

INTERACTION OF pH AND NaCl ON THE RECOVERY
OF HEAT-STRESSED STAPHYLOCOCCUS AUREUS

by

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ABSTRACT

The effect of pH level and NaCl concentrations, alone and in combination, on enumeration of unstressed and heat-stressed cells of three strains of Staphylococcus aureus was determined. A definite narrowing of the optimum pH range for recovery of both unstressed and heat-stressed cells was observed as the NaCl concentration was increased from 0.0 to 7.5%. Recovery levels of unstressed cells diminished only slightly with increases in NaCl, while heat-stressed cells showed a marked sensitivity to NaCl concentrations of 4% and above, regardless of the pH level. Because of this sensitivity to NaCl, recoveries were far poorer than with unstressed cells at NaCl concentrations of 4% and above.

INTRODUCTION

Food-borne illness directly attributable to Staphylococcus aureus is an established problem. In a number of situations, the enumeration of this organism by selective media may become difficult if the food product in question has been subjected to heat treatment or other forms of physical stress sufficient to cause sublethal injury to the organism.

In many cases in which the enumeration of heat-stressed bacteria is attempted, the assumption often is made that conditions satisfactory for the recovery of unstressed bacteria are equally applicable to heat-damaged bacteria. As a result of such a conclusion, errors may be made in regard to the types and numbers of bacteria present in a particular foodstuff.

The injury incurred by bacteria as a result of heat stress usually manifests itself with some type of lesion to the cell. Such cells must be able to repair themselves before they can divide and produce countable colonies and therefore make enumeration possible. As a consequence, the conditions necessary for growth and division may become more restrictive and demanding than those required by unstressed forms. Environmental conditions such as pH, temperature, osmotic pressure, amino acid content of the medium, energy and carbon sources, vitamins, and growth factors may all become more important qualitatively and quantitatively than they are for unstressed bacteria.

The purpose of this study is to ascertain the effects of two environmental conditions interacting with one another, namely, NaCl and pH, on the recovery of heat-stressed S. aureus. Hopefully, the information presented will have practical applications in further defining suitable conditions for the recovery of this organism from food products which have been subjected to physical stress during various processing operations.

REVIEW OF THE LITERATURE

Selective media for the direct isolation of bacteria responsible for food spoilage and food-borne illness are widely used by both the food industry and public health officials (6,7). Such media are used in both quality control measures and as a means of enumerating organisms possibly implicated in illnesses directly attributed to the consumption of food material. Selective media such as staphylococcus medium 110 (12,22), azide blood agar (24), brilliant green bile broth (26), Bacto-Chapman tellurite solution with Bacto-Mitis salivarius agar (13), and eosin methylene blue agar (18) commonly employ a particular inhibitor to discourage the growth of mixed populations of bacteria and thus allow only the bacteria being sought to be enumerated.

In many situations, the food material upon which the bacteriological determination is to be made has been subjected to varying degrees of heat or other forms of physical stress in an attempt to preserve it and/or enhance its flavor. Under such conditions, the heat treatment may be sufficient to kill all of the microorganisms present. Several mechanisms for the killing action, such as coagulation and inactivation of certain enzymes required for metabolism, have been suggested (10). In some cases, however, food processing or cooking procedures may be either insufficient to sterilize the food product or they may be designed, as in pasteurization, only to reduce bacterial populations qualitatively and/or quantitatively. Under these

circumstances the determination of both numbers and types of microorganisms becomes difficult. This problem arises when the common assumption is made that conditions which are satisfactory for the enumeration of unheated organisms are equally satisfactory for the enumeration of heat-stressed organisms.

The type of medium used for recovering heat-stressed organisms is probably the most important single factor in enumerating such organisms. Nelson (27) showed significant differences between four different agar media in their ability to enumerate a variety of heat-stressed bacteria, while the same four media varied only slightly in their efficiencies in enumerating the same organisms when unstressed. This study strongly suggested that bacteria which have been subjected to heat treatment of sublethal intensity are more exacting in their growth requirements than are the unheated control bacteria of the same strain. Furthermore, the gross chemical composition of the recovery medium strongly influences the initiation of growth of heat-stressed bacteria. In a further study, Nelson (28) demonstrated that variations in quantities of several nutritional supplements in an agar basal medium also influence enumeration efficiencies of heat-stressed bacteria.

The literature contains numerous references citing the deficiencies of selective media used to enumerate heat-stressed bacteria. In studies done with Escherichia coli, Maxcy (25) showed bacterial counts obtained on violet red bile agar to be comparable to those obtained on standard plate count agar when used with uninjured cells. The presence of heat-injured cells, however, resulted in a count on the

violet red bile agar that was only 10% of the total count obtained with standard plate count agar. Clark and Ordal (15), in studies done with Salmonella typhimurium 7136, also showed comparable counts using unheated cells plated on trypticase soy agar and Levine eosin methylene blue agar containing 2% NaCl. When the two media were compared using thermally injured cells, 90% of the population was sensitive to the eosin methylene blue-NaCl medium. In comparison, only a slight reduction in plate counts was noted on the trypticase soy agar.

In a study using plate count agar as a reference medium, Nelson (30) compared the ability of five selective media to enumerate unheated and heat-stressed bacteria of the Streptococcus genus. The counts obtained were essentially the same for all five media with the exception of one when unstressed bacteria were used. Plate count agar yielded much higher counts than did the selective media after exposing the bacteria to a temperature of 62.8 C for 15 min.

The presence of S. aureus both as an infectious agent and as the cause of food-borne illness is well known. Staphylococcal food poisoning appears to be more common than any other type of microbial food poisoning (9). The implication of S. aureus as the causative agent in isolated food-borne illness cases may become difficult in situations where sublethal heat was used in processing the food before consumption. In such situations, the effect of sublethal temperatures, the mechanisms of injury and recovery, and the conditions necessary for recovery both before and after thermal stress should be understood in trying to enumerate such an organism.

Thermal injury following heat stress in S. aureus manifests itself in a number of lesions which express themselves by changes in cultural conditions necessary for recovery of the organism. One such lesion is the cytoplasmic membrane. Allwood and Russell (2,3,4,5) noted the leakage of amino acids, proteins, and 260-mu absorbing material from S. aureus cell suspensions following thermal stress. Similar results were obtained by Iandolo and Ordal (20), indicating some type of damage or impairment to the membrane. Ordal (32) also recognized thermal inactivation of cellular enzymes and partial denaturation of protein as other lesions caused by sublethal temperatures. Probably the most prominent injury site which results from thermal injury to S. aureus is the degradation of ribosomal RNA (3,5,36). Ordal attributes this breakdown to the thermal activation of enzymes which degrade rRNA, namely, ribonuclease and polynucleotide phosphorylase (32).

Regardless of the lesion site, the period following thermal stress is the most important if the organism is to survive and multiply. This time period is characterized in S. aureus by a prolonged lag phase (21). In addition, a loss of resistance to sodium chloride occurs (11), and the ability to produce coagulase is impaired (34). During this prolonged lag phase, the organism must initiate the intake of precursor material, resynthesize lost or inactivated cellular components, and effect repair on damaged lesion sites (32,36).

Once these priorities are accomplished, the cells can enter the logarithmic phase and divide at a rate indistinguishable from that of unstressed cells. Ordal (32) suggests that during this

prolonged lag phase the damage to the cytoplasmic membrane is rapidly repaired after the injured cells are placed in a suitable recovery medium. Iandolo and Ordal (20) also noted that inhibitors of protein synthesis, such as chloramphenicol and 5-methyl-tryptophan, do not affect recovery of heat-damaged cells of S. aureus. This suggests that protein synthesis is not an important aspect of the recovery process. It was observed in the same study, however, that actinomycin-D completely suppressed recovery. This result implied that ribonucleic acid synthesis was particularly involved in the recovery process. This conclusion was reached by radioisotope tracer experiments in which the rate of incorporation of the label into the ribonucleic acid fraction paralleled that of recovery and the return of salt tolerance. Such findings strongly implicate RNA as the major lesion site and, if suitable recovery is to be obtained, this lesion must be repaired.

The nutritional requirements for such recovery are an energy source such as glucose, amino acids, and inorganic phosphate (20,32). Stressed cells will recover in a medium which will not support growth or multiplication. In such cases the nutritional requirements for repair are not of the same type as those required for growth and multiplication.

The degree of thermal injury in S. aureus which expresses itself by the length of the prolonged lag phase is influenced by a number of factors. Walker and Harmon (40) studied the thermal resistance of S. aureus in relationship to temperature, time of exposure to the temperature, and the heating menstrum used. Their results indicated

that thermal injury was increased at higher temperatures and if the exposure time to a particular temperature was lengthened. They also noted that thermal injury would vary depending on the menstrum used for heating the cells at a constant time and temperature. Skim milk and cheddar cheese whey protected four strains of S. aureus far better than whole milk and phosphate buffer. In addition, Walker and Harmon noted that the age of cell suspensions was a factor in thermal resistance. Older cells were far more resistant to heat than younger cells of the same strain.

A number of other factors also influence the recovery of heat-stressed cells of S. aureus. Factors such as incubation temperature, plating techniques, recovery media, pH and NaCl concentrations should be kept in mind when trying to obtain maximum recovery of heat-stressed S. aureus. Iandolo and Ordal (20) found that recovery of S. aureus MF-31 following thermal injury was much better at 38 C and 30 C than at 20 C and 45 C. Allwood and Russell (1) list a temperature of 32 C as being far better than 38 C for recovery of heat-stressed S. aureus. In the same study they also noted that counts of heat-damaged cells were lower with surface viable plating as opposed to the pour plate method. Strange and Ness (39) showed a loss of viability following the chilling of aqueous suspensions of unheated coliform bacteria. This is an important factor to consider in enumerating heat-stressed bacteria, since rapid cooling is the most common method employed to quickly stop thermal stress in bacteria during experimentation on heat injury and recovery.

The effect of sublethal temperatures on S. aureus in regard to its NaCl resistance should be considered in enumerating this organism from food products. The discovery by Koch (22) that usually only staphylococci are able to grow in agar media containing 7.5% NaCl has fundamental bearing on the problem of isolating and testing for this organism. Since that time, several workers (12,16,23,33,35) have studied the response to increasing NaCl on the initiation and selection of growth in S. aureus. Based on the initial work of Koch (22), Chapman (12,14) developed a number of selective and differential media which use 7.5% NaCl as at least a portion of the basis of their selectivity. Such NaCl concentrations have little or no effect on staphylococci which are unstressed or have not been subjected to heat. However, Busta and Jezeski (11) found that S. aureus heated at 60 C lost its ability to multiply on staphylococcus medium 110 containing the normal concentration of 7.5% NaCl. In addition, the ability to grow on modified staphylococcus medium 110 (NaCl concentrations 3.75, 2.0, and 1.0%) was dependent on the length of exposure to 60 C. Since then several investigators (15,17,34,36,38) have reported the same observations, indicating a definite salt sensitivity by S. aureus following thermal injury. Such a change in salt sensitivity obviously reduces the effectiveness of media which are designed to selectively enumerate S. aureus on the assumption that the organism is resistant to NaCl.

The pH range for growth of unheated S. aureus varies slightly between individual strains. Iandolo and Ordal (19) list a pH range of 5.0 through 9.0 for S. aureus MF-31. Nelson (29), however, cites a pH

range of 5.0 through 9.5 for Micrococcus pyogenes variety aureus (S. aureus). In the same study Nelson also observed that this pH range was reduced when the organism was exposed to heat-stress. The narrowing of the optimal pH range was also seen in E. coli and Streptococcus durans following exposure to elevated temperatures.

Little information is found in the literature as to the combined effects of NaCl and pH on heat-stressed S. aureus. In a study done on coliform bacteria from 6 IMVIC groups, Nelson (31) pointed out that the inhibitory effects of pH and NaCl on heat-stressed coliform bacteria are additive. The counts on unstressed organisms were unaffected by pH over a range of 5.0 through 9.2. Additions of 3.0% NaCl caused only slight reductions in numbers above pH 9.0. Heat-stressed cells on the other hand gave maximum counts over a narrower pH range of 6.9 through 7.9. Maximum counts obtained with the same stressed forms in the presence of 3.0% NaCl were obtained over a pH range of 6.0 through 7.0.

Genigeorgis et al. (16) examined the effects of pH and NaCl on the probability of initiating growth in four unstressed strains of S. aureus. Their findings indicated an additive inhibitory effect by combining the two parameters. As the NaCl concentration increased, the probability of initiating growth at any pH decreased.

MATERIALS AND METHODS

Organism and Cultural Characteristics

Three strains of S. aureus were employed in this study. Strain UA-112 was obtained from the Department of Microbiology and Medical Technology at The University of Arizona. Strains S-6(B) and FRI-100 were obtained from Dr. M. S. Bergdoll of the University of Wisconsin Food Research Institute. Strain S-6(B) is reported to produce large amounts of enterotoxin B and small amounts of enterotoxin A. Strain FRI-100 is reported to produce enterotoxin A. The enterotoxin productivity of strain UA-112 has not been assayed and is therefore unknown.

The biochemical activities of all three strains appear to be similar. All three strains are catalase and coagulase-positive. In addition, each is capable of fermenting mannitol, glucose, and lactose. Nitrate reduction and growth on agar media containing 7.5% NaCl are additional characteristics shared by all three strains. On the basis of these few cultural and biochemical characteristics, each of these three strains falls under Baird-Parker's (8) staphylococcal classification as being S. aureus subgroup I.

Each strain was grown up on brain heart infusion agar (Difco) slants for 24 hr at 37 C and held at 5 C for subsequent use. Lyophilized cultures were also set up for preservation of essentially unmodified strains.

Media Employed for Growth and Enumeration

Staphylococcus aureus cells of all three strains were grown up by inoculating one loopful of cells directly from brain heart infusion agar slants into test tubes containing 5 ml of nutrient broth (Difco). The medium was incubated for 24 hr at 37 C, after which one loopful of the suspension was transferred to a 125-ml flask containing 50 ml of nutrient broth. This culture also was incubated for 24 hr at 37 C.

Enumeration of S. aureus cells before and after heat stressing was done on nutrient agar (Difco). The medium was prepared in 2-liter quantities and dispensed in 103-ml portions into milk dilution bottles and autoclaved prior to use.

The pH adjustments of the nutrient agar were made by additions of either \underline{N} KOH or \underline{N} H₂SO₄ to 100 ml of sterile melted nutrient agar maintained in a water bath at 45 ± 1 C. The exact amounts added to obtain a particular pH were read directly from a pH titration curve based on volumes of acid or base required per 20 ml of nutrient agar to obtain a desired pH.

After additions were made, samples were poured into plastic petri dishes and allowed to solidify. The pH of the nutrient agar was checked prior to use with a Beckman Expanded-Scale pH meter (Model 76) equipped with a calomel-type reference electrode (Model 39402) and a glass electrode having a low sensitivity to excess sodium (Model 41263). Readings were made at 25 C by direct insertion of both electrodes into the solid medium. The pH levels employed in this study were 5.0, 5.5, 6.0, 7.0, 8.0, 8.5, 9.0, and 10.0 with a variation of ± 0.05 pH units.

Adjustments of the NaCl concentrations of the nutrient agar were done prior to autoclaving separate lots being made up from the regular nutrient agar. Concentrations were expressed on a percentage basis with additions of NaCl being made on a weight/volume basis. Volumes of a particular NaCl concentration were made up by addition of NaCl to 1 liter of distilled water, followed by additions of peptone, beef extract, and agar. The NaCl containing nutrient agar was dispensed into milk dilution bottles in 103-ml quantities and autoclaved. Sodium chloride concentrations used in this study were 2, 3, 3.5, 4, 5, and 7.5%. Adjustments of pH of NaCl containing nutrient agar were done as previously mentioned immediately prior to use.

Heat Stressing and Enumeration

Staphylococcus aureus cells were grown up prior to heat stressing by the method previously described. Following incubation, the flask was placed on a Burrell wrist shaker and agitated at a rate of 105 gyrations per minute for 5 min to limit cell clumping. After agitation, a 1-ml sample was removed and placed in a screw-top 15 X 125 mm test tube containing 5 ml of sterile reconstituted milk (110 g milk-solids-nonfat/liter). The bacteria were uniformly suspended by mixing on a Variwhirl vortex (Van Waters and Rogers, Inc.) after which a 1-ml sample was removed and serially diluted in 99-ml quantities of phosphate buffer (pH 7.2) contained in milk dilution bottles. Using 1.1-ml pipettes, 1.0 and 0.1 ml samples were removed from the appropriate dilutions and placed in plastic petri dishes and pour-plated in duplicate with warm nutrient agar. All dilution and pour-plating procedures

followed those outlined by Standard Methods (7). Plates were incubated at 37 C for 48 hr.

The remaining milk sample was immersed, along with a control milk sample tube containing a thermometer, in a water bath maintained at 56 ± 0.1 C. Timing of the sample commenced when the temperature reached 56 C in the control tube. Less than 4 min were required of any sample to reach the desired temperature. The exposure time of the bacterial suspension to 56 C was selected to reduce the viable population by 99% and varied slightly among the three strains. Staphylococcus aureus UA-112 required 7 min exposure, while strains S-6(B) and FRI-100 required 6 min. Following exposure for the specified time, samples were rapidly cooled in ice water to stop further heat damage to the cell population. The suspension was then agitated on the vortex mixer and a 1-ml sample was removed, diluted, and pour-plated with nutrient agar.

In addition to plating samples on unmodified nutrient agar before and after heat stressing, samples from appropriate dilutions for both groups were also plated on nutrient agar adjusted to specific pH levels and NaCl concentrations.

Determination of NaCl and pH Interaction
on the Recovery of Unheated and
Heated S. aureus Strain UA-112

Viable cell counts of strain UA-112 were obtained by plating on unmodified nutrient agar (pH 7.0, 0% NaCl). In order to compare recovery efficiencies of the organism to various NaCl concentrations at different pH levels, cells were also plated from appropriate dilutions on

nutrient agar containing NaCl concentrations of 0, 2, 3, 3.5, 4, 5, and 7.5%. Counts at each NaCl concentration were made at pH levels of 5.0, 5.5, 6.0, 7.0, 8.0, 8.5, 9.0, and 10.0. Since all combinations of pH and NaCl could not be employed at one time because of the number of plates involved, only a portion of the testing was done at one time. Each portion included a plating on unmodified nutrient agar to provide the necessary control value for calculations. In addition, comparative platings of the organism were done on nutrient agar adjusted to a given NaCl concentration and containing a complete series of pH levels. Enumeration of the organism in a pH series at a given NaCl concentration was done using one lot of cell suspension grown up in its individual flask. All plating for both unstressed and heat-stressed cells was made in duplicate. Furthermore, two trials were run for each NaCl-pH combination.

All counts obtained before and after heat stressing at a specific NaCl combination were divided by their corresponding counts obtained on unmodified nutrient agar and multiplied by 100 to obtain an arithmetic percent recovery. The arithmetic percent recoveries were then converted to logarithms to the base 10 to obtain a \log_{10} of the percent recovery. By such an expression, a comparison of recovery efficiencies at specific NaCl-pH combinations can be made in relationship to the standard base medium of unmodified nutrient agar at pH 7.0 in the absence of NaCl.

Data for heat-stressed cells were treated in the same fashion as those for unheated cells by comparing recoveries obtained on

unmodified nutrient agar to recoveries obtained at a specific NaCl-pH combination and the results expressed in the form of \log_{10} of percent recovery.

Determination of NaCl and pH Interaction
on the Recovery of Unheated and Heated
S. aureus Strains S-6(B) and FRI-100

The procedure for determining recovery efficiencies of S. aureus strains S-6(B) and FRI-100 was similar to that used for strain UA-112. In order to check only those points of greatest importance, fewer NaCl concentrations accompanied by fewer pH levels were used for comparison. Salt concentrations of 0, 2, 3, 3.5, 4, and 5% were used along with pH levels of 5.0, 5.5, 7.0, 8.5, and 9.0. In addition, duplicate plating was only used for counts on the control unmodified nutrient agar for unstressed and heat-stressed cells. Two trials were run for each NaCl-pH combination. Each NaCl-pH combination, as with strain UA-112, was accompanied with plate counts on unmodified nutrient agar both before and after heat stress.

As with strain UA-112, data for both unstressed and heat-stressed cells of both strains were treated so as to compare recoveries obtained on unmodified nutrient agar to recoveries obtained at a specific NaCl-pH combination and the results expressed in the form of \log_{10} of percent recovery.

Statistics

\log_{10} percent recovery values for S. aureus strain UA-112 were statistically treated for significance by placing the values obtained into two separate groups, unheated and heated. The data for each group

were subjected to a completely randomized two-way factorial arrangement using NaCl and pH as the factors.

Recovery values for strains S-6(B) and FRI-100 were also separated into two groups, unheated and heated. The experimental analysis was expanded to include both strains in the heated and unheated groups. Statistical treatment as above involved a completely randomized three-way factorial analysis of variance, with NaCl, pH and strain constituting the factors.

Statistical computation was performed by the CDC 6400 computer at The University of Arizona computer center. The program (37) was supplied by Dr. Lee M. Kelley of the Department of Microbiology and Medical Technology.

RESULTS AND DISCUSSION

Data on recovery of unheated cells of S. aureus strain UA-112 at different levels of pH in the presence of increasing concentrations of NaCl are shown in Figure 1. Strain UA-112 showed no significant differences in its ability to develop countable colonies over a pH range of 5.0 through 8.5 in the absence of NaCl. At NaCl concentrations of 2 and 3%, maximum recovery of cells was obtained over a pH range of 5.0 through 9.0. Recoveries at pH 10.0 were significantly lower at all three of these low NaCl concentrations. At 3.5% NaCl, counts obtained at pH 9.0 were significantly less than maximum and appeared somewhat similar to recoveries made at pH 10.0, while maximum recoveries were not significantly different over a pH range of 5.0 through 8.5. Recoveries at 4% NaCl were essentially the same as those found at 3.5%.

Significant changes in recovery levels occurred at 5% NaCl. Maximum recoveries of cells were obtained at pH levels of 6.0, 7.0, 8.0, and 8.5, while recoveries at pH 5.0 and 5.5 were significantly lower and at essentially the same level. Recoveries obtained at pH levels of 5.0 and 5.5 were still much higher than those obtained at pH 9.0 and 10.0. At 7.5% NaCl, recoveries made at pH 6.0 fell off significantly, while counts made at pH levels of 5.0 and 5.5 were even lower. Maximum recoveries remained essentially the same over a pH

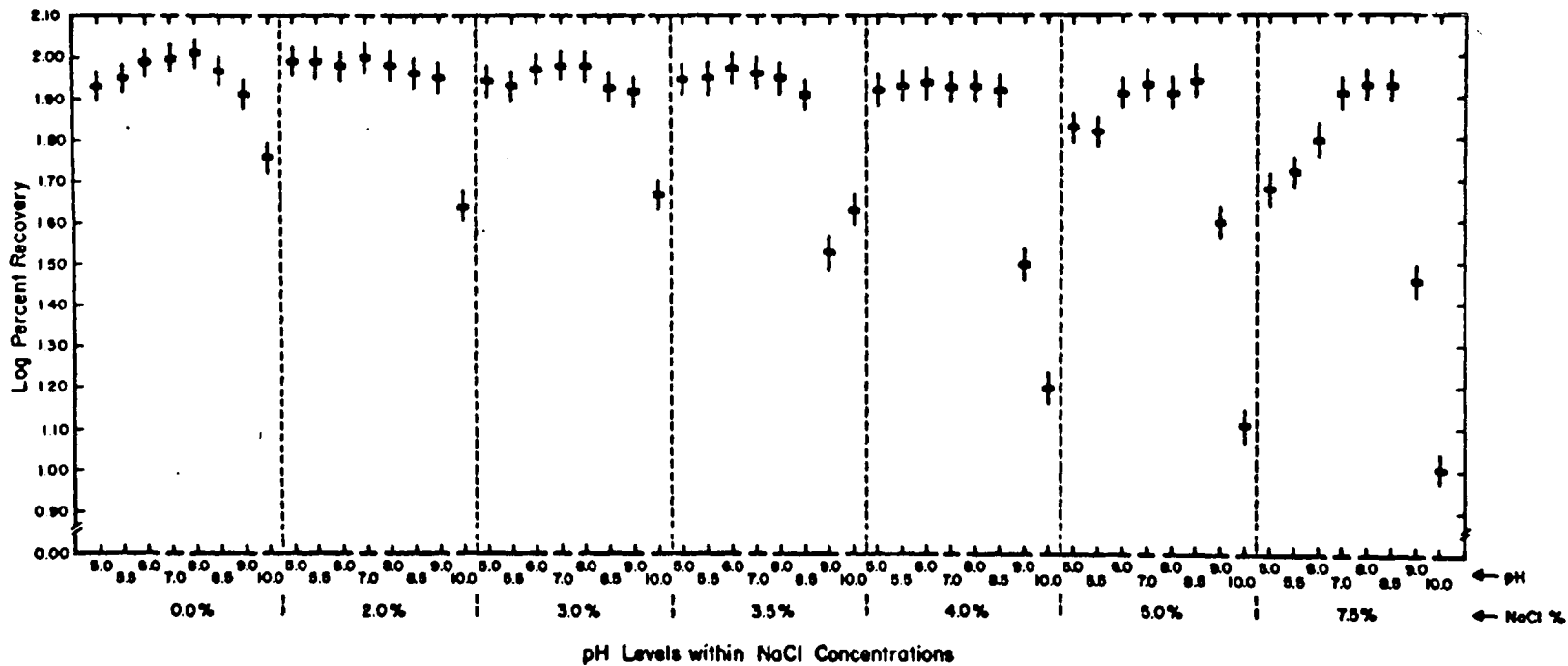


Figure 1. Recovery of unstressed cells of *S. aureus* UA-112 at various pH levels in the presence of increasing concentrations of NaCl.

range of 7.0 through 8.5 and were only slightly lower than counts obtained at the same pH levels in the absence of NaCl.

Data on recovery of heat-stressed S. aureus strain UA-112 at different pH levels in the presence of increasing NaCl concentrations are shown in Figure 2. In comparison to unstressed cells, heat-stressed cells showed greater variation in responses, as indicated by larger confidence limits surrounding average recoveries. At both 0 and 2% NaCl, recovery of cells remained at a maximum over a pH range of 5.5 through 9.0. Recoveries obtained at pH 5.0 and 10.0 were quite low. Recoveries obtained at pH 5.0 with stressed cells were much lower than with unstressed cells under the same conditions.

Increase of the NaCl concentration to 3 and 3.5% was accompanied by a slight drop in recovery at pH 9.0. Maximum recoveries at both of these NaCl concentrations were obtained over a pH range of 5.5 through 8.5.

Recoveries of heat-stressed cells at 4% NaCl showed an overall decrease from counts obtained at 3.5% NaCl. This overall drop in recovery occurred at all pH levels. Maximum recoveries at 4% NaCl were obtained over a pH range of 5.5 through 8.5 and were almost 1 full unit lower than counts obtained at 3.5% NaCl. Such a drop indicates a marked sensitivity of this heat-stressed organism to 4% NaCl. Recoveries made at pH 10.0 in the presence of 4% NaCl were significantly less than recoveries made at pH 10.0 in the absence of NaCl and at 3.5% NaCl. At 4% NaCl and pH 9.0 recoveries were significantly lower than those obtained at pH 9.0 in the absence of NaCl and 3.5%. Recoveries

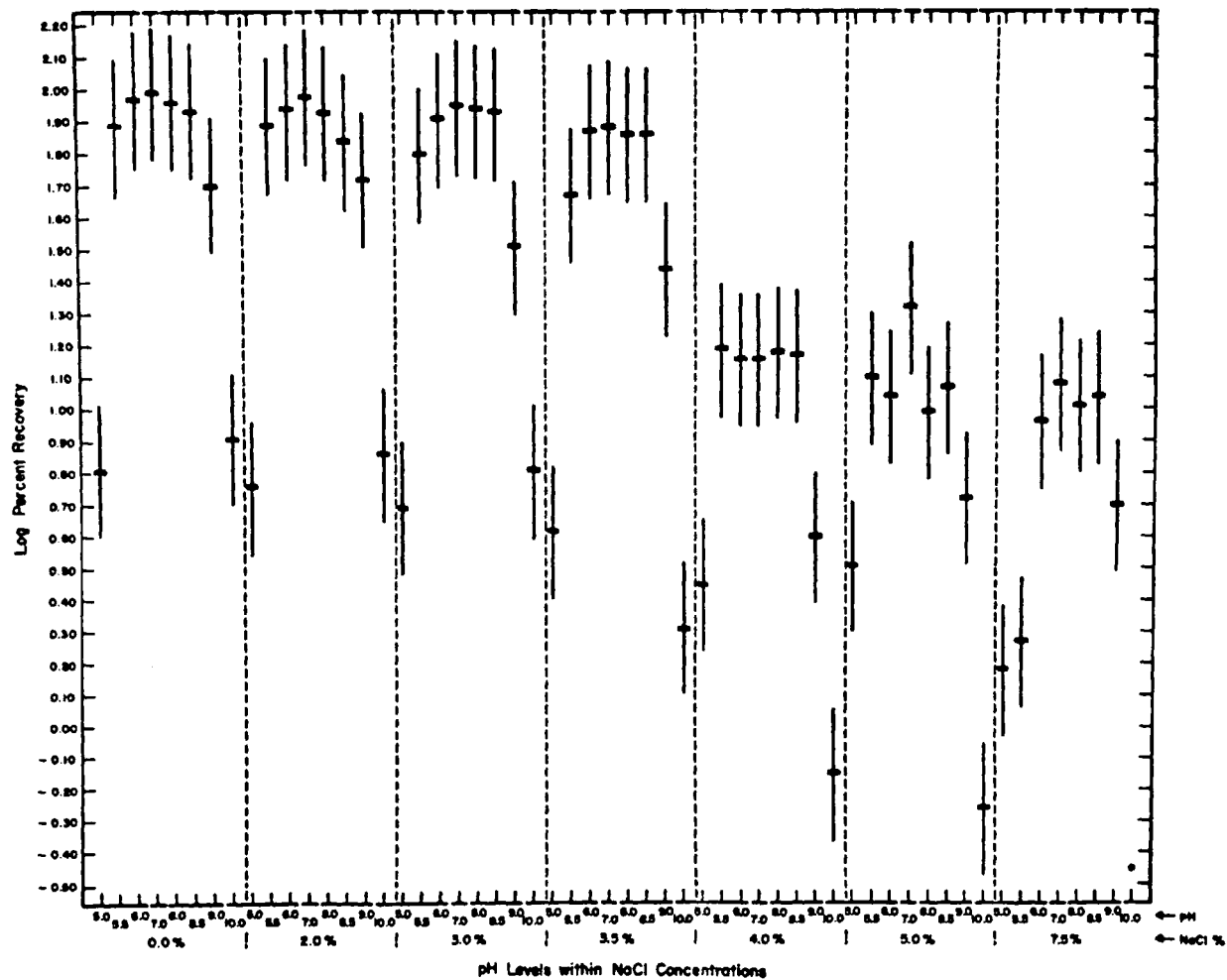


Figure 2. Recovery of heat-stressed cells of *S. aureus* UA-112 at various levels of pH in the presence of increasing concentrations of NaCl.

obtained at 5% NaCl were quite similar to those obtained at 4% NaCl. However, counts obtained at 7.5% NaCl showed a drop in recovery at pH 5.5, while no viable colonies were obtained at pH 10.0.

For the purpose of comparison, the responses of two other unstressed strains of S. aureus, namely, S-6(B) and FRI-100, to pH and NaCl are shown in Figures 3a and 3b. Recoveries of both strains were similar, with minor exceptions, to the recovery profile of unstressed cells of S. aureus strain UA-112. Maximum recoveries of both strains appeared quite similar over a pH range of 5.0 through 9.0 at NaCl concentrations of 0 through 3.5%. At NaCl concentrations of 4 and 5%, recoveries at pH 9.0 became slightly but not statistically significantly lower than at pH 7.0 for strain S-6(B), while strain FRI-100 showed significantly lower recoveries at the same pH level at both of these NaCl concentrations. Both strains showed considerably lower recoveries at pH 5.0 at 5% NaCl; while these recoveries were statistically significantly lower than at pH 7.0 in the absence of NaCl, they were not statistically significantly lower than recoveries at pH 7.0 at the 5% NaCl level. It is apparent for both strains, as with strain UA-112, that, as the NaCl concentration increases, the pH range which permits maximum recovery decreases slightly. Although this narrowing of the optimal pH is not as apparent when compared to strain UA-112, recoveries at pH levels 5.0 and 9.0 at higher NaCl concentrations tend to be slightly lower, indicating a somewhat similar trend.

The responses of heat-stressed cells of strains S-6(B) and FRI-100 at various pH levels in the presence of increasing NaCl

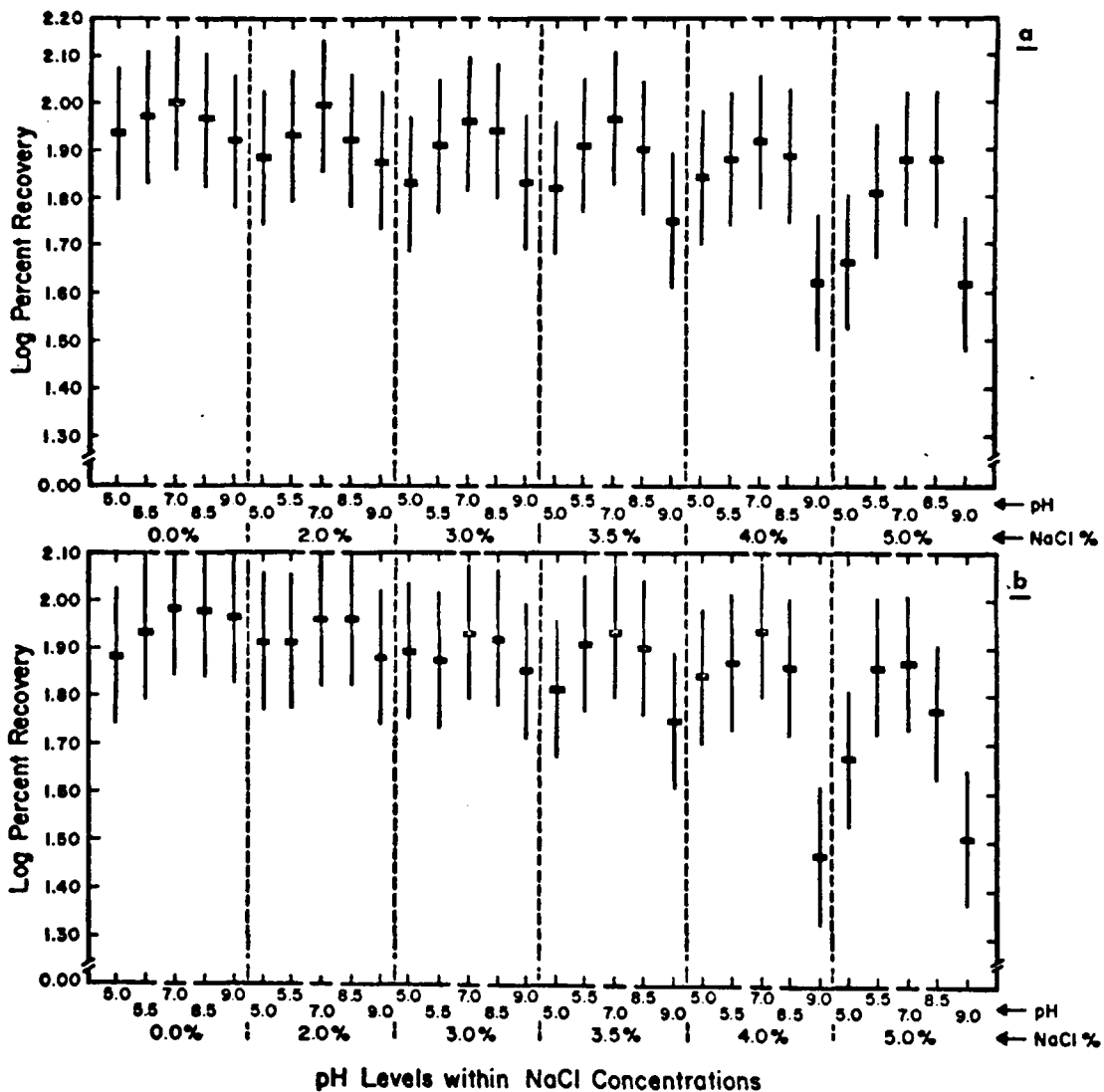


Figure 3. Recovery of unstressed cells of *S. aureus* strains S-6(B) and FRI-100 at various levels of pH in the presence of increasing concentrations of NaCl.

- a. S-6(B)
- b. FRI-100

concentrations are shown in Figures 4 and 5. Both stains appeared quite similar in their responses. Both strains gave maximum recoveries within a given NaCl concentration over a pH range of 5.5 through 8.5 at NaCl concentrations of 0 through 3.5%. Counts made at pH 9.0 over the same NaCl concentrations were somewhat similar but were statistically lower than at pH 7.0. Both strains gave poor recoveries at pH 5.0. Counts at this pH level dropped steadily as the NaCl concentration increased to 5%. At 4% NaCl, both strains showed an overall drop in recovery that was similar to the decreased recoveries displayed by strain UA-112 at the same NaCl concentration. At 4 and 5% NaCl, recoveries at pH 9.0 became greatly reduced in comparison to maximum counts obtained at pH levels 5.5 through 8.5. Recoveries made at these high NaCl concentrations were reduced, as with strain UA-112, by approximately 1 log unit in comparison to counts obtained at 3.5% NaCl.

Unstressed cells of all three strains of S. aureus appeared quite similar in their responses to various levels of pH in the presence of increasing concentrations of NaCl. These responses, however, were quite different from those given by heat-stressed cells of the same strain. A comparison of the salt sensitivities between the two types of cells provides probably the most significant difference. Unstressed cells at pH 7.0 at NaCl concentrations of 0 through 7.5% showed only slight decreases in recovery as the NaCl concentration increases. Heat-stressed cells, however, showed a distinct sensitivity to NaCl at 4%, as indicated by a sharp decrease in counts at that NaCl concentration. This marked sensitivity following thermal injury was similar to the

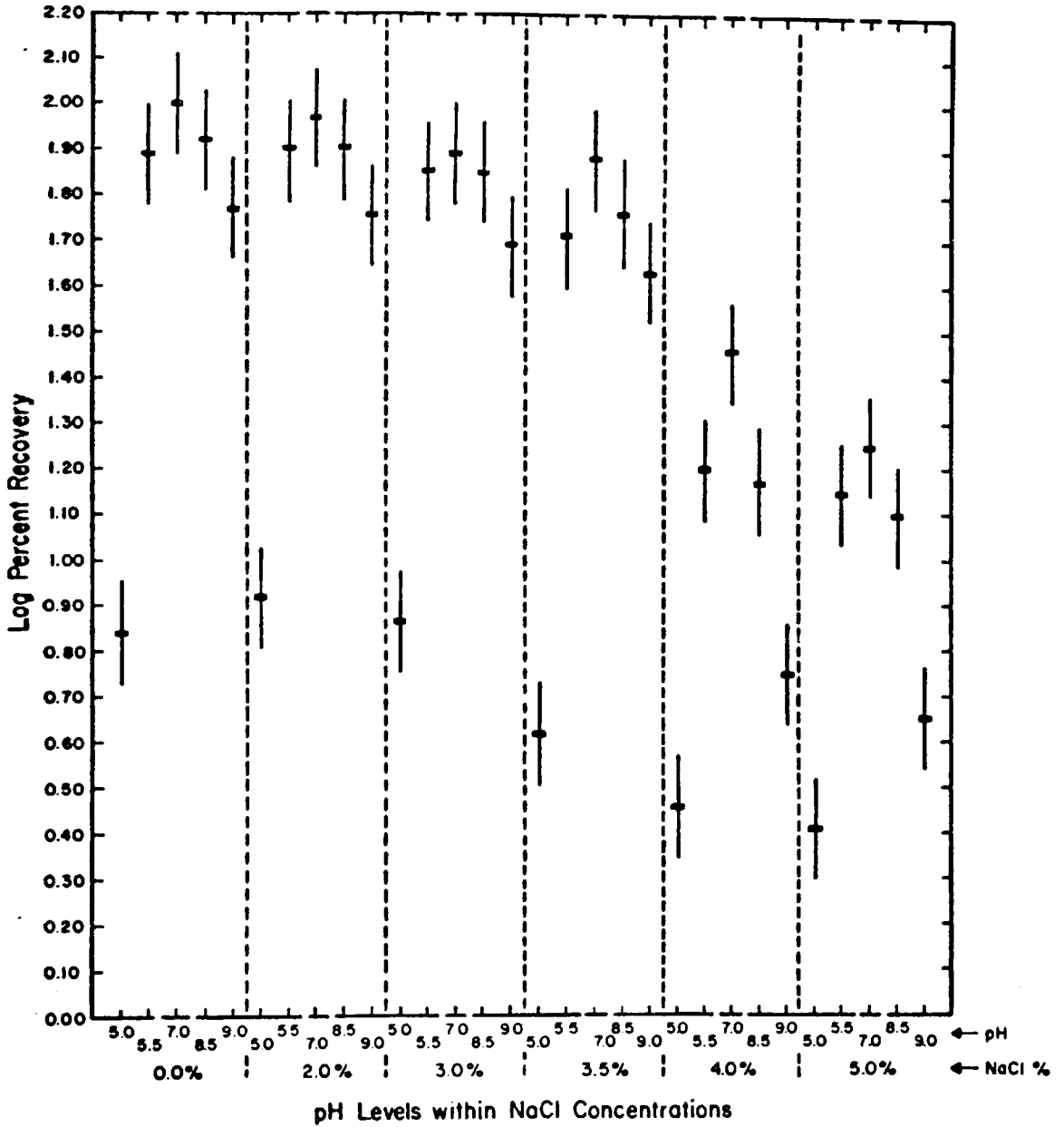


Figure 4. Recovery of heat-stressed cells of *S. aureus* S-6(B) at various levels of pH in the presence of increasing concentrations of NaCl.

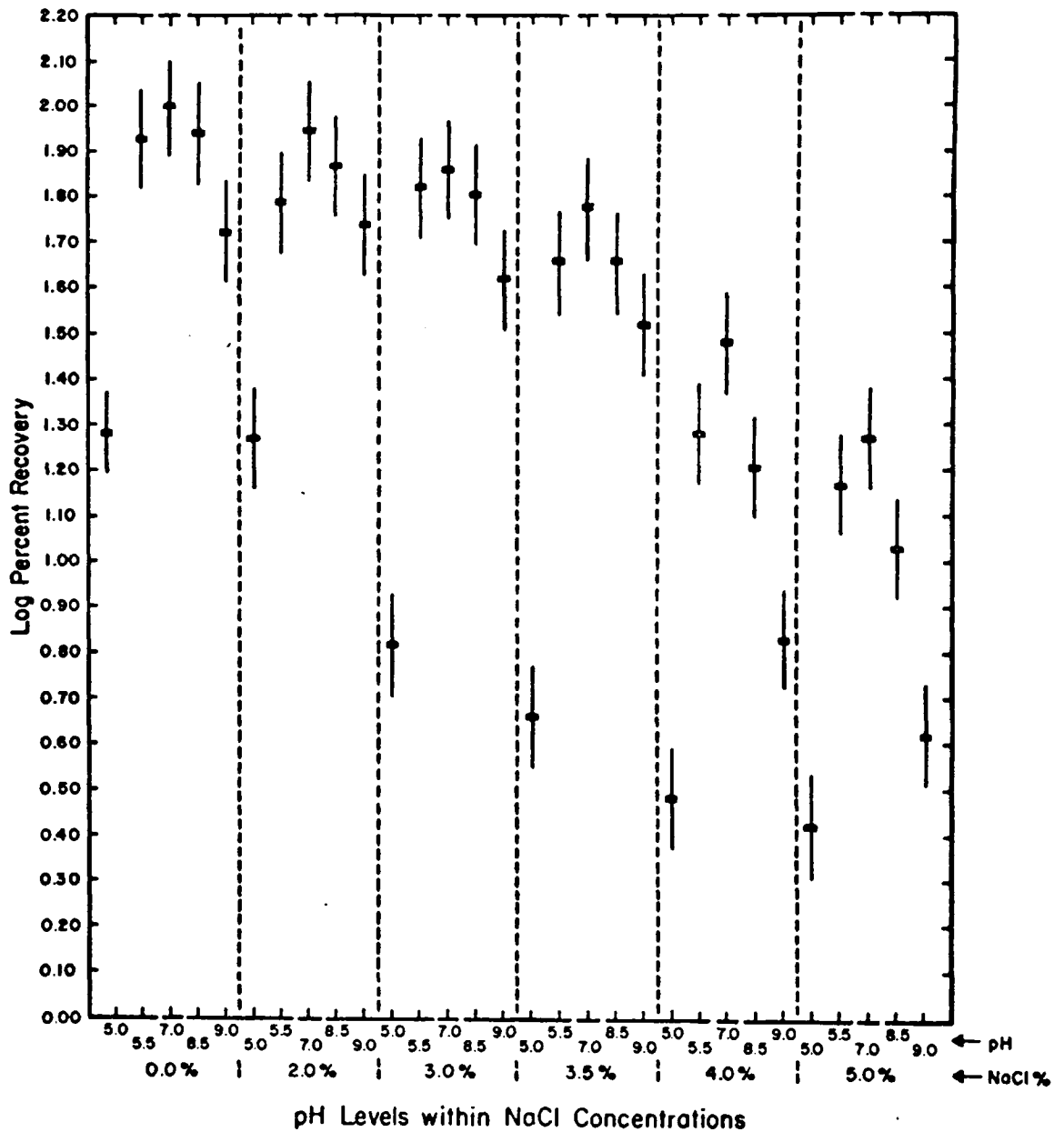


Figure 5. Recovery of heat-stressed cells of *S. aureus* FRI-100 at various levels of pH in the presence of increasing concentrations of NaCl.

observations made by Busta and Jezeski (11), who also noted this sensitivity in heat-stressed cultures of S. aureus. Their finding pointed out that heat-stressed cultures of S. aureus were sensitive to NaCl concentrations below 7.5%. The degree of sensitivity of the culture to these lower concentrations of NaCl was dependent on the degree of thermal stress. Cultures stressed for only brief periods of time could tolerate and grow at higher concentrations of NaCl, while cultures thermally stressed for longer periods of time showed sensitivities to NaCl at 4% and below.

Unstressed cells of all three strains displayed similar maximum recoveries over a pH range of 5.0 through 9.0 in the absence of NaCl, while heat-stressed cells gave maximum recovery counts over a pH range of 5.5 through 8.5. Such findings agree with those of Iandolo and Ordal (19) who list a similar pH range for unstressed cells of S. aureus. The pH range of S. aureus following thermal injury was also in agreement with the findings of Nelson (29), who listed a pH range of 5.3 through 8.5 as a range for obtaining maximum recoveries. Unstressed cells of all three strains maintained their optimal pH range of 5.0 through 9.0 as the NaCl concentration increased through 3%. Recoveries at pH 9.0 at 3.5% NaCl were significantly lower for strain UA-112, while recoveries for the other two strains were only slightly lower. At higher NaCl concentrations, such as 4 and 5%, recoveries fell off at pH 9.0 and somewhat at pH 5.0. At 7.5% NaCl, optimal recoveries were obtained with strain UA-112 over a pH range of 7.0

through 8.5. Recoveries at these pH levels were only slightly lower than those made in the absence of NaCl at the same pH levels.

In contrast, heat-stressed cells maintained their optimal pH range of 5.5 through 8.5 up through 5% NaCl in the case of strain UA-112. Strains S-6(B) and FRI-100 showed similar results, except in a few instances where recoveries were slightly lower at pH 5.5 and 8.5 at 4 and 5% NaCl. At 7.5% NaCl, heat-stressed cells of strains UA-112 showed a drop in recovery at pH 5.5, while maximum recoveries were obtained over a pH range of 6.0 through 8.5. Due to the marked sensitivity of all three strains to 4% NaCl, recoveries at all pH levels at 4% and above were almost 1 log unit lower than recoveries at 3.5% NaCl. In addition, heat-stressed cells of all three strains showed poor recoveries at pH 5.0 at all NaCl concentrations. Furthermore, heat-stressed cells of strain UA-112 showed poor recoveries at pH 10.0 at all NaCl concentrations. At pH 10.0 at 7.5% NaCl strain UA-112 failed to produce any colonies.

Analysis of variance for all three unstressed strains of S. aureus revealed significant first-order interactions between NaCl and pH, indicating an additive effect by the two factors in recovering unstressed cells. In addition, comparison of unstressed cells of strains S-6(B) and FRI-100 showed no significant differences between these two strains in their response to NaCl and pH. Heat-stressed cells of all three strains of S. aureus also showed significant first-order interactions between NaCl and pH, indicating an additive effect of these two factors on recovery. Furthermore, comparison of strains S-6(B) and

FRI-100 to one another revealed two additional first-order interactions. Significant interactions were noted with the factors pH and strain and also NaCl and strain. These interactions indicate that the two strains reacted slightly differently from one another to pH over all NaCl concentrations and to NaCl over all pH levels. In comparison to unstressed cells, heat-stressed cells show more variation from strain to strain in their responses to NaCl and pH, even though both groups show a narrowing of their pH range as the NaCl concentration increases.

Although three strains do not constitute a large sample, the indications are that the responses to pH and NaCl demonstrated here are characteristic of the species. As the degree of adversity, especially pH, was varied, a gradual decline in recovery occurred; this decline was accentuated by increasing NaCl concentration. Both unstressed and heat-stressed cells showed a narrowing of their optimum pH ranges as the NaCl concentration was increased. These findings were in close agreement with the results obtained by Genigeorgis et al. (16) on unstressed cells of S. aureus, indicating a decreased response of the organism to any pH level as the NaCl concentration increases. However, due to the marked sensitivity of heat-stressed cells to NaCl, overall recoveries at 4% and above were far poorer regardless of pH in comparison to unstressed cells. This sensitivity to NaCl, especially as it increased from 3.5 to 4%, by heat-stressed cells of all three strains at near neutral pH's seems to indicate a considerable degree of population homogeneity. Strains UA-112 and S-6(B) both showed very sharp responses to NaCl following thermal stress, while strain FRI-100 showed a

somewhat less pronounced change at 4%. The responses of all three strains to pH were slightly different, as indicated by a small amount of variation occurring from strain to strain.

While dropping the NaCl concentration of a selective medium would obtain better recovery of staphylococci which have been subjected to thermal stress, it would do away with much of the selective character of such a medium. Due to the NaCl sensitivity of S. aureus following thermal stress, it would appear that no combination of pH and NaCl would offer promise of improving recovery of such an organism from mixed populations of bacteria. Improvement of media for this purpose must be sought in other directions.

CONCLUSIONS

Heat-stressed cells of S. aureus are quite different from unstressed cells in their responses to various pH levels in the presence of increasing concentrations of NaCl. Although both types of cells show a narrowing of their optimal pH ranges as the NaCl concentration increases, heat-stressed cells show poorer recoveries at extreme pH levels in comparison to unstressed cells. In addition, heat-stressed cells show a marked sensitivity to NaCl concentrations above 3.5%. Because of these differences, selective media containing high concentrations of NaCl are far less effective in obtaining maximum recoveries of heat-stressed cells than are media low in NaCl content. Unstressed cells are considerably less affected by concentrations of NaCl greater than 3.5%.

Maximum recoveries of unstressed cells of S. aureus can be obtained quite effectively in a medium containing 7.5% NaCl adjusted to a pH range of 7.0 through 8.5. Maximum recoveries of heat-stressed cells cannot be attained on media containing 4% or more NaCl. The pH of the medium should not be adjusted outside a pH range of 5.5 through 8.5.

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