The Aerosol Survival of *Escherichia coli* B in Nitrogen, Argon and Helium Atmospheres and the Influence of Relative Humidity

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SUMMARY

The survival of *Escherichia coli* strain B sprayed from distilled water into atmospheres of nitrogen, argon and helium, as a function of relative humidity (RH) at an aerosol age of 30 min. was good at low RH, while at high RH values regions of marked instability occurred. At high RH differences in survival were observed, indicating that the gas atmospheres were not completely biologically inert. The results indicated that the initial evaporation rates of the aerosol droplets did not influence the long-term survival of *E. coli* B in the aerosol. An alternative reason for the importance of RH is discussed, together with considerations of death mechanisms.

INTRODUCTION

The aerosol survival of *Escherichia coli* depends upon several variables (Cox, 1965, 1966*a*, *b*, 1967; Cox & Baldwin, 1966, 1967). These include an air stress at low relative humidity (RH) caused by the toxic action of oxygen, possibly modified by trace contaminants (Cox & Baldwin, 1967). Hess (1965) found that *Serratia marcescens* and *E. coli* B were also killed by oxygen at low RH. At high RH, regions were found where *E. coli* survival was particularly sensitive to RH, in a manner that was dependent upon the spray fluid and collecting fluid (Cox, 1966*a*, *b*, 1967; Cox & Baldwin, 1966). Relative humidity changes before collection also influenced survival, either beneficially or detrimentally, depending upon the strain of *E. coli* used and the nature of the spray and collecting fluids (Cox, 1966*b*, 1967). These results obtained with different collecting fluids and RH changes showed that the death mechanisms of *E. coli* in nitrogen atmospheres must be influenced by the manner in which water re-enters the bacteria after collection. In certain instances all the deaths can be attributed to the manner in which water re-enters the bacterium, e.g. *E. coli* B and COMMUNE sprayed from raffinose into nitrogen at high RH (Cox, 1966*a*, *b*). Microbial survival in aerosols was reviewed in general terms by Anderson & Cox (1967).

The present paper reports the survival of *Escherichia coli* B sprayed from distilled water into atmospheres of nitrogen, argon and helium. The object of this work was to determine whether these atmospheres were really inert and to examine the possibility that the initial evaporation rates of the aerosol droplet influence the long-term survival of *E. coli* B in the aerosol. A further possibility, namely that protective agents operate by a modification of this rate, was also considered.

METHODS

The techniques used were as previously reported (Cox, 1966*a*), except that only *Escherichia coli* B sprayed from distilled water was studied. Since wet- and dry-bulb thermometer readings were used as a measure of the relative humidity (RH), it was necessary to determine the psychrometric constant for argon and helium so that wet-bulb depression could be converted into RH. As far as is known psychrometric tables exist only for air. The technique was to determine the dew-point as a function of wet-bulb depression in various atmospheres. From these data the psychrometric constants were determined and psychrometric tables calculated. The dew-point hygrometer was very kindly lent by Mr W. R. Sparks of the Meteorological Office, Bracknell.

Anderson (1966) and Cox (1966*a*) provided evidence that *Bacillus subtilis* var. *niger* spores were not altogether satisfactory as a tracer, since under certain conditions they lost viability, especially at high RH. Anderson 1966 developed a tracer technique based on labelling *Escherichia coli* B with ^{14}C . This method was examined as an alternative to the use of *B. subtilis* var. *niger* spores, but the results of 28 experiments showed the technique to be unsuitable for the present work. The reasons for reverting to *B. subtilis* var. *niger* as a tracer are given later in the Results.

RESULTS

The tracer technique

The use of a radioactive tracer necessitates the collection of a much larger number of bacteria than does the spore-tracer technique, so that high aerosol densities and long sampling times are needed to give radioactive counts of ten times (or greater) the background count. As a consequence the ^{14}C technique required a 3-jet Collision spray (Green & Lane, 1957) in place of the 1-jet spray as used previously (e.g. Cox, 1966*a*). During aerosol formation considerably greater cooling occurred in the 3-jet than in the 1-jet spray so that the control of temperature and RH was much inferior to that when using a 1-jet spray. As had been the practice previously (e.g. Cox, 1966*a*), samples were removed from the spray pot both before and after spraying, with the intention of checking for any spray damage to *Escherichia coli*. In all of the 28 experiments it was observed that the radioactive count increased after spraying, often by as much as 30 %, without a concomitant increase in the count of *E. coli* B and *Bacillus subtilis* var. *niger* which was also added as a second tracer. These results tended to exclude the possibility that concentration of the spray fluid occurred through evaporation during spraying. Ultrasonic treatment, or extraction with trichloroacetic acid, of *E. coli* B did not produce a similar increase in radioactive count. The phenomenon thus remains unexplained; it threw considerable doubt on the radioactive count per bacterium. An investigation of the effect of adding sucrose to the phosphate-buffer collecting fluid could not be made because sucrose caused quenching during the scintillation processes involved in the radioactive assay. The extent of quenching was variable and time-dependent and was also related to the degree of radioactivity in the sample to be assayed. For these three reasons the results on aerosol survival presented later were obtained by using a 1-jet spray and *B. subtilis* var. *niger* spores as tracer.

Psychrometric constants

The % relative humidity (RH) is obtained from the relationship,

$$\text{RH} = 100 \times (e/e_a) \%,$$

where e_a is the saturation vapour pressure at the ambient temperature, T_a , and e is given by the expression $e = e_w - A(T_a - T_w)$, where e_w is the saturation vapour pressure at the wet-bulb temperature T_w , and A is the psychrometric constant. According to the theory of August & Apjohn (Penman, 1958) the value of A may be calculated from $A = (p \cdot Cp)/(E \cdot L)$ where p = total pressure, Cp = specific heat of gas at constant pressure, E = ratio of densities of water vapour and dry gas at the same temperature (T_a), and L = latent heat of vaporization of water at the temperature T_a . For air, the value of A calculated as above gives excellent agreement with that used for the derivation of the psychrometric tables of, for example, Marvin (1941) and of the *Handbook of Chemistry and Physics* (1948). The values of A calculated and determined experimentally for the particular conditions used (e.g. a well-ventilated wet bulb) are given in Table 1. The units of pressure were mm. of mercury and of temperature were degrees centigrade. The agreement between the calculated and experimental values of A for air and nitrogen was excellent, while argon gave reasonable agreement; however, the agreement for helium was very poor and suggests that the simple formula of August & Apjohn does not always apply.

Table 1. *Psychrometric constants of the gases used*

Measurements were made at 26.5° over a relative humidity range of 50–16 %.

Gas	A (calc.)	A (expt.)	No. of determinations	S.D.
Air	0.499	0.506	13	0.020
Nitrogen	0.496	0.500	8	0.013
Argon	0.343	0.358	16	0.010
Helium	0.354	0.532	16	0.035

The aerosol survival in nitrogen

Cox (1966*b*) showed that *Escherichia coli* strain COMMUNE gave variants which could have slightly differing survival characteristics. To minimize the possibility that the use of different variants of *E. coli* B might influence the comparisons of the survival in various atmospheres (even though the same stock culture was used throughout) the survival in nitrogen was determined again, both before and after the experiments with argon and helium. In all, 62 experiments in nitrogen were made; Fig. 1 gives the results for the survival at 26.5° and at an aerosol age of 30 min., for collection into phosphate buffer, with and without M-sucrose. For clarity not all the experimental results are given in Fig. 1. The previous data (Cox, 1966*a*; Cox & Baldwin, 1966) showed minima in the survival versus RH curves at 97 %, 85 % and 50 % RH and also poor survival at 100 % RH. The present data are very similar, except that the minimum at 50 % was not detected and that the minimum at 85 % split into a double minimum (or else reproducibility in this region was poor). Since the survival in argon (see later) showed a similar pattern and also the reproducibility of the results in nitrogen at RH values to one side of the minima was good, the former explanation seems more likely.

The aerosol survival in argon

In general terms the survival in argon at an aerosol age of 30 min. for collection in phosphate buffer with and without M-sucrose was very like that in nitrogen, in that instability occurred between 80 % and 90 % RH and between 97 % and 100 % RH, as shown in Fig. 2. Closer examination suggested that the actual position of the minima might be slightly different. A sharp peak (mean of 6 determinations) occurred at 86 % RH, which coincided with a maximum for survival in nitrogen, within the limits of RH measurements. The effect of the collecting fluid was somewhat similar for argon and nitrogen, except in the region of 85 % RH.

Fig. 1

Fig. 2

Fig. 1. The aerosol survival of *Escherichia coli* B sprayed from distilled water into nitrogen as a function of RH, at an aerosol age of 30 min. and at 26.5° ●, Collection into phosphate buffer; ×, collection into phosphate buffer + M-sucrose

Fig. 2. The aerosol survival of *Escherichia coli* B sprayed from distilled water into argon as a function of RH, at an aerosol age of 30 min. and at 26.5° ●, Collection into phosphate buffer; ×, collection into phosphate buffer + M-sucrose

The aerosol survival in helium

Because of the very high cost of helium only limited experiments could be made with it. However, the data (Fig. 3) at an aerosol age of 30 min. for collection in phosphate buffer with and without M-sucrose are sufficient to show great similarity to the survival in nitrogen and argon. Instability occurred between 80 % and 90 % RH, at 97 % RH and at 100 % RH. However, the collection difference between phosphate buffer with and without M-sucrose was probably more marked than with the other

gases in the unstable regions. Like argon and nitrogen, there was a slight peak at 86% RH, within the limits of RH measurements. For all three gases the survivals at low RH were similar.

Fig. 3. The aerosol survival of *Escherichia coli* B sprayed from distilled water into helium as a function of RH, at an aerosol age of 30 min. and at 26.5° ●, Collection into phosphate buffer; ×, collection into phosphate buffer + M-sucrose

DISCUSSION

The results show that the overall survival patterns for *Escherichia coli* B sprayed from distilled water into nitrogen, argon and helium atmospheres were similar. These data are like those previously reported for the survival of this organism in nitrogen (Cox, 1966*a, b*; Cox & Baldwin, 1966), with the exception that the minimum at 50% RH was not detected in the present work. The effect was probably due to the use of variants; *E. coli* strain COMMUNE also gives rise to variants (Cox, 1966*b*). Susceptibility tests to phages T1-T7 inclusive did not show any difference between the present *E. coli* B and that previously used. Anderson (1966) found minima in the survival of *E. coli* B at different RH values, which approximately correspond with those previously reported (Cox, 1966*a, b*; Cox & Baldwin, 1966). A further possible difference between the latter results and those of the present study was that the minimum at approximately 85% RH was in fact a double minimum. Owing to the uncertainty in measuring RH to an accuracy of greater than $\pm 1.0\%$ it is difficult to be sure in the case of nitrogen whether the double minimum existed or whether reproducibility in this region was poor. It is suggested that a double minimum does in fact exist, since the results in argon and helium also indicated a double minimum, and because reproducibility of results at RH values to one side of the minima was good. Results such

as these emphasize the need for an instrument that accurately measures RH to an order of ± 0.2 RH % or better.

Biological inertness of the three gases

A possible difference between the survival in the three gases was that the corresponding RH values for one part of the double minima were 85.7 %, 82.5 % and 82.5 %, respectively, for nitrogen, argon and helium, whereas the other part was 86.8 ± 0.3 % RH for all three gases. The difference between nitrogen and argon is outside the experimental error, while the value of 82.5 % for helium agrees very well with that of argon. Hence there would seem to be a slight difference between nitrogen and argon or helium. Also differences occurred at high RH in terms of the method for collection. As a consequence of these findings it is suggested that slight differences in behaviour do exist for aerosols of *Escherichia coli* B sprayed from distilled water into nitrogen, argon and helium, and that these atmospheres are not truly inert. This indicates that the gaseous atmosphere is involved in the death processes which occur at high RH, and which are independent of the presence of oxygen (Cox & Baldwin, 1967). It is possible that the actual water structure is involved in these death processes, and in which case the gases may operate through modification of this structure. It is known that nitrogen and argon, but not helium, form gas hydrates (clathrates) with water and modify the water lattice (van der Waals & Platteeuw, 1959). Also all three gases can help stabilize clathrates of a more polarizable solute which is also present (van der Waals & Platteeuw, 1959). It is also possible that the gases and water compete for the same sorption sites.

Influence of initial evaporation rate on survival.

Cox (1965; 1966a) discussed the possibility that aerosol survival is influenced by the initial evaporation rate of the aerosol droplet, and Poon (1966) concluded from his investigation of the aerosol survival of *Escherichia coli* that the rate of death and the initial rate of water evaporation followed a similar relationship with respect to RH and temperature. Webb (1959) also tried to correlate rate of death with initial rate of water evaporation.

The data presented in the present paper are suited for a critical examination of the relationship between death and evaporation rate. As shown, at high RH, zones exist where *Escherichia coli* B sprayed from distilled water is particularly susceptible to death in nitrogen, argon and helium. Hence if the degree of killing is related to the initial evaporation rate, then the position of the zones in the survival versus RH curves in helium would have been very different to those in nitrogen and argon, for the reasons outlined below. The approach of Eisner, Quince & Slack (1960) was used to calculate the initial evaporation rate of water droplets, namely the equations (1) and (2) below.

$$\frac{dm}{dt} = \frac{4\pi MD}{RT}(f \cdot p_t - p_0) \frac{a}{(D/aV\alpha + 1)}, \quad (1)$$

where

dm/dt = rate of evaporation of a droplet radius, a ,

M = molecular weight of water,

D = diffusion coefficient for water vapour into the gas,

f = relative humidity as a fraction,

p_t = vapour pressure of water at the ambient absolute temperature, T ,
 p_θ = vapour pressure of water at the droplet temperature, θ ,
 R = gas constant,
 $V = (RT/2\pi M)^{\frac{1}{2}}$
 α = coefficient of evaporation,

and

$$\theta = \frac{LMD(f.p_t - p_\theta)}{KRT(D/aV\alpha + 1)} \quad (2)$$

where L is the latent heat of vaporization of water, and K is the thermal conductivity of the gas.

It is necessary to calculate the equilibrium temperature θ of an evaporating water droplet, because water is comparatively volatile and cooling of the droplet occurs during evaporation, and hence affects the vapour pressure of the water. At 85 % RH and 26.5° the rate of evaporation in helium is approximately twice that in nitrogen. At this temperature the evaporation rate in nitrogen at 85 % RH would be achieved in helium at 93 % RH. Hence if the initial evaporation rate of an aerosol droplet influenced the degree of killing of *Escherichia coli* B, then the position of the minimum in helium would have been at 93 % RH and not at 82.5 %. Such a change would be very readily detected; in fact it clearly did not occur (Fig. 3). Hence it is possible to conclude that viability does not depend upon the initial rate of evaporation. Further support for this statement comes from a study of equation (1), which indicates that the evaporation rate increases as the RH decreases. Yet the present work (Figs. 1-3) and previous work (Cox 1966*a*, *b*, 1967; Cox & Baldwin, 1966) show that in inert atmospheres the survival is greater at low rather than at high RH values. For *E. coli* such studies and comparisons must be made in inert atmospheres because at low RH, oxygen is toxic (Hess, 1965; Cox & Baldwin, 1967) and therefore such comparisons of aerosol survival in air would be invalid.

The rate of evaporation in helium is greater than that in nitrogen at a given temperature and humidity for two reasons: (i) the diffusion coefficient of water vapour into helium is much greater than into nitrogen (Schwartz & Brow, 1951); (ii) the thermal conductivity of helium is very much greater than that of nitrogen (*Handbook of Chemistry and Physics*, 1965).

The influence of relative humidity on survival.

Because the degree of killing does not depend upon the initial rate of evaporation of the aerosol droplet it seems very likely, as suggested by Cox (1966*a*), that protective agents do not operate through a modification of initial evaporation rate. Since the initial evaporation rate has been excluded as being related to the degree of killing, it is of interest to consider in what other way RH can influence survival. Scott (1958), Webb (1960) and Bateman, Stevens, Mercer & Carstensen (1962) have shown that the water content of bacteria is related to RH. The nature of the relationship is fairly typical of the water sorption isotherms of other biological materials (e.g. Bull, 1951). A plot of water sorbed versus RH yields an S-shaped curve, with the slope being greatest between 70 and 100 % RH, i.e. a small change in RH in this range produces a comparatively large change in water content. It is suggested that RH operates by controlling the water content of the bacterium which in turn is related to the

mechanism of death. Such an explanation also provides a reason why the survival of *Escherichia coli* is so dependent upon RH, since the region of critical RH zones is in the 70–100% range where water content changes rapidly with RH. When survival is plotted as a function of water content of the bacterium, rather than RH, the regions where *E. coli* is unstable are much broadened. Cox (1966*a*) suggested that DNA might be affected by aerosolization owing to the formation of hydrates which occurs in a semi-reversible manner and produces a biologically inactive moiety. Benbough (1967) has shown that aerosolization does not impair DNA synthesis in *E. coli*, although RNA synthesis may be slightly affected. The reason that *E. coli* B when collected from the aerosol at high RH and subjected to phage T7 does not reproduce this phage (Cox & Baldwin, 1966) would therefore not seem to be caused by inability to synthesize DNA. The impairment of RNA synthesis would not seem to be sufficiently great to suppress drastically colony formation and phage T7 reproduction. The cause of these phenomena for *E. coli* B collected from the aerosol at high RH remains unexplained. However, the manner in which water re-enters the bacterium after collection from an aerosol can play a very important role in the death mechanisms which occur for aerosolized *E. coli* stored at high RH (Cox, 1966*a, b*).

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