wash bacteria from the membrane. Each suspension was diluted so that the final bacterial count was approximately 2×10^{10} per ml. The utilization of pyruvate was measured as described by Benbough (1967). The rate of reduction of tetrazolium salts was measured by adding to 0.2 ml. suspension, 0.5 ml. of 5% triphenyltetrazolium chloride and 3.0 ml. of a solution of 0.4M-sodium malate (or lactate or succinate) dissolved in 9.0 ml. of 0.1M-tris at pH 8.8 for malate, 7.4 for lactate or 6.8 for succinate. The tubes were incubated at 37° for 15, 30, 45 and 60 min. each and formozan formation measured as described by Benbough (1967).

Very little reduced nicotinamide adenine dinucleotide (NADH) oxidase activity could be detected with whole bacteria. Cell-free extracts were prepared by treating 10 ml. suspension in 50 mM-tris buffer at pH 7.4 by an MSE sonic disintegrator at 0° for 30 min. at maximum power and centrifuging the resulting suspension at 12,000 g for 15 min. The NADH oxidase activity of the supernatant fluid was determined as follows: to 0.2 ml. supernatant fluid, 2.8 ml. of 50 μ M-tris buffer pH 7.4 (containing 0.1 mM-NADH (B.D.H. Chemicals Ltd., Poole, England) were added and rapidly mixed in a 5 $\times$ 1 $\times$ 1 cm. fused silica spectrophotometer cell. After mixing, the extinction at 340 m μ was measured as a function of time on a Unicam spectrophotometer at room temperature.

The effect of oxygen concentration on bacterial colony growth and on activity of enzymes extracted from bacterial colonies on agar surface. About 100 organisms were spread evenly over agar plates which were placed in containers whose oxygen concentration in nitrogen ranged from 0 to 100% (v/v) at atmospheric pressure. After

incubation overnight at 37° the visible colonies were counted. Bacterial colonies on agar were also exposed to atmospheres of nitrogen, air or oxygen for about 6 hr, then they were resuspended in phosphate buffer and pyruvate utilization, formozan production and NADH oxidase activities were measured.

The effect of nitric oxide on the oxygen toxicity of dried bacteria. Immediately after placing bacteria-laden membrane filters in a desiccator at 44% RH, the vessel was evacuated, filled with pure nitrogen, evacuated again and finally partially filled with nitrogen. A calculated quantity of nitric oxide, formed by mixing sodium nitrite and potassium ferrocyanide in dilute acetic acid within an air-free container, was drawn into the desiccator by negative pressure *via* a soda lime tube; the pressure was restored to atmospheric by adding more pure nitrogen. After 15 min. the filter was removed from the desiccator into air, was well ventilated to limit contact with nitrogen dioxide, and transferred to another vessel containing air at 44% RH. The survival of dried bacteria which received the above treatment was compared with controls which did not receive the nitric oxide treatment.

RESULTS

Figure 1 shows that at 70% RH similar survivals were obtained for storage in air or nitrogen. At low RH the survival in nitrogen was much greater than in air or in a 20% (v/v) oxygen + 80% (v/v) nitrogen mixture at atmospheric pressure in which the survivals were similar. Therefore, oxygen must be the toxic atmospheric component towards airborne coliform bacteria. Figure 2 shows that there was a considerable

Table 1. *The influence of growth media on the survival of coliform bacteria sprayed and stored into air at 40% RH for 30 min.*

	Growth medium	
	Chemically defined	Complex tryptone
	Survival (%)	
<i>Escherichia coli</i> B	1.3	16
<i>E. coli</i> JEPP	2.2	5.1
<i>E. coli</i> COMMUNE	33	55
<i>Klebsiella aerogenes</i>	22	53

variation in the sensitivity of different coliform strains to oxygen. Figure 2 shows that the increase in this sensitivity was directly related to the decrease in the test RH between 70 and 30%.

The results in Figs. 1 and 2 were obtained from tests on washed bacteria which were originally grown on chemically defined media. Bacteria grown in this way survived less well at low relative humidities than corresponding strains grown in complex tryptone media (Table 1).

The maximum death rate occurred within the first second of spraying into either

Fig. 1. Death rates of aerosolized: (a) *Escherichia coli* COMMUNE, (b) *Klebsiella aerogenes*, (c) *E. coli* (JEPP), (d) *E. coli* B which was sprayed into nitrogen at 70% RH ($\blacktriangle$ — $\blacktriangle$); sprayed into air at 70% RH ($\triangle$ — $\triangle$); sprayed into nitrogen at 40% RH ($\bullet$ — $\bullet$); sprayed into air at 40% RH ($\circ$ — $\circ$).

Fig. 2. Survivals of aerosolized bacteria in air as a function of RH (aerosol age 10 min.). A, *Escherichia coli* COMMUNE; B, *Klebsiella aerogenes*; C, *E. coli* (JEPP); D, *E. coli* B. Each of the above straight lines represents between 15 and 25 experimental points.

air or nitrogen but was fastest in air at low RH. Also, at low RH the secondary death rates in air did not tend to become zero as compared to those in nitrogen or air at high RH values (Fig. 1). Figure 3 shows that bacteria sprayed and stored in nitrogen for 0.4 sec. before exposure to air were more sensitive to air than those sprayed directly into air in otherwise similar conditions. Small additions of oxygen to nitrogen atmospheres induced large increases in the death rate; the maximum death rate was reached by fairly low oxygen concentrations in nitrogen (Fig. 4).

Fig. 3. The deleterious effect of spraying *Escherichia coli* B into nitrogen before being passed into and stored in air at 50% RH: ○—○, bacteria sprayed into air and stored in air; ●—●, bacteria sprayed into nitrogen and stored in air after aerosol age of 0.4 sec. In the above figure the survival given at 0 min. is in fact the survival at 0.4 sec.

Fig. 4. Effect of oxygen concentration on death rate of aerosolized *Escherichia coli* B sprayed into nitrogen and stored in nitrogen-oxygen mixtures at (a) 50% RH; (b) 70% RH; A, pO_2 is 0.00 atmosphere; B is 0.03 atm; C is 0.36 atm; D is 0.60, 1.20, 3.00 and 4.50 atm.

Table 2. *The effect of some compounds included in the spray fluid on the survival of aerosolized Escherichia coli B at 50% RH*

Compound	Concentration (mm)	Aerosol age (min.)			
		1	2	5	10
		Survival (%)			
Distilled water*		85	80	80	76
Distilled water*		32	14	6	3
Thiourea	0.7	68	59	51	45
KNO ₃	0.5	43	31	24	24
KI	0.3	42	38	35	35
KCNS	1.2	40	32	18	15
2-aminoresorcinol	0.3	58	38	22	20
Methylene blue	0.05-5.0	42	20	6	1
Thymolindophenol	0.05-5.0	29	5	1	—
Ascorbic acid (pH to 7.0 with NaOH)	1.2	45	22	12	9
Catalase	0.01 %	38	16	10	5
H ₂ O ₂	2.0	35	13	9	5
Raffinose	300	68	62	40	20
Raffinose	3.0	30	16	4	3
Glucose	2.8	31	10	4	2
Sucrose	2.8	38	20	6	3
Glycerol	2.5	29	12	5	4
Glycerol	250	48	42	26	16
MnCl ₂	0.4	40	24	19	12
MnSO ₄	0.5	48	35	24	16
CoCl ₂	0.2	37	21	15	10
ZnCl ₂	0.4	30	10	5	1
KCl	0.5	31	10	3	1
MgCl ₂	0.4	32	18	8	6
MgSO ₄	0.6	37	23	9	7

* Sprayed into N₂; all others sprayed into air.Table 3. *Polysaccharide contents of coliform bacteria grown in various chemically defined media*

Strain	Growth medium	Polysaccharide dry weight
<i>Escherichia coli</i> B	<i>a</i>	0.13
<i>Escherichia coli</i> COMMUNE	<i>a</i>	0.23
<i>Escherichia coli</i> JEPP	<i>a</i>	0.10
<i>Klebsiella aerogenes</i>	<i>a</i>	0.22
<i>Escherichia coli</i> B	<i>b</i>	0.23
<i>Escherichia coli</i> B	<i>c</i>	0.19
<i>Escherichia coli</i> COMMUNE	<i>b</i>	0.38
<i>Klebsiella aerogenes</i>	<i>b</i>	0.42

a, denotes the chemically defined medium described in Methods.*b*, denotes the above medium except that the ammonium citrate concentration was decreased to 50 µg./ml.*c*, denotes the above medium except that the magnesium sulphate concentration was decreased to 0.24 µg./ml.

The death rate of bacteria in the aerosol at low RH is decreased when certain chemicals are included in the spray fluid (Benbough, 1967; Cox, 1966). The protective effects of more chemicals are listed in Table 2; most of these were effective in millimolar quantities. Redox dyes were tested because in the reduced form their ability to combine with oxygen might limit death in the aerosol. Methylene blue decreased the initial death rate substantially, but the secondary death rate was accelerated; thus it appeared to delay the toxic effect of oxygen. Thiourea, ascorbic acid, 2 aminoresorcinol, like cysteamine (Benbough, 1967), protected aerosolized bacteria at low RH only; these compounds were toxic between 60 and 80% RH. In contrast to the protective compounds already described, sugars and other polyhydroxycompounds were needed in high concentrations to protect aerosolized bacteria. Catalase did not protect, hence toxic amounts of H_2O_2 were not produced; H_2O_2 included in the spray fluid was not toxic.

Fig. 5. Death rates of aerosolized: (a) *Escherichia coli* COMMUNE; (b) *Klebsiella aerogenes*; (c) *E. coli* B sprayed into air at 40% RH. ○—○, Bacteria grown on chemically defined media as described in Methods; ●—●, bacteria grown in the same medium except that the ammonium citrate was reduced to 50 μ g./ml.; ▲—▲, bacteria grown in the same medium except that the $MgSO_4$ was decreased to 0.24 μ g./ml.

Strains of coliform bacteria with the higher polysaccharide content can withstand the oxygen effect much better in the aerosol (Table 3). By increasing the polysaccharide content of each strain by growing and incubating in carbon-rich and nitrogen- or magnesium-limiting media (Wilkinson, 1959), the following result was obtained: the initial death rate was as fast as corresponding strains grown on the complete defined medium but the secondary death rate was considerably slower (Fig. 5).

Enzymes concerned with reduction of 2:4:6-triphenyl tetrazolium chloride, NADH oxidase and pyruvate degradation were inactivated by oxygen in partially dried bacteria (Table 4). Results on Table 4 also show that hyperbaric oxygen inactivated the same enzymes in bacterial colonies. The effects of oxygen on partially dried bacteria and on colonies may therefore be basically the same; however, much higher oxygen concentration was needed to produce the same effect in the latter case. Double atmospheric oxygen pressure produces a threshold bacteriostatic effect on *Escherichia coli* colonies whilst five times atmospheric oxygen pressure causes 50% and 30% inhibition of colony formation from single *E. coli* B and *E. coli* COMMUNE bacteria respectively.

When partially dried bacteria on membrane filters were exposed to nitric oxide for a few minutes before exposure to oxygen the number of bacteria killed was greatly diminished. The maximum protective effect was produced by 50 parts per million nitric oxide in nitrogen (Table 5).

Table 4. *The inactivation of three bacterial enzyme systems by oxygen*

(a) Enzymes concerned with the utilization of pyruvate

Treatment	Uptake of pyruvate by suspension $\mu\text{M}/\text{min.}/10^{10}$ bacteria
<i>E. coli</i> B dried at 44% RH for 1 hr at 1 atm. in	
Nitrogen	31 ± 1
Oxygen	21 ± 1
Air	22 ± 1
<i>E. coli</i> B colonies exposed for 18 hr at 1 atm. in	
Nitrogen	33 ± 1
Oxygen	28 ± 1
Air	33 ± 1

(b) Enzymes concerned with the reduction of 2:4:6-triphenyltetrazolium chloride to formozan

Treatment	Incubated with		
	Succinate	Malate	Lactate
	Rate of increase of extinction/hr; <i>n</i> -butanol extract		
<i>E. coli</i> B dried at 44% RH for 1 hr at 1 atm. in			
Nitrogen	0.120	0.033	0.042
Oxygen	0.048	0.000	0.015
Air	0.045	0.000	0.019
<i>E. coli</i> B colonies exposed for 18 hr. at 1 atm. in			
Nitrogen	0.132	0.072	0.074
Oxygen	0.061	0.039	0.048
Air	0.140	0.065	0.075

(c) NADH oxidase

Treatment	Activity n-mole NADH oxidized/min.
<i>E. coli</i> B dried at 44% RH for 1 hr at 1 atm. in	
Nitrogen	190 ± 10
Oxygen	90 ± 10
Air	110 ± 10
<i>E. coli</i> COMMUNE dried at 44% RH for 1 hr at 1 atm. in	
Nitrogen	140 ± 10
Oxygen	90 ± 10
Air	90 ± 10
<i>E. coli</i> B colonies exposed for 18 hr at 1 atm. in	
Nitrogen	220 ± 10
Oxygen	130 ± 10
Air	230 ± 10

Table 5. *Effect of nitric oxide on the survival of dried Escherichia coli B at 44% RH exposed to air*

NO conc. p.p.m. at 1 atm.	Proportion of bacteria surviving after 30 min. exposure to air
0	0.3 ± 0.1
10	0.5 ± 0.2
50	0.6 ± 0.2
60	Less than 0.001
100	0

DISCUSSION

The lethal action of oxygen appears to depend on the loss of water from bacteria. Bateman, Stevens, Mercer & Carstensen (1962) measured the water content of *Serratia marcescens* at many RH values and found that it decreased rapidly between 100% and 70% RH, but below 70% the decrease was very much less. This shows that strongly bound water is lost when bacteria are exposed to RH values of 70% and less. Bound water has a structural significance and its loss may cause changes in accessibility and reactivity of macromolecules. Freeze-dried *Escherichia coli* show electron spin-resonance signals (Dimmick, Heckley & Hollis, 1961; Lion, Kirby-Smith & Randolph, 1961; Lion & Bergmann, 1961); these signals are emitted by free radicals and the signals were greatly enhanced when the freeze-dried bacteria were exposed to oxygen, presumably because paramagnetic molecules such as oxygen react readily with free radicals to form peroxyradicals. Such events probably occur when bacteria are sprayed into air at low RH values. The peroxyradicals formed in this way would inactivate many of the oxidative enzymes. Protection by nitric oxide can be explained by its paramagnetic character: it would react with free radicals formed during bacterial hydration and thus prevent peroxyradical formation.

Airborne bacteria can be protected in two ways. First, polyhydroxy compounds limit the loss of water from bacteria in the same way as those bacterial strains that possess a high polysaccharide content. Secondly, other compounds may act directly on the oxygen inactivating site; these compounds are effective in micromolar quantities and they include paramagnetic ions such as Mn^{2+} , NO_2^- , I^- and compounds with labile hydrogens such as ascorbic acid, aminothiols and reduced dyes.

Comparison of the extent of enzyme inactivation in bacterial colonies by hyperbaric oxygen to inactivation in dried bacteria by air confirms the role of cellular water in limiting oxygen toxicity.

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