Thermal Inactivation of Bacteriophages Active Against Lactic Streptococci

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Abstract
Thermal destruction studies on two bacteriophages active against *Streptococcus lactis* were carried out in milk at pH 6.2. Inactivation curves were similar at all temperatures studied; a relatively rapid initial decline in titer was followed by a slower reduction and leveling off of numbers, until complete inactivation was approached. When inactivation was nearly complete, the populations sometimes again declined rapidly. A longer time was required for complete destruction of the bacteriophage than would be anticipated from a straight-line extrapolation of the initial portion of the thermal inactivation curve. Destruction times for one bacteriophage at 70, 72.8, 75, and 77.8°C were 200, 70, 20, and 6 min, respectively, and, for the other, 170, 60, 25, and 9 min, respectively. Plotting the thermal death times of the two bacteriophages yielded z values of 8.75 and 10.5.

Relatively little information is available in current literature concerning the thermal destruction of lactic streptococcal bacteriophages. Much of the early data on heat destruction of bacteriophages refers mainly to thermal death points and no attempt is made to define specific thermal inactivation characteristics. Whitehead and Hunter (12), Nichols and Wolf (9), Nelson et al. (8), and Nowfl (10) all reported data of this type. The studies of Cherry and Watson (2), Wilkowske, Nelson, and Parmelee (13), and Daoust, El-Bisi, and Litsky (3) have dealt with, in the main, the kinetics of the heat inactivation of lactic streptococcal bacteriophages. This study was undertaken to establish some parameters of the heat destruction of two lactic streptococcal bacteriophages in milk at several temperatures.

Materials and Methods

*Propagation of bacteriophage.* Two strains of *Streptococcus lactis*, 144F and 240, and their homologous bacteriophages were used in this study. Initial tests had demonstrated that these two bacteriophages were the most heat-resistant of the races available from the collection maintained at this laboratory. The bacteriophages were propagated on a sterile milk (10% reconstituted NFD) culture of their host organism at 31.1°C. After lysis of the host organism, the culture was handled in the following manner, to obtain a bacteria-free bacteriophage suspension: The pH of the milk was adjusted to 4.5 with 10% lactic acid. The precipitated milk protein was removed by centrifugation and the resulting serum was filtered through a sterile Selas filter unit (Selas no. VFA 86-2, 03) to remove the bacteria. The serum containing the bacteriophage was stored at 4°C until used. The bacteriophages were propagated in this manner one to seven days prior to use. Host organisms were maintained by daily transfer in sterile milk.

*Determination of the number of active bacteriophages.* The number of active bacteriophages in the serum as well as in the heat-treated suspensions was determined, using a modification of the Adams (1) overlay method. The procedure was changed to provide a period of 10 min at 33.3°C for adsorption of the heated bacteriophage by the host organism in a 0.5% CaCl₂ solution.

The medium used in the overlay method and throughout the study was suggested by Jezeski (6) and consisted of the following ingredients per liter: yeast extract (Difco), 10.0 g; glucose, 10.0 g; tryptone (Difco), 5.0 g; sodium acetate, 1.5 g; agar (Difco), 15 g; and tomato extract, 30.0 ml. The overlay agar formula was modified by reducing the glucose and agar content to 5 g each per liter. The tomato extract was obtained by filtering peeled, whole, canned tomatoes twice through three layers of cheesecloth. Before sterilization, the pH of the medium was adjusted to 7.2 with 1-N NaOH.

*Heat-treatment procedures.* Two systems were employed for heating. The first consisted of an apparatus similar to that described by Daoust, Read, and Litsky (4). A 1:10 bacteriophage suspension to milk dilution was used. The heating menstruum (in this instance, milk) was allowed to attain a temperature slightly higher than the test temperature and, upon addition...
of the bacteriophage suspension, the test temperature was obtained; thus, no time-lag correction was necessary. Ten-milliliter samples were removed at predetermined intervals and placed in screw-capped test tubes in an ice bath until the assay for active bacteriophage was carried out.

The second procedure utilized was similar to that described by Wilkowske, Nelson, and Parmelee (13). Ten milliliter quantities of a 1:10 dilution of bacteriophage suspension to milk were placed into sterile 16- by 125-mm screw-capped test tubes. Heating was accomplished by submersion of the tubes up to the bottom of the screw-cap in a hot-water bath maintained at the desired test temperature. Time-temperature lag correction factors were determined and used at each of the temperatures studied. At predetermined intervals, tubes were removed from the hot-water bath and placed in an ice bath until the assay for surviving bacteriophage was conducted.

Use of litmus milk subcultures. In instances where the quantity of phage-milk suspension made determining the presence of plaques on the overlay difficult, a litmus milk subculture was used. In most experiments this subculturing was carried out when 0.1-ml and 0.01-ml quantities of sample were being examined for the presence of active bacteriophage. Subculturing was carried on for at least three transfers, to determine the presence or absence of bacteriophage. The subculture was done by transferring the test quantity of heated phage suspension to a tube containing 10 ml of sterile litmus milk plus one drop of host culture. The tubes were incubated 16 hr at 22.2 C.

Results and Discussion

Initial studies were carried out at temperatures close to those utilized in the pasteurization of milk. The bacteriophage-milk suspension was heated at 60, 61.7, and 62.8 C for 1 hr with samples taken every 10 min. The number of active bacteriophages present in the samples was determined as described. The thermal reduction curves (TRC) for the two bacteriophages at the three temperatures are presented in Figures 1 and 2. It is apparent from these figures that there was little reduction in numbers of active bacteriophages after 1 hr.
of heat treatment at these temperatures. The thermal reduction rate of the two bacteriophages at the three temperatures is very similar. It appears that heat treatments in this range have little effect on completely inactivating bacteriophages present in milk. These results suggest that normal pasteurization temperatures employed in the processing of milk and milk products would not reduce substantially the numbers of bacteriophages present in the milk.

To carry out a successful thermal inactivation study, it is necessary to establish a series of time and temperature relationships resulting not only in inactivation but also in survival of the bacteriophage. To obtain these parameters in a reasonable time span it was necessary to utilize heat-treatment temperatures of 70, 72.8, 75, and 77.8 C for time periods sufficient to obtain nearly complete inactivation of the bacteriophage.

In Figure 3 are given the thermal reduction curves for the two bacteriophages at 70 C. The two curves are similar; a rapid initial decline in numbers was followed by a reduction in rate of destruction which, in turn, was followed by leveling off of the population at relatively low numbers.

At 72.8 C, the thermal reduction curve for Bacteriophage 240 is similar to that obtained at 70 C; whereas, Bacteriophage 144F demonstrated no apparent slowing down or leveling off of population as occurred at 70 C. The TRC for Bacteriophage 144F at 72.8 C is almost a straight line. These curves are presented in Figure 4.

Lack of a linear semilogarithmic relationship between number of active bacteriophages and heating time was also apparent at 75 C. The TRC for the two bacteriophages at this temperature are given in Figure 5. Again, there was a rapid decline in numbers, followed by a leveling off of the populations at relatively low numbers.

At the highest temperature investigated, 77.8 C, the methodology employed in heating the phage-milk suspension limited the time of heat-treatment increment to 2 min. It was not possible to study holding times of shorter duration. However, the thermal reduction curves at this temperature, shown in Figure 6, indicate behavior similar to that observed at other temperatures.

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Fig. 6. Thermal inactivation of the bacteriophages active against *Streptococcus lactis* 144F and 240 when in a milk medium at pH 6.2 and heated at 77.8°C.

Maximum survival times and minimum inactivation times for the two bacteriophages are given in Table 1. A thermal death-time curve was constructed from these values and is shown in Figure 7. Z values of 8.75 and 10.5 were determined for Bacteriophages 240 and 144F, respectively. These z values are similar to those suggested by Daoust, El-Bisi, and Litsky (3) for the fast and slow component in the lactic streptococcal bacteriophage they studied. It is of interest to note that the relative heat resistance of the two bacteriophages changed as the temperature of heat treatment increased. At the lower temperatures, 70 and 72.8°C, Bacteriophage 240 was the more heat-resistant; whereas, at the higher temperatures, 75 and 77.8°C, Bacteriophage 144F exhibited the greater heat resistance.

Similar thermal reduction curves for lactic streptococcal bacteriophages have been reported by Wilkowske, Nelson, and Parmelee (13). Other investigators (3, 5, 7, 11, 13) have also reported curves of a similar nature for the thermal inactivation of other types of bacteriophage. However, none of these investigators has been able to explain the failure of bacteriophage to exhibit a linear semilogarithmic relationship between numbers of survivors and heating time, as is observed when the thermal destruction of bacteria is studied. The failure of the bacteriophage particle to follow first-order kinetics when heated suggests that there may be more than one component associated with host adsorption and lysis which is heat-labile.

The thermal-reduction curves reported here...

### Table 1

Maximum survival and minimum inactivation times at 70, 72.8, 75, and 77.8°C in milk for Bacteriophages 240 and 144F

<table>
<thead>
<tr>
<th>Temperature (°C)</th>
<th>Bacteriophage 240 Survival time (min)</th>
<th>Inactivation time (min)</th>
<th>Bacteriophage 144F Survival time (min)</th>
<th>Inactivation time (min)</th>
</tr>
</thead>
<tbody>
<tr>
<td>70</td>
<td>180</td>
<td>200</td>
<td>150</td>
<td>170</td>
</tr>
<tr>
<td>72.8</td>
<td>60</td>
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<td>50</td>
<td>60</td>
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<tr>
<td>75</td>
<td>15</td>
<td>20</td>
<td>20</td>
<td>25</td>
</tr>
<tr>
<td>77.8</td>
<td>3</td>
<td>6</td>
<td>6</td>
<td>9</td>
</tr>
</tbody>
</table>

*For thermal death-time curves based on these data, see Figure 7.*

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and by other investigators (3, 7, 11, 13) can be fitted into two linear components and, in some instances, three components. This observation suggests that there is more than one entity affected by the heat treatment of the bacteriophage particle and that these entities vary in their heat susceptibility. Daoust, El-Bisi, and Litsky (3) suggest that the lack of linearity results from either protein denaturation or nucleic acid inactivation. Data presented here and elsewhere (3, 5, 7, 8, 11, 13) seem to suggest that the lack of linearity could be caused both by the denaturation of bacteriophage particle protein and by nucleic acid inactivation. However, much more specific information relative to the heat destruction of these two entities is necessary before this conclusion can be considered correct.

The information presented in this paper shows that these two bacteriophages are not destroyed by normal pasteurization temperatures. This factor should be considered when milk or milk products which may contain bacteriophages are processed; particularly if the resulting product will be used for, or may come in contact with, a cultured dairy product. This information should also be given consideration when establishing heat treatments for the processing of whey likely to be heavily contaminated with bacteriophage.

References