# THE SURVIVAL OF AIRBORNE MICROORGANISMS

## II. EXPERIMENTS WITH *ESCHERICHIA COLI* NEAR 0°C

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*Summary*

The humidity/death rate relationships of two types of airborne *Escherichia coli* were studied at approximately 0.5°C over the range 45-100 per cent. relative humidity. Support was obtained for previous findings with psychrophilic bacteria of minimal death rates at about 70 per cent. R.H.

Evidence suggests that after spraying into the air from an aqueous suspension, bacterial cells come rapidly into equilibrium with the atmosphere and that measured death rates are associated with these equilibrium conditions.

At least two separate lethal mechanisms operating near 50 per cent. and 90 per cent. R.H. respectively seem to be potentially available for the destruction of airborne bacteria. For *Esch. coli,* at least, the lower-humidity mechanism is probably a direct water activity effect. The high-humidity mechanism seems to be neither a water activity effect nor a "cold shock" reaction.

Death of *Esch. coli* at low humidities was not affected by the age of the cells, but at high humidities death was most rapid with young cultures.

Differences in behaviour at high humidities were observed between rough and smooth, and aerated and anaerobic cultures.

## I. INTRODUCTION

It was shown previously (Brown 1953) that certain bacteria when airborne at a temperature near 0°C are likely to have a minimal death rate in the region of 70 per cent. relative humidity (R.H.). The bacteria used were capable of growth at 0°C. Therefore experiments were made near 0°C using an organism with mesophilic temperature requirements. Although it has no significance in chilled meat storage, *Escherichia coli* was selected for this investigation as it has the advantages of a well-studied physiology and has been used in air disinfection studies at higher temperatures. In the present paper attention is also directed towards the elucidation of factors that may affect the sensitivity of a bacterial cell to atmospheric conditions.

## II. MATERIALS AND METHODS

The same equipment as described in the previous paper (Brown 1953) was used, but slight modifications in technique were made, as described.

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### (*a*) *Humidity and Temperature Control*

A vessel of water heated electrically by an immersion unit was substituted for the trays of water in the high humidity range. This change facilitated a more continuous selection of humidities and permitted extension of the range to 100 per cent. R.H. The immersion heater was also used to raise the humidity 10-15 per cent. during the course of some experiments.

In later runs at 90 per cent. R.H. room temperature was raised to 1°C to avoid freezing of the wet thermometer bulb. This change had no measurable effect on death rate.

### (*b*) *Sampling*

Because of evidence of an early change in death rate in some cases, the sampling schedule was modified so that the first six exposures were of 30 sec duration at intervals of 1 min. These plates were rotated at 2 r.p.m. In the later work at 90 per cent. R.H. all plates were exposed for 30 sec and rotated at 2 r.p.m. to a time schedule most suited to the death rates encountered. Incubation was at 37°C for 18-22 hr.

### (*c*) *Organisms*

*Esch. coli*, types 1 and 2, were used. The organisms were grown at 37°C in the following ways:

(1) As standing cultures in tubed nutrient broth (6-8 ml) for 18 hr. These cultures were also used as inocula.

(2) As surface cultures on nutrient agar for 18 hr.

(3) In nutrient broth (60 ml) aerated by the principle described by Skerman, Lack, and Millis (1951).

(4) Anaerobically in nutrient broth (6 ml) in evacuated glass ampoules.

Standing tubes and agar cultures were inoculated with approximately $10^6$ cells. In the other cases the inoculum is given with the appropriate results.

In order to observe time schedules etc., it frequently became necessary to store a culture for up to 4 hr at $-0.5$°C before use. This treatment was found to have no effect on subsequent results. Broth cultures were harvested by centrifugation and resuspended in an equal volume of sterile glass-distilled water. The turbidity of this suspension was determined and a calculated volume diluted to 100 ml with sterile glass-distilled water. For some types of experiment turbidity was measured in the original broth culture. Agar cultures were dispersed into glass-distilled water (6 ml) and thence treated identically with the broth-grown cells. Suspensions obtained in this way were sprayed for 1 min into the test room.

In the course of the work it was found that frequently, but not invariably, the death rate of airborne cells was not a simple logarithmic function. In such cases a high death rate was obtained in the first 10-20 min of a run, after which the rate decreased to a value which was usually the same as that obtained when no change in slope was observed. An expression of the initial rapid death as a rate was difficult as in most instances plots of log survivors against time were best joined by a smooth curve which merged into the "normal"

linear regression. During the early part of the investigations the following observations concerning this change in slope were made:

(1) The change in slope could not be associated with any change in the atmospheric conditions in the test room.

(2) Of the organisms examined in this and the preceding paper (Brown 1953) the type 2 and one type 1 strain of *Esch. coli* were the only ones in which the phenomenon was observed.

(3) Conditions which caused an increase in "normal" death rate also caused an increase in the initial high death rate.

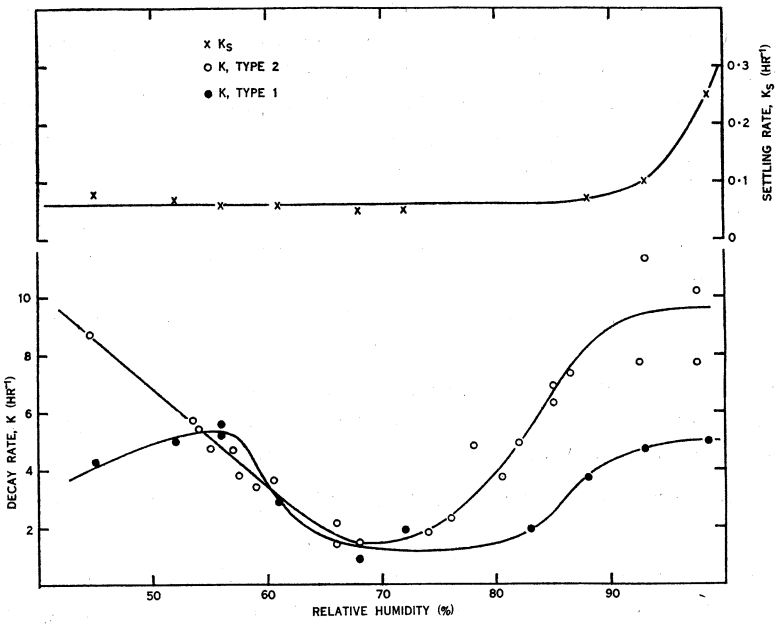


Fig. 1.—*Esch. coli*, types 1 and 2. Decay rate ($K$) and settling rate ($K_s$) *v.* humidity, relationships of standing broth cultures.

It was subsequently realized that the type 1 strain which gave an initial high death rate, also produced a slightly rough mutant, whereas a second type 1 strain, which showed only a smooth-mucoid transformation, always gave logarithmic death.

A little later in the programme the type 2 organism at high humidities began to give atypical results, which included complete departures from logarithmic death for the duration of an experiment. At this stage it was realized that rough colonies were present and subseqently at least three different variants were isolated, ranging from smooth to very rough. The smoothest variant was selected for extension of the investigations. While it remained smooth, logarithmic death was always obtained (at high humidities) and, conversely, departures from a straight line were always associated with colony heterogeneities. On the other hand, colony heterogeneities were not always associated with non-

logarithmic death. Departures with this strain were most commonly observed in old cultures (e.g. 48 hr).

Values of $K$ which follow represent the "normal" values in all cases. The calculation of $K_s$ was complicated when non-logarithmic death occurred and values given are derived from runs in which the change in slope was least significant.

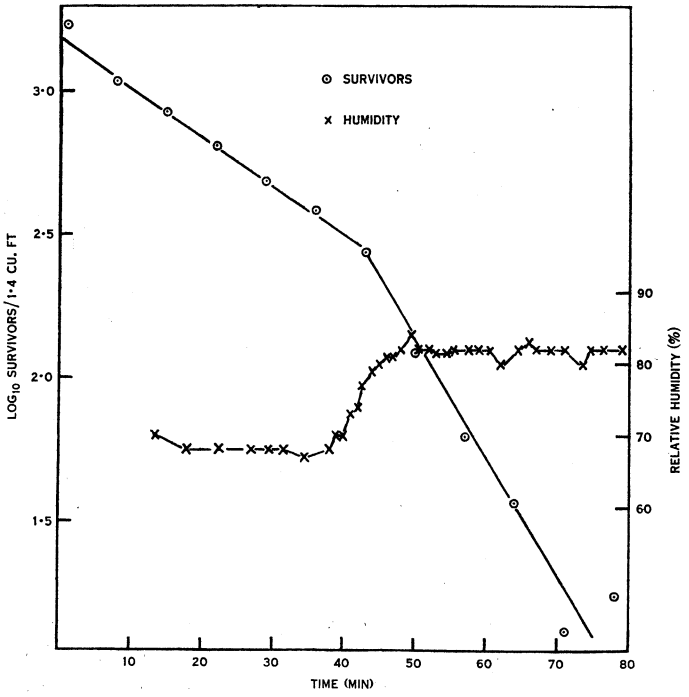


Fig. 2.—*Esch. coli*, type 2. Effect on survival of increasing humidity from 68 to 82 per cent. R.H. Standing tube culture (cf. Fig. 1).

## III. Results

### (*a*) *Effects of Relative Humidity*

(i) *Death/Humidity Relations.*—The relations between decay rate ($K$), settling rate ($K_s$), and relative humidity are shown in Figure 1 for *Esch. coli* types 1 and 2. The occurrence, in both strains, of minimal death rate in the 70 per cent. R.H. region and the magnitude of $K$ at this minimum are both features of similarity with the psychrophilic bacteria already described. In view of the relatively small number of points obtained the location of the maximum in the type 1 curve should not be regarded too rigidly. Because of the greater magnitude of the effects on type 2, this strain was used in the extension of the work.

(ii) *Reversibility of Desiccation.*—In experiments in which humidity was raised some 40 min after delivery of the bacterial aerosol, it was found (Figs.

2 and 3) that desiccation of the cells was readily reversible (as measured by death rate) and that the measured death rates were associated with equilibrium conditions.

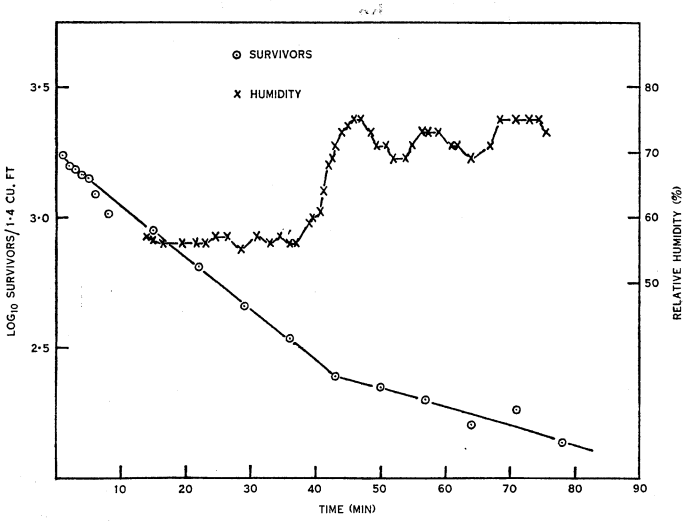


Fig. 3.—*Esch. coli*, type 2. Effect on survival of increasing humidity from 56 to 73 per cent. R.H. Agar surface culture (cf. Fig. 1 and Section III (*b*) (i)).

### (*b*) *Effects of Growth Conditions and Changes in Physiology*

(i) *Growth on Agar Surfaces.*—In further experiments in which growth conditions were varied, cells grown on agar surfaces were found to have the same death rates below 70-75 per cent. R.H., but were much more resistant at high humidities than broth-grown cells (Table 1).

TABLE 1

*ESCHERICHIA COLI* TYPE 2. COMPARISON OF DEATH RATES OF BROTH- AND AGAR-GROWN CELLS WHEN AIRBORNE AT HIGH HUMIDITIES

| Relative Humidity (%) | $K_d$(Hr$^{-1}$)* | |
|---|---|---|
| | Broth-grown | Agar-grown |
| 75 | 1·6 | 1·2 |
| 80 | 4·0 | 1·6 |
| 85 | 6·5 | 2·1 |
| 90 | 9·0 | 3·5 |
| 95 | 9·4 | 5·2 |

* Values for $K$ are interpolated from Figure 1 for the broth-grown cells and from a similar curve (not shown) for the agar-grown cells.

(ii) *Effect on Survival of Aeration During Growth.*—The foregoing results suggested a possible implication of oxygen in determining sensitivity at high humidities. Accordingly a number of comparisons was made at high and low humidities using cells grown for 18 hr in four different ways ranging in effect from complete anaerobiosis to full aeration. The results of this comparison are given in Table 2, from which it is seen that, again, little or no effect was apparent at low humidities, while at 90 per cent. R.H. greatly increased resistance was associated with aeration during growth.

TABLE 2

EFFECT OF OXYGEN DURING GROWTH ON SURVIVAL OF AIRBORNE *ESCH. COLI* TYPE 2

| Growth Conditions (18 hr at 37°C) | Inoculum (Approx. No. Cells) | Turbidity (Arbitrary Units) | Relative Humidity (%) | $K$ ($Hr^{-1}$) | Standard Deviation |
|---|---|---|---|---|---|
| Anaerobic broth | $10^6$ | 2-3 | 90 | 13·1 | ±0·76 |
| | | | 52 | 5·8 | ±0·90 |
| Standing tubed broth | $10^6$ | 3-10 | 90 | 8·8 | — |
| | | | 52 | 6·6 | — |
| Aerated broth | $5\times10^6$ | 27-31 | 90 | 2·8 | ±0·06 |
| | | | 56-57 | 3·8 | ±0·07 |
| Agar surface | $10^6$ | — | 90 | 3·6 | — |
| | | | 56-57 | 4·4 | — |

(iii) *Effect of Age of Culture.*—The above results could be attributed either to a direct effect of oxygen on sensitivity of the cell or to an indirect effect on the physiological age of the culture. To test this latter possibility additional runs were made in which comparatively high death rates ($K_d = 11\text{-}17$) were found at 90 per cent. R.H. for young (4-8 hr) aerated cultures and comparatively low death rates ($K_d = 3\text{-}4$) were found at the same humidity for old (2 days) anaerobic cultures. These findings were extended by an examination of a complete growth cycle of the organism, grown anaerobically and aerobically for examination at 90 per cent. R.H. and aerobically only for examination near 50 per cent. R.H.

At the lower humidity, death rate was independent of the age of the culture.

The results at the high humidity are illustrated in Figure 4. From this it appears probable that the maximal death rate occurred during the transition from lag to logarithmic growth phase, or else very early in logarithmic growth. Because of the uncertainty in locating this maximum it would be unwise, at present, to comment on the identity or otherwise of anaerobic and aerated cultures in this part of their growth cycle. It will be noted, however, that these two types of cells showed quantitative differences after the cessation of logarithmic growth, the airborne anaerobic cells dying quite rapidly until comparatively late in their growth cycle.

(iv) *The Smooth-Rough Change.*—Because of the previously described (Section II) association of rough colonies with non-logarithmic death, a very

rough strain of *Esch. coli* type 2 was examined at 90 per cent. R.H. over a complete growth cycle. The findings are summarized in Table 3, from which it will be seen that, whereas resistance of the smooth culture increased rapidly during the logarithmic growth phase (see also Fig. 4), resistance of the rough culture was constant over that phase but subsequently increased to the same level as that of the smooth.

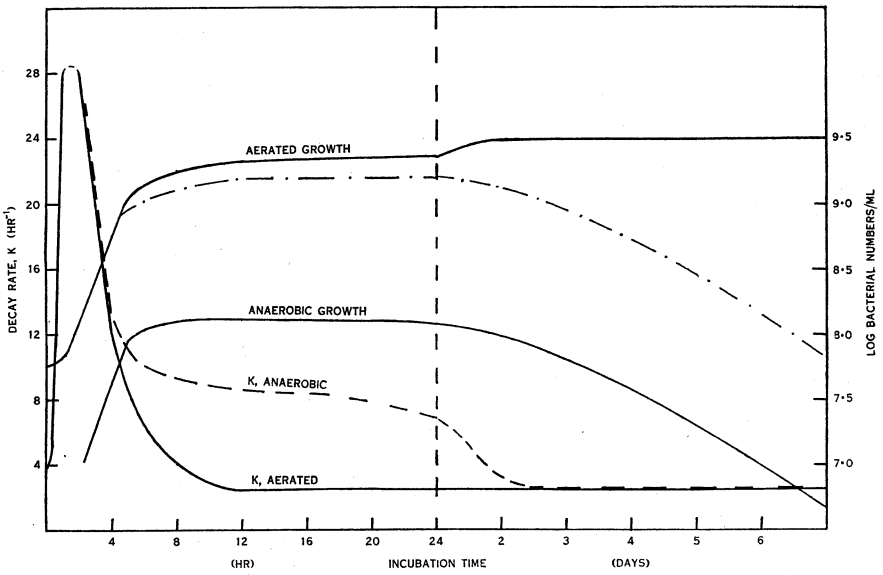


Fig. 4.—*Esch. coli*, type 2. Decay rate ($K$) *v.* culture age, relationship at 90 per cent. R.H. This figure is intended to depict the relationship of death of airborne cells to their phase of growth, not necessarily to their absolute age. The continuous aerated growth curve represents bacterial mass expressed as $\log_{10}$ number of cells. The broken line is the approximate course of the aerated viable count. The anaerobic growth curve also represents viable count. Anaerobic growth was followed by means of a series of ampoules inoculated simultaneously and broken as required for air sampling and viable count determinations. Inocula were: aerated growth, 3 ml standing culture ($10^9$ cells); lag phase (aerated), $10^{10}$ cells (centrifuged); anaerobic growth, 0.05 ml standing culture ($2 \times 10^7$ cells).

### (*c*) *The Lethal Mechanisms*

(i) *Death Rates in Aqueous Solutions.*—Survival was measured at 0.5°C in aqueous solutions of glycerol at concentrations corresponding to the humidities at which high and low death rates were observed in air (see Fig. 1). The results of these measurements are given in Table 4 and imply that the death rates in air at humidities below 70 per cent. R.H. are very likely due to a simple water activity effect. The rates of death in solution at 93 per cent. R.H., however, which were subsequently confirmed with solutions of NaCl, KCl, and sucrose, were markedly different from the values obtained in air and suggest that an explanation involving factors other than water activity should be sought for the high death rates at high humidities.

(ii) *Cold Shock.*—The close relationship found between culture age and resistance at high humidities suggested that the lethal mechanism in this zone may involve a "cold shock" such as described by Hegarty and Weeks (1940). It was felt that the foregoing results of survival in solutions were sufficient to discount prolonged exposure to cold as a lethal process, but the effect of the

TABLE 3

*ESCH. COLI* TYPE 2. DEATH RATES AT 90 PER CENT. R.H. OF ROUGH AND SMOOTH STRAINS

| Growth Phase (In aerated culture) | Approximate $K_d$ ($Hr^{-1}$) | |
|---|---|---|
| | Smooth | Rough |
| Early logarithmic | 28-30 | 18-20 |
| Late logarithmic | 8-10 | 18-20 |
| Stationary and decline | 2·5 | 2·5 |

sudden initial cooling during atomization remained to be considered. Accordingly, aerated *Esch. coli* cultures at the beginning and middle of their logarithmic growth phases were cooled rapidly from 37°C by making 100-fold dilutions with nutrient broth and quarter-strength Ringer's solution maintained at 0 and 0.5°C. Viable counts were made immediately before dilution, immediately after dilution, 5 min after, and at intervals of 10 min from 15 to 35 min after dilution. The results of these measurements may be summarized as follow.

TABLE 4

DEATH RATES* OF *ESCH. COLI* IN AIR AND GLYCEROL SOLUTIONS AT 0.5°C

| Relative Humidity (%) | Death Rate, $K_d$ ($Hr^{-1}$) | |
|---|---|---|
| | Air | Solution |
| 93 | 8-10 | 0·32-0·39 |
| 70 | 1·2± | 0·6-1·8 |
| 55 | 5·2± | 4·0-5·0 |

* Death rates in solution were determined by making five viable counts at intervals of 15 min. Glycerol concentrations were obtained from International Critical Tables (1928).

Early log phase cultures suffered destruction ranging from 0 to 40 per cent. immediately on cooling, but underwent no further change on holding at the

lower temperature. Mid-log phase cultures suffered no measurable destruction on cooling.

Additional results were obtained in which identical death rates were observed at 90 per cent. R.H. for mid-log phase cultures atomized from (*a*) suspensions at 20-25°C and (*b*) suspensions allowed to equilibrate with the test atmosphere (*c.* + 1°C).

(iii) *The Possibility of Specific Toxic Concentrations of Endogenous Substrates.*—At neither 50 nor 90 per cent. R.H. was any difference found between the death rates of unwashed cells with high endogenous respiration and cells washed with phosphate buffer to have little or no endogenous respiration.

## IV. Discussion

Before discussing individual aspects of the foregoing results, it is important to consider two possible complications arising from the fact that the investigation was conducted near the freezing point of water.

The first of these is the possible effect on sedimentation figures of freezing in the settling plates. It was usual for some plates to freeze at humidities of about 70 per cent. and all plates invariably froze at 50 per cent. R.H. The $K_s$/humidity curve of Figure 1, which is flat over the range where freezing occurs, and a comparison of counts of frozen and unfrozen plates from many experiments, have provided evidence that freezing of the medium does not measurably affect the survival of cells which have deposited on it.

The second possibility is that the airborne cells themselves may freeze. It will be apparent from the outset that this question does not arise in the experiments at 90 per cent. R.H. in which the wet bulb temperature of the room remained above 0°C. The existence of ice crystals in the cell is also impossible after thermodynamic equilibration with the atmosphere. The possibility of freezing is therefore limited to the initial period of desiccation of the particles and this time may be shown to be very short in relation to sampling intervals. The ability of ice to form during this period will, of course, be determined by the wet bulb temperature of the room, heat transfer rates, and the freezing point of the cell, which, for cells grown in nutrient broth has been placed between $-0.2$ and $-1.7$°C (W. J. Scott, personal communication).

The time of evaporation of external water from the bacteria-carrying particle may be gauged from a theoretical equation of Langmuir (1918) as used by Dunklin and Puck (1948). From this equation the author has calculated a time of 0.026 sec at 0°C and 80 per cent. R.H. for spherical droplets of 2 $\mu$ diameter containing a rod-shaped cell 2 by 1 $\mu$, to lose all extra-cellular water, assuming no change in evaporation rate with change in shape of the droplet and no diffusion of solutes from the cell to the external water. In actual fact the time is probably somewhat longer than this owing to local rises in humidity caused by the aerosol, but not so much longer as to affect the validity of the dependent conclusions.

It may be inferred from experimental results that the complete period of equilibration (i.e. including loss of intracellular water) is also quite short. If

this period were of sufficiently long duration, relative to sampling intervals, the death rate over the first part of a run at low humidity would be expected to be represented by a curve. The slope of the curve at any time would then be equal to the equilibrium $K$ corresponding to the higher humidity to which the cell had dried out at that time. Nothing of the kind has been observed, although it must be presumed to happen over the period of equilibration. The high initial death rate of *Esch. coli* bears no relationship to "normal" death rates at any higher humidity.

Similarly, Figures 2 and 3 show that the regressions of log survival against time for each humidity were best joined by straight lines. The rate of readjustment of the cell to the new condition therefore was again short in relation to the intervals between samples (in this case 7 min). It may be concluded therefore that, under conditions such as described, airborne bacterial cells come into thermodynamic equilibrium in less time than is measurable by death rate response. This conclusion differs from that of Robertson and Lester (1951), who contend that "desiccation" at 15 per cent. R.H. may continue for up to 20 hr.

The effect of humidity on the survival of both types of *Esch. coli* is in general agreement with the trend of results in the preceding paper (Brown 1953). It will be noted that in spite of the similarities in minimal $K$ values of all the organisms examined, death rate ($K_d$) is still the major component of $K$. This conclusion is supported by additional, unquoted, sedimentation figures obtained by the use of a direct microscopical method and by experiments with bacterial spores.

While the differences between agar and standing broth cultures (Table 1) may be explained in the simple terms of differences in physiological age, the additional difference between aerated and anaerobic cultures shown in Figure 4 may also be implicated. At present no explanation is offered for this difference.

The immediate cause of the non-logarithmic death rates has not been discovered. Certain associations have been observed but do not cover all occurrences of the phenomenon. A sufficiently high proportion of the rough strain in a late logarithmic culture at high humidities (Table 3) would produce such a change in slope but this is an isolated case.

Some tentative conclusions may be drawn regarding the factors affecting death of airborne cells. At least two distinct mechanisms seem to be potentially available for the destruction of cells. In *Esch. coli* the mechanism operating near 50 per cent. R.H. is very likely a simple water activity effect. Only negative information is available regarding the high-humidity mechanism, which on the evidence is neither a water activity nor a cold shock reaction nor is it affected by washing the cells with phosphate buffer. The results of the cold shock experiment are not intended to be quoted in opposition to those of Hegarty and Weeks (1940), as in all probability the discrepancies may be explained by the different strains of organism they used.

## VI. Acknowledgments

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## VII. References

Brown, A. D. (1953).—*Aust. J. Biol. Sci.* **6**: 463.
Dunklin, E. W., and Puck, T. T. (1948).—*J. Exp. Med.* **87**: 87.
Hegarty, C. P., and Weeks, O. B. (1940).—*J. Bact.* **39**: 475.
Langmuir, L. (1918).—*Phys. Rev.* **12**: 368.
Robertson, O. H., and Lester, W. (1951).—*Amer. J. Hyg.* **53**: 69.
Skerman, V. B. D., Lack, Jean, and Millis, Nancy (1951).—*Aust. J. Sci. Res.* B **4**: 511.