EFFECTS OF INCUBATION TEMPERATURE ON THE SALT TOLERANCE OF *SALMONELLA*

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ABSTRACT

Salt has been shown effective in preventing growth of salmonellae in foods. Many of the studies reported in the literature have been on the lethal action of high levels of salt as used in curing brines. Little information is available on the interaction of incubation temperature and low levels of salt on the growth of salmonellae. The growth of *Salmonella heidelberg, Salmonella typhimurium,* and *Salmonella derby* in nutrient broth containing 0 to 8% added NaCl (in 0.5 or 1% increments) has been tested by shake cultures at 8, 12, 22, and 37 C. In addition, *S. heidelberg* has also been tested in 0 to 9% added NaCl at 39, 41, 43, and 45 C. At 8 C, growth of *S. heidelberg* took place in 1 and 2% added NaCl; *S. typhimurium* increased in numbers in 1% added NaCl; and *S. derby* failed to increase. When incubated at 12 C, the three serotypes were all able to increase in numbers in the range of 0 to 4% NaCl. At 22 C, this range increased from 0 to a maximum of 5 to 8%. When incubated at 37 C, the organisms were able to increase in numbers in up to 7 to 8% NaCl. The salinity of the medium was not found to increase the maximum growth temperature of *S. heidelberg* as has been reported in the literature for other organisms. Low levels of salt were found to stimulate growth of salmonellae. This stimulation was more pronounced at low temperatures than near the optimum for the organisms. Since salt is used to preserve foods, these data are important in the preservation of perishable foods. Salt concentrations preventing growth of salmonellae at low temperatures may not be sufficient to prevent growth of these pathogens at higher temperatures.

Salmonellae are frequently associated with foods of animal origin and with foods handled or contaminated by man. Small numbers of salmonellae may increase to infective levels in these foods if proper processing and storage conditions are not maintained.

Salt has been shown effective in preventing growth of salmonellae in foods. The effect of salt is often similar to that of drying, and the effects can be equated in terms of the activity of the water in the system *(10)*. The lethal action of salt, like that of other disinfectants, is known to be reduced at low temperatures, but the influence of temperature on the inhibitory (as distinct from lethal) action of salt is not well documented *(5)*.

Shipp *(13)* reported that *Salmonella enteritidis* is rapidly killed at room temperature in curing brines, but survives for weeks at the lower temperatures (5 C) which occur in curing cellars. Similar results were reported by Koelensmid et al. *(6)*, who showed that salmonellae may survive for up to 70 days at 5 C but are rapidly destroyed at 20 C. These authors also studied growth of salmonellae at 5 C and 20 C in media containing salt. They reported no growth in any concentration at 5 C, and this is in accord with the general observation that the minimum growth temperatures for *Salmonella* species lie above 5 C *(8)*. Growth at 20 C was observed in 6% but not in 8% NaCl. Other authors have also indicated a maximum tolerance between 6% and 8% NaCl *(5, 12)*.

Most authors have reported that bacterial growth occurs over the widest range of salt concentrations near the optimum temperature for each species tested. There have, however, been reports of growth in higher salt concentrations at low temperatures near the minimum rather than at the optimum, and it has also been suggested that the temperature optimum and maximum temperature for growth may be raised when an organism is grown in the presence of salt.

Since many foods contain salt at levels which are sublethal for *Salmonella*, it was felt important to determine the potential for *Salmonella* growth in presence of salt at various temperatures.

MATERIALS AND METHODS

Medium

Nutrient broth was used in these studies. The medium was prepared by dissolving the nutrient broth (dehydrated) and sodium chloride in distilled water and diluting to volume with distilled water to give the desired concentrations. Volumes were carefully tested before and after autoclaving to ensure that the salt concentrations had not increased due to loss of water during sterilization.

Test organisms

Salmonella heidelberg, ATCC 8325; *Salmonella typhimurium*, ATCC 6994; and *Salmonella derby*, ATCC 6966, were used in these studies because information was available on the growth of these organisms at low temperatures *(7, 8, 9)*.

Inoculum

The inoculum in all instances was a nutrient broth culture without added salt, incubated at the temperature to be used in the test. Overnight incubation was used for 37 C samples, but several days' incubation was required for inoculum cul-

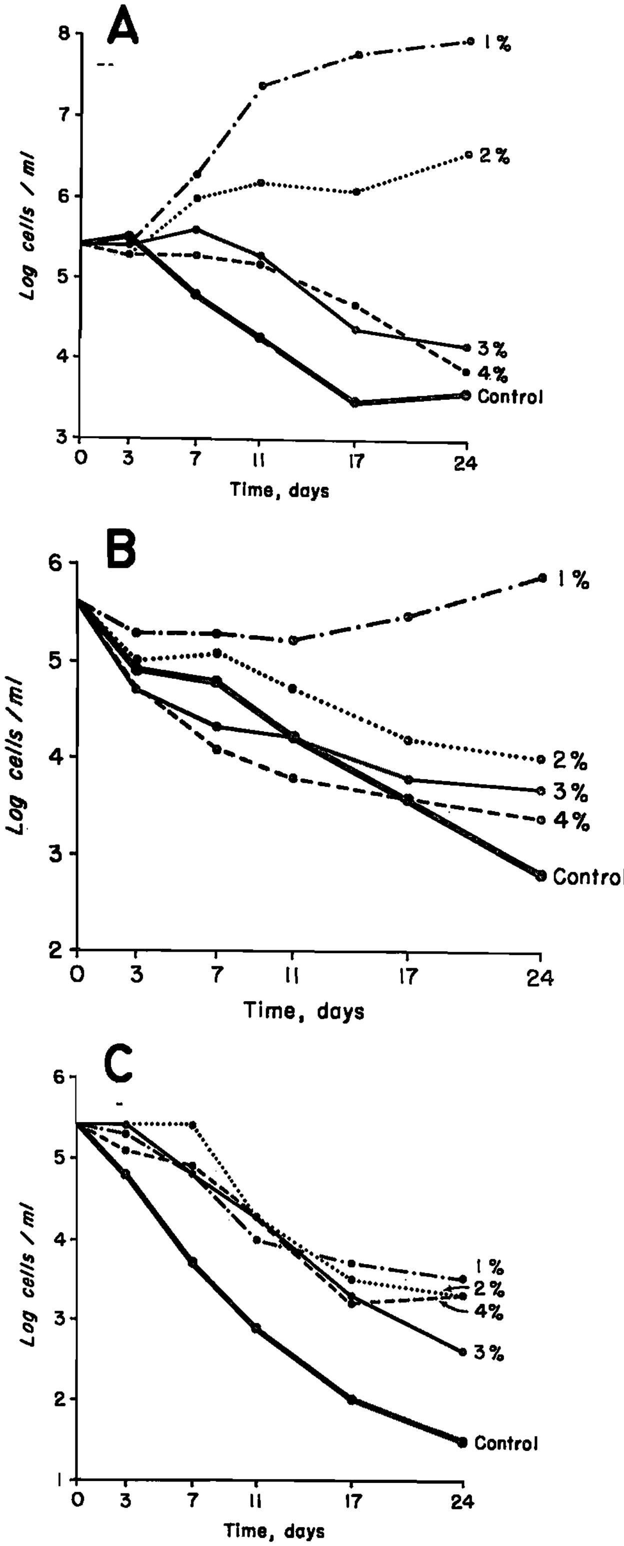

Figure 1. Growth of *Salmonella heidelberg* (A), *typhimurium* (B), and *derby* (C) at 8 C in 0 to 4% NaCl.

tured at 8 C. Cultures were diluted to 55% transmittance at 660 nm with a Bausch & Lomb Spectronic 20 spectrophotometer, yielding approximately 10^8 cells/ml. Serial decimal dilutions were prepared, using 0.1% peptone water, and the test medium was inoculated with 0.1 ml, yielding approximately 5 × 10^6 cells/ml. The large inoculum was used so that absorbance readings would increase when cell numbers began to increase.

Growth test

In these studies salmonellae were inoculated into 20 ml of nutrient broth containing the desired levels of sodium chloride in 50-ml Erlenmeyer flasks fitted with side arms. The flasks were incubated at the test temperatures in refrigerated and/or heated water bath shakers. Increases in numbers of cells in samples incubated at 8 C were determined by the drop plate method. Duplicate or triplicate 0.1 or 0.01 ml volumes of the appropriate dilutions from each sample were inoculated onto the surface of trypticase soy agar. The plates with duplicate or triplicate samples were incubated at room temperature until counted.

Increases in number of cells in samples incubated at 12, 22, 37, and 41 C were determined by turbidity measurements at 660 nm, using a Bausch and Lomb Spectronic 20 spectrophotometer. Absorbance was expressed as the negative log of transmittance.

RESULTS

Growth of salmonellae in a medium containing added sodium chloride between 0 and 8% is greatly influenced by the incubation temperature. The slowest growth and the lowest salt tolerance are expressed at the low incubation temperatures. As the incubation temperature is increased, the organisms are able to grow in the presence of higher levels of sodium chloride. At 8 C, *S. heidelberg* was able to increase in only 1 and 2% sodium chloride during 24 days' incubation (Fig. 1a). At this temperature, the rate of growth is slow in the nutrient broth test medium. At the higher salt concentrations (3 and 4%), the organisms were unable to increase in number during the 24 days' incubation, and the number of cells actually decreased. *Salmonella typhimurium* was able to increase only in the medium containing 1% sodium chloride (Fig. 1b), and *S. derby* was unable to increase in any of the concentrations of salt tested between 0 and 4% at 8 C for 24 days (Fig. 1c).

It is interesting that growth was obtained with both *S. heidelberg* and *S. typhimurium* in low levels of salt, but no growth was obtained in the controls without added salt. In fact, the overall rate of decline was greater for the three organisms in the control medium than in the media containing salt. This does not appear to be an acquired ability to tolerate salt, but rather an expression of the organisms of a specific osmotic requirement or a requirement for sodium chloride. The effects of lithium or other cations in satisfying this requirement were not tested.

At 12 C, growth was obtained at higher salt concentrations and growth was much more rapid than that obtained at 8 C (Fig. 2). Both *S. heidelberg* and *S. derby* increased in number in salt concentrations up to 5% but not above (Fig. 2 a, c). *Salmonella*

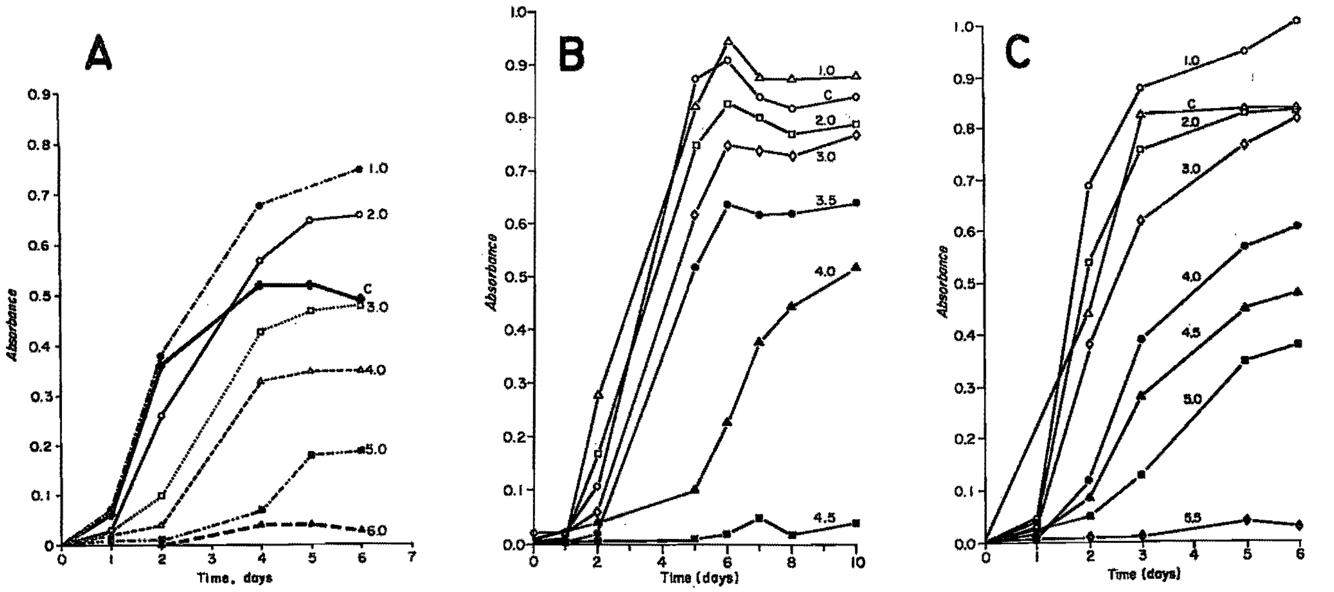

Figure 2. Growth of *Salmonella heidelberg* (A), *typhimurium* (B), and *derby* (C) at 12 C in 0 to 6% NaCl.

typhimurium (Fig. 2b) increased in salt concentrations only up to 4%. The salt requirement or growth stimulation exhibited at 8 C is also evident at 12 C for S. *heidelberg* but less pronounced for S. *derby* and S. *typhimurium.*

As the salt concentrations are increased, the rates of growth decrease and the length of lags are increased. Conversely, as the incubation temperature is increased, the rates of growth increase and the lengths of lag decrease for the same salt concentration. This is evident at 22 C for the organisms tested (Fig. 3). The most rapid growth was obtained with S. *heidelberg* (Fig. 3a), followed closely by S. *derby* (Fig. 3c). *Salmonella heidelberg* increased in numbers in medium containing 8% salt after a lag of 25 hr. *Salmonella derby* increased in numbers in the medium containing up to 7.5% salt, but only after a 48-hr lag at 7.5% salt. *Salmonella typhimurium,* which increased in 5% salt and below (Fig. 3b), failed to increase in salt concentrations between 5.5 and 8% for the duration of the 112-hr incubation period, and increased in 5% salt only after a 22-hr lag. Only concentrations between 5.5% and 7.5% were tested with S. *derby,* but concentrations between 0 and 8% were tested with S. *heidelberg* and S. *typhimurium.* The salt requirement of the organisms evident at 8 and 12 C also show up at 22 C for S. *heidelberg* and S. *typhimurium* (Figs. 3a, b) but is not evident for S. *derby* (Fig. 3c) because salt levels between 1 and 5% were not tested.

The ability of salmonellae to tolerate higher salt concentrations as the incubation temperature is increased, which shows up at 12 and 22 C, is even more pronounced at 37 C. At this higher temperature the lag period was greatest in samples containing the highest salt concentrations, while the rate of growth and number of cells obtained were greatest at the lower salt concentration. *Salmonella heidelberg* tested in salt concentrations ranging from 0 to 8% (Fig. 4a) increased at all levels of salt tested. The rate of growth and the final number of cells attained decreased with an increase in salt concentration. Growth at 8% salt did not take place until 38 hr after incubation. *Salmonella derby* (Fig. 4c) was also able to increase in salt concentrations up to 7.5% salt, but not at 8%. The rate of growth between 0 and 3% salt were very similar; however, above 3% the rates of growth decreased.

Salmonella typhimurium (Fig. 4b) showed similar growth patterns, but the rate of die-off after maximum cell numbers were obtained was more rapid than with the other two serotypes.

The temperature optimum and the maximum temperature for growth reportedly are raised when halophilic or marine organisms grow in the presence of high concentrations of salt *(3, 14). Salmonella heidelberg* was grown at 41 C in the presence of salt ranging between 0 and 9% (Fig. 5). The rate of growth at this temperature, as measured by change in absorbancy/time, was not as rapid as that obtained at 37 C. As a further test of the reports that the optimum temperature for growth and maximum growth temperature are raised at high salt concentrations, S. *heidelberg* was grown in nutrient broth containing 0 to 9% salt and incubated at 37, 39, 41, 43, and 45 C. The increase in cell number, as measured by increased absorbancy readings, was followed for 70 hr. At each of the five incubation temperatures, the greatest increase in cell number took place in samples containing 0, 1, and 2% NaCl. As the salt concentrations were increased beyond 2%, at each of the incubation temperatures, the increase in number of cells became less; thus the optimum temperature for S. *heidelberg* was not increased above 37 C by increasing salt.

DISCUSSION

It is obvious from the literature that in high concentrations of salt, as used in curing brines (i.e. 10 to 30% NaCl), the effects of temperature on the survival of *Salmonella* is considerable, and there is a possibility that some of these pathogens might survive at low temperatures. At higher temperatures, salmonellae are rapidly destroyed. The interaction of low or inhibitory salt concentrations and incubation temperatures on growth of salmonellae and other non-halophilic organisms has not been well defined.

The experiments described in this report show that at the lower non-lethal salt concentrations (<10%) the maximum salt concentrations in which growth occurs are greater at higher incubation temperatures near the optimum. At lower temperatures, the salt concentrations and temperatures interact to increase the length of the lag phase and decrease the rate of growth. This becomes more evident as the salt concentration is increased and as the incubation temperature is decreased.

The rate of growth of bacteria in media containing salt or other solutes is a function of the water activity and generally not of the concentration of a particular solute or solutes *(10, 11, 16)*. As the solute concentration is increased, the water activity (a_w) is decreased. With a decreased a_w, the lag phase of the organism increases and the rate of growth decreases. A lowering of the incubation temperature from the optimum, while not materially affecting the a_w, at a given salt concentration *(1)* does raise the lower limit of a_w for growth. Wodzinski and Frazier *(16, 17, 18)* reported that both *Pseudomonas fluorescens* and *Aerobacter aerogenes* exhibited the greatest tolerance for low a_w at the temperature which was nearly optimal for rate of growth of the organisms. The reverse was true with *Lactobacillus viridescens*, which showed the greatest tolerance to low a_w at the temperatures below the optimum. Lactic acid bacteria isolated from curing brines also tolerated the highest sodium chloride concentrations at the optimum temperature for growth *(2)*. However, as the temperature was increased, the optimum sodium chloride concentrations also increased. Growth was not obtained with *Lactobacillus* and *Pediococcus homari* at 40 C unless sodium chloride was added to the medium, but growth was obtained at 30 C without the addition of sodium chloride. No other substances were found that would replace the NaCl requirement at higher incubation temperatures.

The salinity of the growth medium was also found to have a marked effect on the maximal growth temperature of four bacteria isolated from the marine environment *(14)*. Two strains of *Vibrio marinus* and unidentified strains of a gram-positive coccus and a gram-negative bacillus were found to possess increased maximum growth temperatures as the salt concentrations were increased from 0 to 3.5%. *Vibrio marinus* MP-1 had a salt requirement of at least 0.7% and when grown in a defined medium a decrease in the maximal growth temperature was observed at both low and high concentrations of NaCl.

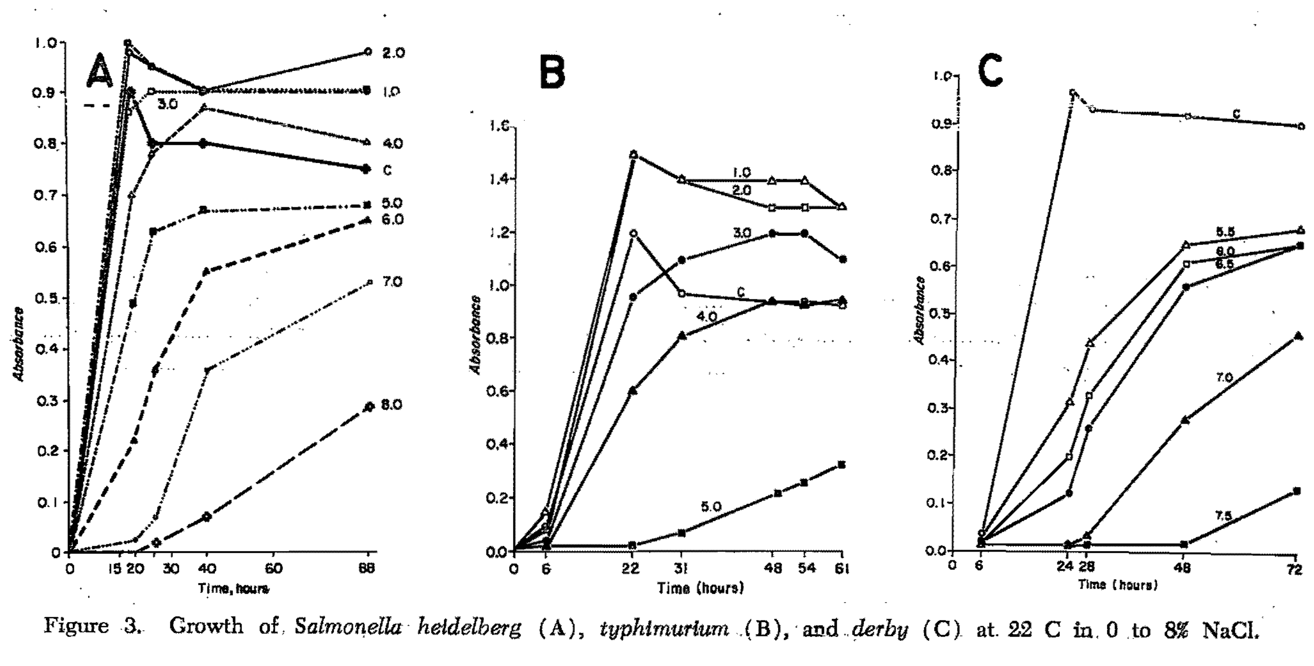

Figure 3. Growth of *Salmonella heidelberg* (A), *typhimurium* (B), and *derby* (C) at 22 C in 0 to 8% NaCl.

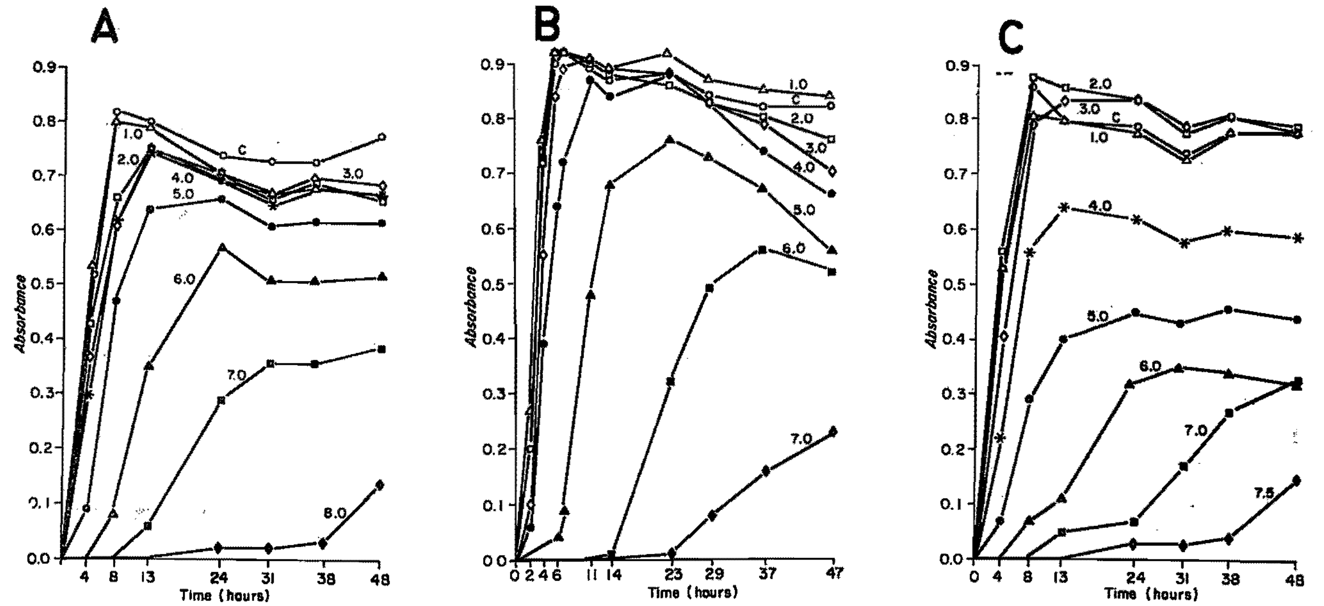

Figure 4. Growth of *Salmonella heidelberg* (A), *typhimurium* (B), and *derby* (C) at 37 C in 0 to 8% NaCl.

As a rule, relatively low concentrations of salt will stimulate microorganisms, while higher concentrations inhibit them *(5)*. This stimulation has been borne out with S. *heidelberg*, S. *typhimurium*, and S. *derby*, used in this study. At the lower suboptimum temperatures below 37 C, the organisms appear to grow more rapidly when low levels of NaCl are present. This effect is evident in salt concentrations ranging from 0.5 up to about 2.5% salt. In most instances the final number of cells attained is also higher at these salt concentrations than in the control samples, as measured by absorbance. This stimulation, which shows up at the lower temperatures, diminishes as the incubation temperature approaches optimum for the organisms and is not evident at temperatures above optimum. The growth of *Escherichia coli* in a dilute medium was reported to be greatly stimulated by low concentrations of sodium chloride *(15)*. The test organism decreased in number when grown in media containing 0 to 0.2% NaCl, but growth was stimulated at NaCl levels of 0.4% and 0.8%, with maximum stimulation at 2.8% NaCl.

Halophilic bacteria often incubated at 25 C have considerably higher optimum temperatures in solutions of high salt concentration. This same general phenomenon has been reported with osmophilic yeasts on high sugar media *(4)*. A shift in optimum temperature was not evident when the incubation temperature of S. *heidelberg* was raised from the optimum of 37 C to 41 C or above in the presence of high and low NaCl concentrations. The rate of growth at 41 C and above was, in fact, slower than at 37 C. Growth was rapid at both temperatures in salt concentrations up to about 5%, but rapidly decreased thereafter, indicating that the temperature optimum for S. *heidelberg* remains close to 37 C. When S. *heidelberg* was incubated at temperatures between 37 and 45 C in the presence of 0 to 9% NaCl, a shift in optimum temperature could have been de-

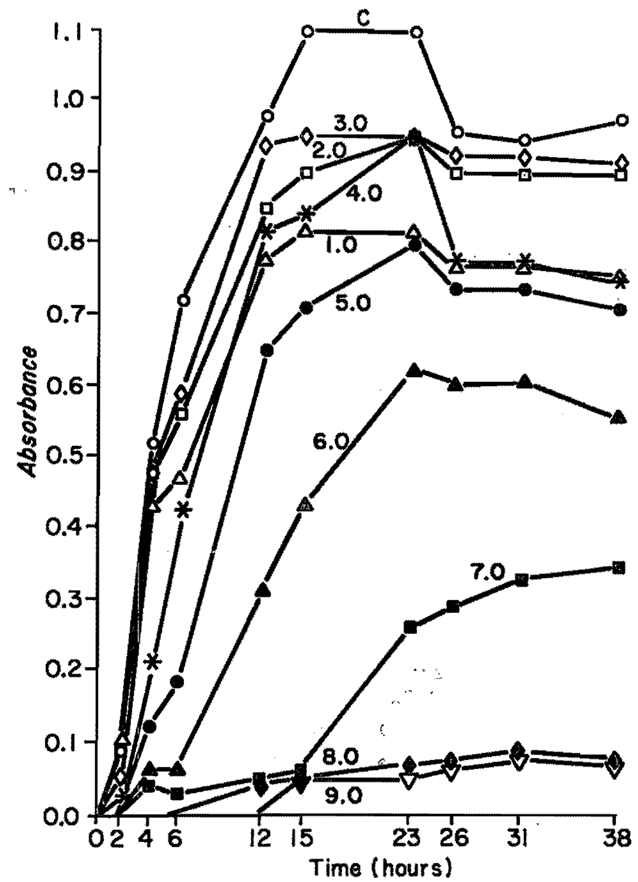

Figure 5. Growth of S. *heidelberg* at 41 C in 0 to 9% NaCl.

tected. Since a shift did not take place, it is reasonable to assume that under the test conditions this organism does not possess a higher optimum temperature in the presence of higher levels of salt. Work reported in the literature was done with marine isolates or halophilic bacteria, and it is possible that this phenomenon is peculiar to these organisms.

The amount of water in a food necessary to allow growth of spoilage and pathogenic bacteria varies with the types of organisms present. Each organism has a specific water requirement. The effects of temperature on these water requirements for *Salmonella* as regulated by the amount of salt present are large, especially at low temperatures far below the optimum for the organisms. At higher temperatures these effects are not as great with Salmonella growing over a wider range of salt concentrations.

From a practical point of view, application of these data to the storage of perishable food products is important. Foods with a low salt content may not support the growth of *Salmonella* or other pathogens at low temperatures. If, however, storage temperatures are raised, conditions may become favorable for growth of these pathogens.

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tection, personal hygiene, pest control and establishment and equipment sanitation. The NRA works with technical organizations in the fields of public health and safety to ensure that new developments of importance to the foodservice industry are evaluated and reported through bulletins, seminars and programs.

The foodservice industry has been represented in the development of the Public Health Service Food Service Sanitation Manual and will continue to be represented in the development of revisions to this manual, in order that the food protection and sanitation guide will be reasonable, applicable and will be beneficial to both public health departments and to the restaurant industry as a pattern for local codes and ordinances.

State and local restaurant associations are encouraged to promote the adoption and the use of the model code to the maximum extent appropriate for state and local application, excepting those provisions which pertain to the grading, regrading and placarding of establishments.