The Effects of Oxygen on the Possible Repair of Dehydration Damage by *Escherichia coli*

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SUMMARY

Proflavine in the post-aerosolization plating medium lowered by over 80% the number of viable *Escherichia coli* B/R organisms recovered from aerosols of 55% relative humidity (RH) and above, but had no effect on the recovery of viable *E. coli* WP2_s organisms. Oxygen enhanced the lethal action of semi-dehydration on *E. coli* strain B/R, held at 50% RH and below, but had less effect on the survival of *E. coli* WP2_s. In addition, *E. coli* B/R organisms displayed an increased rate of respiration, and discharged 260 m μ absorbing material, when held at intermediate values of RH. These phenomena were not observed with *E. coli* WP2_s organisms. Thus *E. coli* B/R seems to possess an energy consuming mechanism by which it is able to survive semi-dehydration above 55% RH by a discharge of damaged cell components. This mechanism is very slowly destroyed when the organisms are semi-dehydrated in nitrogen but, below 50% RH, it is rapidly inactivated by oxygen. It is suggested that the mechanism is a membrane-bound system, possibly involving the cytochrome chain.

INTRODUCTION

When bacteria are held in an aerosolized state for prolonged periods, the rate at which they lose viability depends on the quantity of water vapour and oxygen present in their gaseous environment and on the environmental temperature. Much of the relevant work done between 1920 and 1953 has been summarized by Wells (1955) and by Webb (1965). From these investigations it was concluded that one of the main factors which determines the rate of loss of viability is the partial pressure of water vapour. Between 10 and 40° the partial vapour pressure and temperature, usually expressed as a relative humidity (RH), controls the rate at which organisms die, rather than the absolute water content of the air (Wells, 1955), apparently because the RH determines the quantity of water bound to macromolecules within the cell (Webb, 1960). Most investigators have found organisms held at 25° and at relative humidities above 75% to be stable. In contrast, however, Cox (1967, 1968) found that *Escherichia coli* died rapidly when held in an atmosphere of nitrogen at relative humidities between 85 and 75%.

Oxygen appears to enhance the rate at which organisms die only when they are held at RH values below 40% (Webb, 1965, 1967; Hess, 1965), suggesting that the presence of water prevents the oxidation of cell constituents. The mechanism by which they are killed at relative humidities between 80 and 40%, therefore, appears to differ from that responsible for their death at 40% RH and below. Results are described here

which indicate that the respiratory mechanisms of bacteria are damaged by oxygen when they are held below 40% RH but that, at higher RH values, death appears to be due, in part, to the destruction of a mechanism by which some strains are able to repair the dehydration damage which otherwise would impair their ability to manufacture protein and nucleic acid.

METHODS

Organisms. (a) *Escherichia coli* B/R (NCIB 9485), a strain resistant to ultraviolet radiation (u.v.) due to its ability to dark repair DNA damage, and (b) *Escherichia coli* WP2_s, a tryptophan requiring mutant of *E. coli* B/R, which is unable to perform host cell reactivation of bacteriophage, is highly sensitive to u.v. and has a reduced ability to dark repair u.v. damage (Hill, 1965; Ashwood-Smith & Bridges, 1966).

Preparation of aerosols in air. *Escherichia coli*, strains B/R and WP2_s, were grown for 24 hr at 37° in Bacto Nutrient Broth (NB), harvested and washed twice in de-ionized distilled water and resuspended in either (a) distilled water or (b) 0.3 M-raffinose or 0.3 M-myo-inositol to a concentration of 1×10^{10} bacteria/ml. These organisms were then aerosolized for 5 min. into a 800 l. stainless steel rotating drum by using a Collison spray operating at 25 p.s.i. The air was supplied from a carbon ring compressor and, before use, was passed through two bacterial filters (to remove fine particles) and two columns each of activated charcoal and silica gel. The RH value was pre-set by spraying distilled water into the drum until a given RH value had been obtained. Samples were collected from the aerosol by using a liquid impinger containing 9 ml. 0.5 M-glucose in 0.85% (w/v) NaCl. Serial ten-fold dilutions then were made in nutrient broth and 0.2 ml. of each dilution plated onto nutrient agar and nutrient agar containing 5 µg. proflavine/ml. To the remainder of the initial sample 1 ml. 0.1% (w/v) casamino acids in potassium phosphate buffer (pH 6.9) was added and immediately a 2 ml. sample withdrawn. The rest was placed in an incubator at 37° and allowed to incubate for 45 min. The 2 ml. portion originally removed was passed through a Millipore filter to remove bacteria, and the same treatment was given to the incubated bacteria following the 45 min. incubation period. The difference between extinction at 260 mµ. of filtrates from the initial and the incubated samples was determined with a Beckman DB spectrophotometer.

One of the difficulties in the estimation of the respiration rate of bacteria after periods of storage in air is the rapid loss of viability encountered at low RH values. To overcome this problem the storage time in aerosols was decreased to 5 min., samples were taken as above and the organisms in each sample concentrated in 0.1 M-phosphate buffer, to 5×10^8 bacteria/ml., by centrifugation. The bacterial concentration was standardized with a Coleman nephelometer and, therefore, the bacterial count at the time the O₂ uptake measurements were made was a total count, not a viable one. Viable counts were made by serial dilution, plating on nutrient agar and counting the colonies after 24 hr of incubation. After the bacteria had been concentrated, 2 ml. portions were placed in the main chamber of a Bronwell Warburg flask and 0.5 ml. of a solution of 0.1 M-glucose + 500 µg. casamino acids/ml. placed in the side arm. Oxygen uptake was measured at 37° with double capillary manometers.

Preparation of aerosols in nitrogen. The bacterial suspensions were prepared as above, but using deaerated (boiled) distilled water. The suspension was then placed into a Collison spray and nitrogen gently bubbled through it for about 60 min. For

use in the aerosol studies the nitrogen was washed successively with (a) alkaline pyrogallol, (b) water, (c) $\text{N-H}_2\text{SO}_4$, (d) N-NaOH , (e) water, and then passed through the silica driers and charcoal columns to the aerosol drum. The drum was purged with the cleaned nitrogen for 2 hr and then a Collison spray, containing water free from dissolved oxygen, was introduced into the system. The spray was operated with nitrogen until a given RH value was reached and then replaced by the spray containing the bacterial suspension which was 'atomized' into the drum for 5 min. All other procedures were as above. The reasons for the extensive washing of the nitrogen will be evident from the text.

Measurement of release of thymidine-2- ^{14}C . Bacteria of both strains were grown at 37° for 24 hr in a medium consisting of: 0.1 M-glucose, 500 μg . casamino acids/ml. and 0.8 μC . [^{14}C]thymidine (34 mc./m-mole)/ml. all in 0.1 M-phosphate buffer (pH 6.9). The [^{14}C]-labelled thymidine was purchased from New England Nuclear Corp., Boston, Mass., U.S.A. The bacteria were harvested, washed, aerosolized and collected as above. After 15 min. of storage, triplicate 10 ml. samples were taken and casamino acids (to 500 μg ./ml.) and, in some cases, proflavine (to 5 μg ./ml.) added to each. The samples were incubated for 45 min. at 37° and the bacteria removed by passage through Millipore filters of 0.45 μ porosity. Each filtrate was evaporated to dryness *in vacuo*, resuspended in 0.5 ml. of distilled water and placed into a 1 in. (2.54 cm.) stainless steel planchette containing a 0.75 in. (1.9 cm.) diameter filter paper disc previously glued to the bottom of the planchette. The samples were then dried under an infrared lamp and the radioactivity present determined with a Phillips Gas Flow Counter.

RESULTS

Effects of oxygen and relative humidity

The two strains of *Escherichia coli* responded differently to changes in the RH value of the atmosphere in which the organisms were held. *E. coli* B/R, both in nitrogen and in air, was considerably more stable than the WP2_s strain, especially at relative humidities above 40% (Fig. 1). In addition, the ratio between the percentage recoveries of strains B/R and WP2_s after 15 min. in air or nitrogen showed that the difference in stability of the two strains was greatest when the bacteria were held at intermediate humidity values (Fig. 1c). Oxygen affected the survival of strain B/R to a greater extent than it did that of strain WP2_s. However, oxygen enhanced the death rate only at relative humidities below 50% (Fig. 1).

Effect of proflavine

Webb & Tai (1968) showed that the ability of airborne organisms to repair u.v.-induced damage was gradually destroyed when they were held at RH values below 55%. Since the two strains of *Escherichia coli* used vary in their ability to repair damage induced by u.v. radiation, it seemed possible that the added resistance of strain B/R to dehydration at RH values above 50% might reside in an ability to repair comparable damage induced by semi-dehydration. Proflavine in the plating medium, as well as related acridine dyes and caffeine, apparently inhibit the repair system (Witkin, 1963; Lieb, 1964). Following aerosolization between 55 and 75% RH proflavine in the growth medium greatly diminished the recoverable number of colony-forming organisms of *E. coli* B/R; the recovery of *E. coli* WP2_s was not affected (Fig. 2).

This decrease in the number of surviving bacteria of strain B/R, due to proflavine, occurred both when the bacteria were held in air or in nitrogen at RH values between 75 to 55%. Holding them in air at RH values below 55%, however, resulted in a rapid decrease in the action of proflavine (Fig. 2 *c, d*).

Fig. 1. The effect of relative humidity and oxygen on the survival of airborne *Escherichia coli* strains B/R and WP2s after storage for 15 min. (a) *E. coli* B/R (Δ) bacteria stored in N_2 , ($\times$) bacteria stored in air. (b) *E. coli* WP2s (Δ) bacteria stored in N_2 , ($\times$) bacteria stored in air.

Release of 260 m μ -absorbing material and [^{14}C]thymidine

Since it was possible that the proflavine-sensitive function was similar to that by which the bacteria were able to repair u.v. damage, the presence of 260 m μ -absorbing material in the filtrate of bacteria incubated after periods of storage was examined. *Escherichia coli* WP2s held at RH values between 90 and 10% and incubated for 45

min. following aerosolization showed no significant increase in 260 $m\mu$ -absorbing material in supernatant or filtrate fluids. However, an increase in u.v.-absorbing material in the filtrate fluids of strain B/R was observed. This reached its maximum quantity after organisms had been stored at about 60% RH (Fig. 3), coinciding with both the relative sensitivities of the two strains used and the effect of proflavine in the plating medium; maximum differences between the two strains occurred when the bacteria were held at 55 to 60% RH.

Fig. 2. The effect of relative humidity and proflavine on the recovery of *Escherichia coli* STRAINS B/R and WP2s from aerosols in N_2 and air. (a) *E. coli* B/R (Δ) bacteria held in N_2 plated on nutrient agar; ($\blacktriangle$) bacteria held in N_2 plated on nutrient agar containing 5 μ g. proflavine/ml.; ($\times$) bacteria held in air plated on nutrient agar; ($\bullet$) bacteria held in air plated on nutrient agar plus 5 μ g. proflavine/ml.; (b) *E. coli* WP2s ($\blacktriangle$) bacteria held in N_2 plated on nutrient agar; (Δ) bacteria held in N_2 plated on nutrient agar + proflavine; ($\bullet$), ($\times$) bacteria held in air and plated on nutrient agar and nutrient agar + proflavine, respectively; (c) *E. coli* WP2s; relative recovery = fraction surviving on nutrient agar/fraction surviving on nutrient agar plus proflavine; (d) *E. coli* B/R relative recovery same as for (c) above. N_2 and air = gas in which bacteria were held. Storage time, 15 min.

In an attempt to ascertain whether the released u.v.-absorbing material was DNA, the radioactive counts/min. from a standard number of [^{14}C]thymidine-labelled bacteria, before and after 45 min. of incubation, was determined. This procedure failed to reveal DNA loss because the quantity of radioactive material released represented considerably less than 1% of the total cell DNA and the experimental error in radioactive counting was greater than 1%. The radioactivity found in the filtrates, however, simulated their 260 $m\mu$ extinction value; the amount present in filtrates from *Escherichia coli* B/R suspensions was greatest after storage at 60% RH and decreased as the RH was decreased, especially when the organisms were held in air.

A similar trend was found with strain WP2₈, but the quantity of released material was small. Proflavine decreased the radioactivity in filtrates of *Escherichia coli* B/R, but not that in *E. coli* WP2₈ filtrates (Table 1). It appeared, therefore, that the survival of *E. coli* B/R at RH values above 60% depended on its ability to reject damaged material.

Fig. 3. The survival and release of 260 mμ.-absorbing material from *Escherichia coli* strains B/R and WP2₈ after being held for 15 min. in aerosols of various relative humidities with protective additives. (a) The release of 260 mμ.-absorbing material from bacteria held in air for 15 min. *E. coli* B/R aerosolized from a suspension in 0.3 M-myoinositol (△), 0.3 M-raffinose (○), H₂O (×); *E. coli* WP2₈ aerosolized from a suspension in 0.3 M-myoinositol (▲), 0.3 M-raffinose (●), H₂O (×); (b) ratio of the surviving fractions of *E. coli* B/R obtained from aerosols in air and N₂ in the presence of myo-inositol and raffinose.

Effect of protective compounds

To examine the above possibility, the ability of two compounds to preserve the mechanism by which damaged material was discharged from the organism was examined. Myo-inositol, a compound able to enter the cell, and raffinose, a compound unable to permeate through the cell membrane, were both able to preserve this mechanism at all relative humidities studied (Fig. 3). There was, however, a difference in the ability of these two compounds to preserve the viability of the organisms. Myo-inositol afforded protection to bacteria held either in nitrogen or in air at all the RH values studied. Raffinose, on the other hand, offered little protection to organisms held

at high RH value, but displayed a high protective ability at the low RH values (Fig. 3b). This particular finding with raffinose agrees with the observations of Cox (1968). Raffinose afforded *Escherichia coli* strain WP2₈ organisms only a small measure of protection at low relative humidities when they were held in either air or nitrogen and the protection afforded them by myo-inositol was not as great as that afforded to *E. coli* strain B/R organisms.

Table 1. *The influence of relative humidity on the release of [¹⁴C]thymidine from Escherichia coli after storage in aerosols for 15 min.*

E. coli strain B/R and WP2₈ were held for 15 min. in N₂ or air at set values of relative humidity (RH), resuspended in glucose + casamino acids with (†) and without (*) 5 µg. proflavine/ml., incubated at 37° for 45 min. and the bacteria removed by Millipore filtration. The filtrates were evaporated *in vacuo*, the solids dissolved in 0.5 ml. water, deposited into stainless steel planchettes, dried under an infrared lamp and radioactive counts determined with a gas flow counter. The numbers presented are the radioactive counts/min. from 10 ml. of culture filtrate and are the averages of six independent determinations.

Strain	Holding gas	Non-dried control	Holding aerosol relative humidity					
			30	40	50	60	70	80
			radioactivity (counts/min.)					
B/R	Air	*21	26	101	214	621	533	241
	N ₂	*14	246	543	510	640	430	284
	N ₂	†16	44	61	66	88	43	51
WP2 ₈	Air	*16	22	19	31	21	33	69
	N ₂	*14	42	53	71	92	66	48
	N ₂	†21	48	62	59	74	76	52

Effects on respiration

The rate at which *Escherichia coli* B/R consumed oxygen increased after they had been stored in air or nitrogen at relative humidities between 80 and 55%. At RH values below this, however, storing the organisms in air substantially decreased the respiration rate, but storage in nitrogen did not. *E. coli* WP2₈, on the other hand, displayed no increase in respiration rate but a decrease, which seemed to parallel the loss in viability (Fig. 4 & Table 2).

Influence of N₂ purity

Throughout these investigations differences seemed to exist between the behaviour of the two strains of *Escherichia coli* used and that of *E. coli* K12 as reported by Cox (1968). Both of the strains used here were stable at relative humidities above 80%, which contrasted with the behaviour of the *E. coli* K12 strain used by Cox, which apparently is unstable in aerosols at high RH values. The reasons for this discrepancy are not understood, but such effects may result from traces of gaseous toxic materials in the local air emanating from newly painted laboratories, the use of laboratory insecticides, and from the presence of toxic materials in crude nitrogen. Often traces of these toxic materials cause a tendency to high death rates at high relative humidities (Webb, 1965). The effects of using the nitrogen directly from the supply tank and of washing through alkaline pyrogallol only are shown in Fig. 5. When the bacteria were held in aerosols generated with the crude unwashed nitrogen they tended to die more

rapidly in atmospheres of 70 to 80% RH. This particular effect was greatly enhanced when the nitrogen was washed through alkaline pyrogallol only; presumably small amounts of pyrogallol in the nitrogen led to the high death rates.

Fig. 4

Fig. 4. The relative rate of glucose oxidation by *Escherichia coli* strains B/R and WP2_s after storage in air or N₂ for 15 min. *E. coli* B/R in N₂ (▲), in air (×); *E. coli* WP2_s in N₂ (●), in air (○).

Fig. 5

Fig. 5. The effect of the N₂ washing procedure on the deaths of *E. coli* B/R held for 15 min. in aerosols at various relative humidities.

Table 2. *The influence of relative humidity and storage gas on the uptake of O₂ by Escherichia coli after 5 min. in aerosols*

After aerosolization the bacteria were resuspended in 0.1M-potassium phosphate buffer to a total bacterial concentration of 5×10^8 /ml. The number was determined by nephelometry. Triplicate samples of 2 ml. were used to measure O₂ uptake in a glucose + casamino acids medium (A) and 1 ml. portions for viable counts on nutrient agar (B). A, O₂ uptake μ l./hr; B, % recovery.

		Holding aerosol relative humidity													
		Non-dried control		30		40		50		60		70		80	
Strain	Storage gas	A	B	A	B	A	B	A	B	A	B	A	B	A	B
B/R	Air	58	100	18	21	44	38	76	61	104	81	87	88	73	96
	N ₂	53	100	74	63	81	76	88	79	93	88	86	89	61	94
WP2 _s	Air	56	100	17	22	18	24	16	29	31	48	62	74	61	86
	N ₂	54	100	16	30	17	35	24	40	36	59	59	68	58	81

DISCUSSION

It seems clear that the survival of these two strains of *Escherichia coli* in aerosols of above 50% RH is independent of the presence of oxygen. The greater sensitivity of *E. coli* WP2_s over *E. coli* B/R to semi-dehydration at RH values above 60% seems to be due, in part, to an inability of the latter strain to carry out some kind of repair involving the elimination of 260 μ .-absorbing material from the cell. *E. coli* B/R, on the

other hand, seems capable of overcoming damage produced at high relative humidities by some mechanism which is inhibited by the presence of proflavine in the plating medium. The increased respiration of *E. coli* B/R organisms after a period of storage above 60% RH, a phenomenon which does not occur with *E. coli* WP₂₈, suggests that the mechanism requires energy. Moreover, part of the protective action of both raffinose and myoinositol appears to be to prevent the destruction of this particular mechanism in *E. coli* B/R cells. The fact that both the increased respiration and the release of u.v.-absorbing and [¹⁴C]thymidine labelled material from the organisms rapidly decreases when they are held in air below 50% RH and, at this particular RH value, oxygen begins to have a lethal action, all suggests that at low RH values the repairing mechanism is inactivated by oxygen. The ability of raffinose to preserve the integrity of this system when the bacteria are held at low RH values, plus the apparently necessary enhanced respiration, suggests that the mechanism of dehydration repair is controlled by some function of the cytoplasmic membrane.

Bacteria do not possess mitochondria and the mitochondrial function may be exercised by the cytoplasmic membrane. In addition, portions of the bacterial chromosome are known to be in contact with the cytoplasmic membrane at various places. It is possible, therefore, that a compound unable to enter the cell, but able to penetrate partially into the cytoplasmic membrane, would be able to preserve the integrity of that portion of the membrane system involving energy production and the possible repair of those segments of the bacterial chromosome associated with the membrane. The release of u.v.-absorbing materials from organisms held in aerosols at high RH values and those protected by myo-inositol at low RH values has been reported earlier (Webb, 1965). The liberation of this material from the bacteria has been found to be associated with survival rather than with death. To the author's knowledge, however, this release of material has not been suggested to be characteristic of particular strains. The nature of this repair system remains to be elucidated, but it is possibly the same mechanism as that responsible for the 'dark' repair by u.v.-induced damage. Because oxygen seems to be required to inactivate the system, some easily oxidizable cellular constituent appears to be involved in the repair mechanism. These types of compound are involved also in the respiratory systems of the organism which seem to be stimulated during the process of repair. Such considerations, along with the diminished respiration of the organisms after storage in air of low relative humidities, points to death resulting from the inactivation at low RH values of some part of the mitochondrial or cytochrome system which, in some strains, is able to repair semi-dehydration damage. The apparent action of oxygen on this repair mechanism may also explain why oxygen seems to play no role in the inactivation of airborne viruses (deJong & Winkler, 1968). Certain experimental procedures, such as the exposure of bacteria to 365 m μ . light (Kelner, 1949), holding them in water or at elevated temperatures (Stein & Meutzner, 1950) will induce some cells to repair u.v. damage of DNA. Whether or not these procedures stimulate the operation of parts of a single metabolic process is uncertain; however, all of them, including the 'dark' repair of u.v. or semi-dehydration damage, seem to rely on an enhanced activity of the cytochrome chain produced either by an increase in respiration rate or the absorption of 365 m μ . light by the quinones or flavenoid pigments of this chain. The finding that some bacteria possess a system by which they are able to overcome semi-dehydration damage presents the interesting possibility that those repair systems able to cope with damage due to 254 m μ . u.v.

radiation, a wavelength not present in sunlight, may have evolved in response to other physical stresses such as semi-dehydration and extremes of temperature. Indeed, in some cases, these systems now may have evolved to a point where the continuity of biological life cycles, especially the germination of spores or seeds, depends on the activation of the system through exposure to periods of semi-dehydration, extremes of temperature and even sunlight itself (Borthwick *et al.* 1952).

The apparent difference between the behaviour at 80% RH of the two strains of *Escherichia coli* used here and that used by Cox (1968) may be due to the condition of the air existing in respective laboratories or the purity of the nitrogen used. Alternatively, the strain used by Cox may lack a repair system or its prophage may be a defective one. The latter is suggested since semi-dehydration at RH values above 70% will induce some prophages and, should the prophage be a defective one or made defective by the dehydration process, simple plaque counts would not detect induction (Webb & Dumasia, 1967*a, b*).

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