STUDIES ON THE MECHANISM OF SORBED WATER KILLING OF BACTERIA

G. W. MONK,[1] P. A. McCAFFREY, AND M. S. DAVIS

Chemical Corps, Fort Detrick, Frederick, Maryland

Received for publication November 19, 1956

The bacterium *Serratia marcescens* is particularly sensitive to small amounts of sorbed water. It has already been shown (Monk and McCaffrey, 1957) that the logarithmic death rate is negligible for very wet and very dry cells and is maximal for a water concentration of about .33 per cent, which corresponds to only about one-eighth of the amount found inside a normal cell suspended in water. It is the purpose of this paper to report an experimental determination of the temperature dependence of this death rate and various other processes involving this species, and to discuss the possible application of these findings to an understanding of the process by which sorbed water kills the organisms.

MATERIALS AND METHODS

S. marcescens strain 8 UK was grown on tryptose agar for 18 to 24 hr at 31 C, harvested in distilled water, washed in distilled water, and assayed for viability exactly as already described (Monk and McCaffrey, 1957). The washed cell suspension was stored between experiments for not more than 4 days in a refrigerator. The samples were freeze dried in the apparatus already described, and then were rewetted to about 33 per cent moisture by back lyophilizing 0.020 ml of water. Tubes containing identical moist samples were then stored for 2 to 3 hr at various temperatures and the surviving number of cells determined. The water content was determined from the loss in weight suffered by similar samples during drying at about 100 C in a vacuum oven.

RESULTS AND DISCUSSION

Since it had already been shown that 33 per cent sorbed water causes the viability to decrease exponentially with time it was considered valid to calculate the death rates in the present experiment from the original and final viabilities. The calculated logarithmic death rates are plotted as a function of temperature in figure 1. Even considering the rather scattered points at low temperatures it is apparent that the death rate is roughly proportional to the temperature above 0 C. While at 25 C the rate given is near the maximum for the water content death rate curve, the values of lower temperatures may not necessarily be maximal. This point will be discussed later.

If we assume that the killing process is governed by a unimolecular reaction which proceeds according to the usual transition state theory (Glasstone, Laidler, and Eyring, 1941), we can characterize the activated state by the dependence of the death rate on temperature. In an ideal system the rate of reaction k depends on the absolute temperature T, the energy of activation E, and the entropy of activation ΔS, according to Eyring's equation:

$$k = (ek'T/h)e^{-E/RT}e^{\Delta S/R} \qquad (1)$$

where k' is Boltzman's constant, h is Plank's constant, and R is the gas constant per M. The usual method of evaluating E and ΔS is to plot log k against $1/T$ and fit a straight line to the experimental points. As can be seen by taking the logarithm of equation (1), the slope and intercept of this line will be determined by E and ΔS, respectively. The data from figure 1 have been replotted in this fashion in figure 2 and the solid straight line through the points near room temperature used for the calculations. The deviations from the theory at low temperatures can probably be explained in terms of the decreased solubility of the reacting substance.

Using the solid line, the energy of activation has been calculated as 7,700 cal/mole and the entropy of activation as -49 cal/mole°K. These low values tend to indicate that a typical protein denaturation is not the governing process during killing by sorbed water. To illustrate this point, we have also determined corresponding values for the thermal death of this organism.

[1] Present address: American Machine & Foundry Company, 1101 North Royal Street, Alexandria, Virginia.

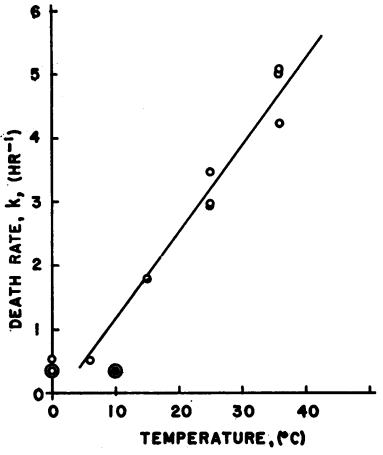

Figure 1. The logarithmic death rate of *Serratia marcescens* due to sorbed water at various temperatures. At all temperatures the sorbed water was about 34 per cent of the moist cell weight.

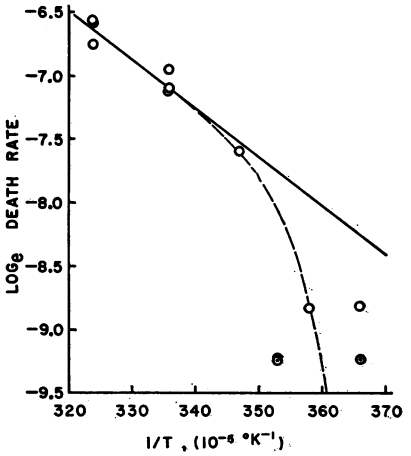

Figure 2. Arrhenius type plot of the data from figure 1. The solid line through the points near room temperature was used to compute the energy and entropy of activation for sorbed water killing of *Serratia marcescens*.

A washed suspension of *S. marcescens* was injected into a flask of water maintained at different temperatures for different experiments. By extracting and plating aliquots from the flask after various times the viability as a function of time of exposure was determined. In general, the viability decreased exponentially at a high rate for several orders of magnitude and then decreased at a much lower rate as though some heat resistant cells were present in the suspension. This latter effect was disregarded and the initial slopes

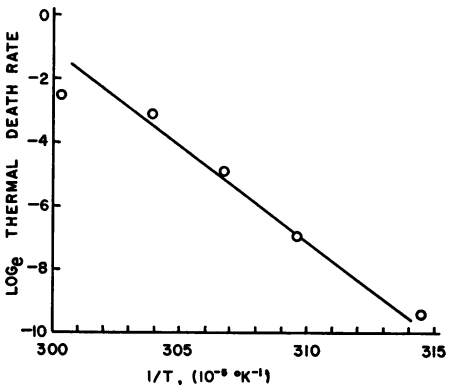

Figure 3. The thermal death rate of *Serratia marcescens* at various temperatures.

taken as the thermal death rate. In figure 3, the natural logarithm of the death rate has been plotted against the reciprocal of the absolute temperature. The death rate at the highest temperature was so great that it was difficult to determine accurately and little weight was given that point in drawing the straight line in figure 3. From this line, the energy of activation for thermal death was found to be 120,000 cal/mole and the entropy of activation found to be +300 cal/mole°K. Both these values are much higher than the corresponding values for sorbed water death.

In an effort to find if any reactions carried out by this organism could have a low energy and entropy of activation, the endogenous respiration rate of this bacterium was investigated.

The oxygen consumption of 1 ml of the washed cell suspension was measured at 20, 25, 30, and 36.6 C. Using the rather crude assumption that the rate of uptake was proportional to the concentration of oxygen in the intracellular water and not limited by diffusion, the absolute rate was calculated by dividing the measured rate by the amount in solution in the cells. Such an assumption affects the entropy but not the energy of activation. It can be shown that even a factor of 10 change in the absolute rate would not change the entropy significantly. These absolute rates are plotted in figure 4. The solid line corresponds to an energy of activation of 9,000 cal/mole and an entropy of activation of −39 cal/mole°K.

The growth of bacteria during the log phase is probably limited by a process that can be described by its energy and entropy of activation

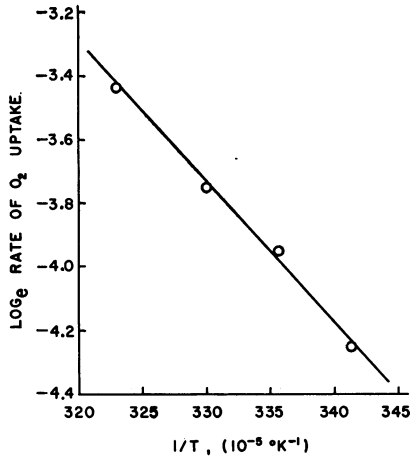

Figure 4. The rate of oxygen consumption by *Serratia marcescens* in distilled water.

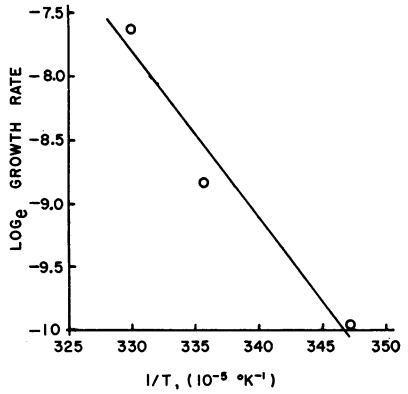

Figure 5. The growth rate during the log phase of *Serratia marcescens* in tryptose broth incubated at various temperatures.

even though the nature of the process is unknown (Johnson, Eyring, and Polissar, 1954). For this particular organism, crude growth curves in tryptose broth were determined at 15, 25, and 30 C. The growth rate during the log phase is plotted in figure 5 and from the solid line the energy of activation was found to be 26,000 cal/ mole and the entropy of activation +12 cal/mole °K.

The four sets of determinations are compared in table 1, from which it is apparent that sorbed water killing is similar to those processes involving enzymatic reactions rather than to protein denaturation. The values given are comparable to those given in the literature for a wide variety of biological processes (Huennekens, 1953).

TABLE 1

Energy and entropy of activation of various processes involving Serratia marcescens

Bacterial Process	E	ΔS
	k cal/mole	*cal/mole °K*
Sorbed water death	7.7	−49
Thermal death	120.0	+300
Endogenous O_2 uptake	9.0	−39
Growth	26.0	+12

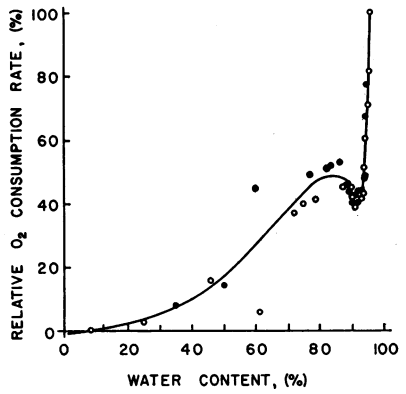

Figure 6. The relative endogenous oxygen consumption rate of *Serratia marcescens* following drying to various water contents. The open and solid points are data from different runs.

The cells are so dry at the lethal concentration of water that it is not obvious that ordinary chemical reactions could proceed. Of course, it is possible, as discussed before, that while the average water content is only 33 per cent, certain more hygroscopic materials in the cell may have a much higher percentage of water associated with them. In order to demonstrate that reactions can proceed in the presence of only small amounts of water, the endogenous respiration rate was measured as a function of water content. Several aliquots of a washed cell suspension in Warburg flasks were dried in a desiccator. After various periods of drying, the water content in a sample was determined by weighing and the rate of O_2 uptake was measured in the Warburg apparatus. In this manner the rate could be determined as a function of water content. The results of two runs are shown in figure 6, in which the rates are expressed as a percentage of the normal endogenous rate of suspended cells. For the purposes of this discussion, the important point is that significant oxygen is consumed at 33 per cent water

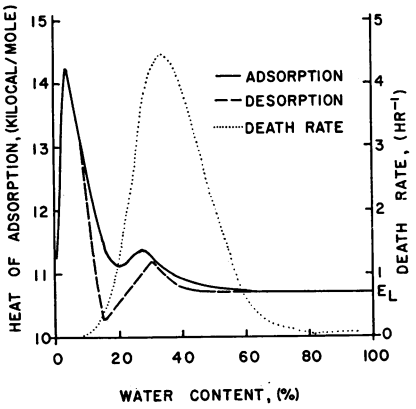

Figure 7. The heats of sorption (Stevens, C. L., *private communication*) and death rate (Monk and McCaffrey, 1957) plotted on the same water content scale.

content. The peculiar behavior of the curve around 90 per cent water may be associated with a change in the physical nature of the suspension that might affect the access of the oxygen to the cells. While cells suspended in an aerosol would surely consume oxygen, it must be remembered that this is not a necessary condition for death since the death rate curve reported for washed cells was done in the absence of air. The effect of oxygen on the death rate of this organism has not been determined. As indicated in figure 6, a gas of unknown nature is given off by very dry cells.

Additional evidence on the nature of the killing process in the presence of sorbed water can be obtained by comparing the heats of sorption with the death rate curves. In figure 7 the heats of sorption determined by Stevens (1956. *Private communication*) are replotted on the same water content scale as the death rate curve determined by Monk and McCaffrey (1957) for washed cells. As pointed out by Stevens, the secondary maximum in his curves possibly could be due to the heat of solution as intracellular material is dissolved. It is interesting to note the correspondence of this peak with the death rate peak. This suggests that at least some of the cellular material may have sufficient water to become active at the most lethal water content. One particularly simple explanation is that a hygroscopic substance goes into solution at low water contents and acts as a toxin because of its high concentration. At 33 per cent water it is saturated and has the maximum effect. At higher water contents it is diluted, and the system achieves more nearly its normal metabolic balance. At lower water contents smaller amounts of this substance are in solution. On the other hand, the toxic effect could be due to a reaction which is ordinarily going on in a normal cell, but due to the fact that not all of the components of the metabolic system are hydrated to activity the reaction in a damp cell results in the depletion or excess of an intermediate. This unbalanced condition may cause death when the cell is put in nutrient.

The concept of a saturated solution at 33 per cent water is substantiated by figure 2. If the death rate depended only on the thermal energy available according to the Arrhenius equation, the data would follow the solid line. However, if a saturated solution exists at 33 per cent water and 25 C, smaller amounts would be in solution at any lower temperature. Hence the decreased death rate below the expected value may support the assumption that a saturated solution exists at the most lethal water content.

As a matter of fact one could determine from figure 2 the relative solubility of the unknown substance as a function of temperature by assuming that the death rate is proportional to the amount in solution. A corollary of this unproved proposition would be that, while the maximal value of the death rate should decrease linearly with the reciprocal of the absolute temperature, the position of the peak should shift to higher water contents as the temperature is lowered. This follows from the fact that at the lower temperatures more water is needed to dissolve all of this unknown substance. This might have important practical significance in freeze drying where the local sample temperature may rise to values where the death rate is appreciable. It would be interesting to see if experiments would bear out the shift in the peak of the death rate water content curves.

CONCLUSIONS

While the evidence given here is not conclusive, it indicates that death due to sorbed water results from a rather subtle change in the cell and suggests that of the three explanations offered in an earlier paper the concept of an unbalanced metabolic system is not untenable. The idea of death being caused by drastic physical changes in the cell is contraindicated by our failure to find evi-

dence of such changes, either in preliminary electron microscope studies or in observations on cell extracts in the ultracentrifuge. In like manner, we have so far failed to find a protein that is sensitive to sorbed water, as suggested in the third explanation offered.

ACKNOWLEDGMENT

The authors wish to acknowledge the technical assistance of F. E. White.

SUMMARY

The energy and entropy of activation for the killing of *Serratia marcescens* by sorbed water compares more closely to the values for endogenous respiration and growth than to those for thermal killing. At the most lethal water content of about 33 per cent, the existence of measurable endogenous respiration indicates that at least part of the metabolic system is operative in the presence of only small amounts of water. A simple explanation is offered which assumes that a hygroscopic substance which is toxic under these conditions is all in solution at 33 per cent water. In dryer cells less of this substance is in solution, and in wetter cells it is diluted and the cells can resume a more normal metabolic balance.

REFERENCES

GLASSTONE, S., LAIDLER, K. J., AND EYRING, H. 1941 *The theory of rate processes.* McGraw-Hill, New York.

HUENNEKENS, F. M. 1953 *Investigation of rates and mechanisms of reactions.* Edited by S. L. Friess and A. Weisberger. Interscience Publishers, Inc., New York.

JOHNSON, F. H., EYRING, H., AND POLISSAR, M. J. 1954 *The kinetic basis of molecular biology.* John Wiley and Sons, New York.

MONK, G. W. AND MCCAFFREY, P. A. 1957 The effect of sorbed water on the death rate of washed *Serratia marcescens.* J. Bacteriol., **73,** 85–88.