Effect of Water on the Thermal Death of a Hydrocarbon Bacterium in a Nonaqueous Fluid

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Received for publication 12 August 1974

A bacterium that grows in oil was tested for survival at elevated temperatures in menstruums of varying water content. For each doubling of the water concentration, the surviving fraction decreased by a factor of approximately 3.0. A minimum value of 0.02% water is required before enhanced killing occurs.

It is well known that dry cells are considerably more heat resistant than wet cells, and that cells suspended in a nonaqueous medium have a response to heating similar to that of dry cells (1, 2). In a previous publication, we described the kinetics of thermal death of a hydrocarbon-oxidizing bacterium in several nonaqueous menstruums (3). Bacterial cells heated in a variety of oils had greatly enhanced survival when compared with cells heated in menstruums containing water. The addition of as little as 0.51% water to an oil-heating menstruum completely overcame the protective effects of the oil and promoted exponential killing of the cells. However, the rate of killing at this low water content was less than that observed when the cells were heated in an aqueous menstruum. As the presence of water strongly influenced thermal killing of bacteria, it was of great interest to determine the relationship between the water content of a heating menstruum and its effect on thermal death.

The bacterium used in this study confined its development to the oil phase of an oil-water mixture and as such was an ideal experimental organism. A more detailed description of the organism and its oleophilic growth pattern has been published elsewhere (4). Details of culture methods, heating procedure, and enumeration of survivors have been described (5). The principal difference in the work reported here is that the heating menstruum contained varying amounts of tritiated water in a mixture of hexadecane and 1-octanol. The octanol functions as a carrier to disperse the water in the hexadecane. The micelle formed is composed of a central water molecule surrounded by four to five alcohol molecules (3). The alcohol molecules are oriented with their hydrophobic ends outward mediating dispersal in the oil (3). A stock solution of a water-saturated 1-octanol was prepared by shaking the alcohol with tritiated water for 72 h at room temperature. The specific activity of the $^3$H$_2$O in both phases was measured, and the percentage of water content of the octanol was calculated and adjusted to 5% with dry octanol (5-WO). To prepare a menstruum with a specified water content, the 5-WO solution was further diluted with dry octanol to yield a solution with a water content 10 times that desired in the final menstruum. Next, 1 ml of this water-octanol mixture was added to 9 ml of hexadecane to give an oil menstruum with a specific water content. To obtain a menstruum containing 1% water, 2 ml of the 5-WO was added to 8 ml of hexadecane. A temperature of 88.6$^\circ$C was used in all heat killing experiments.

Previous work (5) indicated that the survival curve for a dry hexadecane-octanol mixture (9:1, vol/vol) was identical to those obtained with octane, dodecane, hexadecane, and a mixture of paraffin oil and kerosene (1:1, vol/vol). The survival curve for anhydrous menstruums consisted of a shoulder for the initial stages of heating followed by a logarithmic die-off. The shoulder in the survival curves represents a protective mechanism that delays the onset of logarithmic die-off. The shoulder is also a consistent feature of all heat killing experiments in anhydrous menstruums regardless of the temperature of heat application, the age of the culture, or the type of dry oil menstruum used (5).

The survival curves for cells heated in menstruums containing 0 to 1% water are shown in Fig. 1. As the water content of the heating menstruum was increased to 0.1%, the slope of the shoulder became more negative, indicating more rapid killing and a gradual elimination of the protective mechanism. No break, however, was noted in the survival curves when water was added, suggesting that different mechanisms for thermal killing may exist for dry and water-
containing menstruums. The data points at 25 min for the 0.03 and 0.06% survival curves suggest further that a small amount of water mediates rapid killing for short heating intervals, but also selects for a population that has a heat-resistant component on prolonged heating. At water concentrations greater than 0.1%, the survival curves became concave upward with a rapid killing in the first 5 min of heating followed by a slower rate of killing at longer heating times. Survivor curves obtained for the 0.2 to 1.0% menstruums are considered to indicate a heterogeneous population with respect to heat resistance (2). The heat-resistant fraction of this population, however, also showed increased mortality as the water content of the heating menstruum increased.

The relationship between the water content of the heating menstruum and its effect on thermal death is seen in Fig. 2. The data were derived from Fig. 1 by plotting the surviving fraction at a 15-min exposure for each curve against its respective water content. Extrapolating the curve back to the surviving fraction obtained with a dry menstruum (0.72 for a 15-min heating period) indicates the presence of a threshold value of 0.022% water. Below this concentration the presence of water will have no apparent effect on survival. Higher concentrations of water, however, will accelerate thermal killing.

The choice of using an exposure period of 15 min to compile the data for Fig. 2 was arbitrary. If the data for exposure periods of 10 or 20 min were used to generate the curve of Fig. 2, the threshold water concentration becomes 0.027 and 0.019%, respectively. Furthermore, the slope of the line in Fig. 2, and hence the apparent rate of killing, will vary slightly depending on which data points are used. Curves plotted for exposure periods of 10, 15, and 20 min have slopes of -1.54, -1.80, and -1.98, respectively. For each doubling of the water content in the heating menstruum, the surviving fraction will decrease by a factor of 2.9 for the 10-min data, 3.5 for the 15-min data, and 3.8 for the 20-min data.

**Fig. 1.** Survival curves for a bacterial culture suspended in oil with varying concentrations of water. The percentage of water content of each menstruum is indicated with its respective survival curve. All killing experiments were carried out at 88.6°C.

**Fig. 2.** Effect of water content of the heating menstruum on thermal death.
The conclusions to be drawn from these experiments are that: (i) the introduction of small quantities of water into a nonaqueous heating menstruum will greatly accelerate thermal death in direct proportion to the amount of water added; and (ii) a minimum or threshold level of water is required for enhanced thermal killing of approximately 0.02%. Previous findings with the *Brevibacterium* culture indicated that the type of oil in which the organism is suspended at the time of heating is not important and that all dry oils afford the same degree of thermal protection. Water, however, greatly accelerated thermal death. If other microorganisms follow the pattern of the *Brevibacterium* culture, then the inclusion of a small quantity of water can greatly affect the pasteurization of oil-rich foods.

This study was supported by grant NGR 10-004-041 from the National Aeronautics and Space Administration. The expert technical assistance of Lee Motyka is gratefully acknowledged.

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