

THESIS



ABSTRACT

SURVIVAL OF HEATED STREPTOCOCCUS FAECALIS AS AFFECTED BY AGE, INCUBATION TEMPERATURE, AND RECOVERY MEDIA

by Larry R. Beuchat

The objective of this study was to investigate the properties relating to thermal resistance of Streptococcus faecalis and to examine the changes brought about by exposure of S. faecalis to heat.

Three strains of S. faecalis, S21, S23, and S28, were studied. All strains were cultured in All Purpose plus Tween broth (APT) before subjecting them to heat in fresh APT broth.

The effect of age on heat resistance was determined by exposing cells in different phases of the growth cycle to heat and then recovering the viable cells on APT agar. Survivor curves for the three strains were plotted after heating the organisms for various lengths of time and then counting survivors on APT agar. Incubation was at 30 C.

To investigate the effect of incubation temperature on the recovery of heated S. faecalis, heated organisms were incubated at 7.3, 10, 15.6, 21, 25, 30, 37 and 45 C. APT agar was used as the recovery medium.

APT recovery media containing various added amounts of either sodium chloride (NaCl), potassium chloride (KCl), or magnesium chloride (MgCl_2) were used to assess the effect of salts on the recovery of thermally injured S. faecalis.

Analyses were performed to determine the effect of heat on the ribonucleic acid (RNA) content of cells. Total cellular RNA of S. faecalis heated for various lengths of time at 60 C was quantitated by a modified phenol extraction procedure.

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to my parents

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INTRODUCTION

A clear assessment of the significance of Streptococcus faecalis as a potential food poisoning organism cannot be drawn from the literature. Many workers (Cary et. al., 1938; Buchbinder et. al., 1948) have considered alpha-type streptococci responsible for food poisoning outbreaks when they were predominant in suspected foods. Reports of food poisoning in which fecal streptococci have been implicated have invariably involved numbers of greater than one million organisms per gram of food.

As long as the food poisoning potential of S. faecalis exists, caution should be taken when processing, handling, or storing foods which are subject to contamination by the organism. To help define those measures which might be taken to reduce the risk of poisoning by S. faecalis, the recovery of three strains of the heated organisms was evaluated in media containing various concentrations of sodium chloride, magnesium chloride, and potassium chloride. The effect of age of the cells on resistance to heat and the effect of incubation temperature on the recovery of heated S. faecalis was also assessed. Finally, in an attempt to discover a possible metabolic mechanism related to survival, ribonucleic acid analyses of heated and unheated cells were carried out.

LITERATURE REVIEW

The rate of recovery as well as the total recovery of heated microorganisms is dependent upon the physiological condition of the cells before heating, the nature and extent of the heating process, and the environmental conditions to which the heated cells are subjected after exposure. The coordination or noncoordination of many factors within each of these areas as well as the relationship of factors between specific areas decide the extent of thermal inactivation of a given culture.

Physiological Condition of the Cells Before Heating

Elliker and Frazier (1938) noted that growth of Escherichia coli at and above the optimum temperature resulted in cultures whose heat resistance during the maximum stationary phase was distinctly greater than that of cultures incubated at temperatures below the optimum for growth. A continuous decline in resistance was noted during the period when cultures of E. coli were incubated at about their optimum temperature. A thermoduric streptococcus in the lag and early logarithmic phases of growth was shown by Anderson and Meanwell (1936) to have an increased resistance to heat when the incubation temperature was reduced below the optimum.

White (1963) observed that the pattern of changing heat resistance of Streptococcus faecalis was the same for incubation at 27 as for 37 C. A similar pattern was found with incubation at 45 C, the outstanding feature being that the resistance in the final stationary phase was very great in the 45 C culture and was retained at a high level for at least 18 hr. Very young cultures at 27 were slightly more resistant than those at 37 which in turn were slightly more resistant than those at 45 C.

Elliker and Frazier (1938) demonstrated that cultures of E. coli exhibited a decided increase in heat resistance while in the initial stationary phase of growth, in comparison to the heat resistance determined for log phase cells. The increase in heat resistance was more marked in cultures incubated at 28 than in those incubated at 38.5 C. It was noted that the heat resistance of all of the cultures decreased as reproduction commenced and their resistance fell to a minimum during the period of most active cell division. The resistance then increased again to a second peak as the rate of reproduction decreased and the culture entered the maximum stationary phase of growth. The same type of phenomena were observed by White (1953) and Lemcke and White (1959). The log survivor/time graphs of young cultures of S. faecalis as determined by White (1953) varied in shape with age of culture and often showed an initial rapid death rate, passing suddenly into a slower one. Resistance was increased by transfer to fresh medium

but fell during the lag phase of growth, reaching a minimum as rapid reproduction began. Lemcke and White (1959) demonstrated (from mortality curves) that E. coli cells harvested from broth cultures 0 to 8 hr old were more susceptible to heat than those from more mature cultures. The time of commencement and the approximate duration of the logarithmic growth phase of the organism in broth were determined from growth curves, and the heat resistance was found to be minimal during the growth phase.

Strange and Shon (1963) conducted experiments to determine the death rate of washed Aerobacter aerogenes in an aqueous suspension at 47 C as affected by the nature of the growth medium, the composition of the liquid used to wash and resuspend the bacteria, the bacterial growth phase, the oxygen tension, and the composition of the diluent in which the bacteria were heated. The relative resistance of bacteria in different growth phases was found to differ according to the growth medium and the washing fluid; stationary phase bacteria were not more resistant than exponential phase organisms under all conditions. Starvation increased the thermal resistance of exponential and stationary phase bacteria.

Walker (1964) reported that the age of culture had a marked influence on the thermal resistance of Staphylococcus aureus. Cultures grown for 60 or 228 hr in broth were several times more resistant than the same cultures incubated for only 12 hr.

Mortality curves shown by Lemcke and White (1959) indicate that in many cases the death rate for E. coli was

not constant, especially with cells from cultures incubated for less than five hr. Reasoning was that cells do not develop at the same rate and, hence, pronounced morphological and cytological changes take place. Some cells which develop at a slower rate are more resistant than those growing more rapidly. White (1953) observed a more uniform death rate for young cultures of S. faecalis after the culture had undergone four serial subcultures at two-hour intervals.

Stark and Stark (1929) stated that the resistance of S. faecalis increased during incubation up to 24 hr, after which the resistance decreased up to 48 hr.

Sensitivity to cold shock varies with the age of the culture. Hegarty and Weeks (1940) reported that sensitivity to cold is not great during the beginning of the log phase, however, immediately after the first apparent multiplication the number of sensitive cells increased rapidly, and throughout the logarithmic phase a majority of cells were susceptible to cold shock. The number of sensitive cells decreased at the inflection point of the growth curve.

Luedecke and Harmon (1966) reported that Pseudomonas fragi cells grown in skim milk had a greater thermal resistance ($D_{52} = 3.0$ to 3.1) than those grown in milk containing fat ($D_{52} = 1.9$ to 2.5).

Nature and Extent of the Heat Treatment

The results of Watkins and Winslow (1932) and Strange and Shon (1964) indicate that higher bacterial concentrations

tend to have a protective effect against thermal inactivation. Watkins and Winslow (1932) suggested that the influence of cell concentration may be attributed to the production by the cells of substances which form a protective zone about each cell and which will obviously remain more concentrated when in the neighborhood of similar zones surrounding adjacent cells. Presumably the zone of protective substances reacts upon the cell walls to make them more resistant to the action of heat. The reason for low resistance of cells from young cultures was attributed to the inability of such cells to form the protective substances in question or to other factors associated with physiological youth. Kaufmann et. al., (1959) observed that an increase in size of the initial inoculum reduced a "lag extension effect" induced by exposure of Micrococcus MS 102 to ultra high temperatures.

Morita and Burton (1963) attributed the loss of permeability control as a reason for death of a marine psychrophile at temperatures above their maximal growth temperature.

The leakage of ribonucleic acid (RNA) from cells is thought to be partly responsible for the death of heat treated microorganisms. Haight and Morita (1966) studied the leakage of various cellular components into the surrounding menstruum when Vibrio marinus was subjected to temperatures above 20 C. The materials listed in decreasing rates of leakage were identified as protein, deoxyribonucleic acid (DNA), RNA, and amino acids. The RNA in the supernatant resulting from heat was both polymeric and non-polymeric.

A large fraction of RNA from the supernatant was of high molecular weight, but a significant quantity of RNA of low molecular weight was also noted. The studies did not show whether degradation occurred during or prior to heating or whether the low molecular weight RNA was part of the nucleotide pool of the cell. The possibility was suggested that heating induced degradation of RNA inside the cell, which preceded leakage, or that the RNA was hydrolyzed enzymatically after leaving the cell. Strange and Shon (1963) using A. aerogenes reported that the shape of the death and leakage curves provided evidence that RNA breakdown does occur during heating. Conditions which accelerated the death rate of A. aerogenes at 47 C also increased the rate of degradation of endogenous RNA. It was concluded that depletion of RNA is probably not the primary cause of death at 47, but the effect on bacterial metabolism by a rapid increase in endogenous pool constituents resulting from RNA degradation may contribute to the lethal effect.

The effects of different temperatures on the viability of, and leakage of 260 m μ -absorbing substances from a strain of E. coli have been studied by Russell and Harris (1967). Although the initial damage inflicted on the cells was to the permeability barrier, it was concluded that evidence available for supporting the hypothesis was inadequate.

The liberation of substances from Bacillus subtilis has been studied (Pollock, 1961; Demain et. al., 1965). Pollock (1961) stated that at least 40% of the penicillinase

liberated from cells is accomplished without gross disorganization of cell structure. Demain et. al., (1965) found that B. subtilis MB-1480 produced several 5'-ribonucleotides in the extracellular medium in addition to the previously found 5'-guanosine monophosphate and guanosine diphosphate. Excretion of RNA closely paralleled growth and was accompanied by DNA and protein excretion. The outward excretion of such large molecules without significant death of the microorganism involved is not entirely understood.

Moats (1961) followed chemical changes in bacteria heated in milk as related to loss of stainability. Loss of stainability was associated with extraction from the bacterial cells of nitrogen- and phosphorous-containing materials identified as nucleic acids.

The effects of various concentrations of sodium chloride (NaCl) and potassium chloride (KCl) in the heating menstruum were investigated by Strange and Shon (1963). The survival of A. aerogenes at 47 C under aerobic conditions differed, K^+ concentrations above 0.1 M being more lethal than equivalent concentrations of Na^+ ; the lethal effect of heating in mixtures of these salts (total M > 0.1) increased with K^+ concentration.

Gossling (1958) studied the lethal effect of a changing environment on the viability of E. coli. An 80% loss of viability was observed when the ionic environment was changed from phosphate buffer to Ringer's solution. Similar loss in viability was found to occur when environmental

changes were in the reverse direction. The presence of 1% NaCl in place of Ringer's solution modified the effect; electrolyte concentrations below about 0.02 M had no effect.

Mitchell (1951) stated that salts, depending upon the concentration, may establish a more favorable osmotic pressure difference between the cell interior and the suspending medium and thereby decrease the leakage of essential components from the cells during heating.

Hansen and Riemann (1963) note the relationship of Na^+ to pH fluctuation and state that the presence of other electrolytes must be taken into consideration when the effect of pH on the heat resistance of microorganisms is tested. Using phosphate and citrate-phosphate buffer solutions at various levels of pH, White (1963) measured the heat resistance of three strains of S. faecalis at 60 C. All three strains were more susceptible at high and low pH than at pH near neutrality. Hersom and Hulland (1963) state that an increase in hydrogen ion concentration usually causes a corresponding decrease in heat resistance.

The presence of clumps of spores in a suspension is known to affect heat resistance (Hersom and Hulland, 1963). Hansen and Riemann (1963) tested the heat resistance of Streptococcus faecium in skim milk and found that clumping was the factor mainly responsible for the initial flat part of the survival curves they obtained.

Jordan and Jacobs (1947) demonstrated that when E. coli cultures were exposed to lethal temperatures, an initial period of slow death occurred followed by a period of rapid death. The effect of heat was less marked than when the lethal agent used was phenol. Hayes (1965) theorizes that such "multi-hit" curves indicate a multiplicity of targets within each bacterium. The extent of the "shoulder" increases with multiplicity.

A widely held view is that death results from the coagulation of cell protein and there is evidence that factors which affect protein coagulation have a marked influence on bacterial heat resistance (Hersom and Hulland, 1963; Hansen and Riemann, 1963). Acid or alkaline conditions increase heat denaturation of protein and cause a decrease in the heat resistance of bacteria.

Sugiyama (1951) reported that the presence of fatty acids, particularly longer chain members in sporulating medium, tended to increase the heat resistance of Clostridium botulinum. Jensen (1954) indicates that there are great differences in the heat resistance of streptococci depending on whether the heating menstruum is fatty or aqueous in nature. When organisms were heated in moist melted butter at 100 C they were destroyed in 15 min; in dry butter the killing time at 115 C was 50 min.

Environmental Conditions After Heat Treatment

The log survivor/time graph for mature cultures of S. faecalis as determined by White (1953) was not always straight over the entire length. A preliminary period of slow death was frequently observed; a final similar period was less often observed. A similar initial lag period was observed with heated cultures of other bacteria (Tobias et. al., 1955; Kaufmann, 1957; Jackson and Woodbine, 1963). Jackson and Woodbine (1963) subjected an enterotoxigenic strain of S. aureus to sublethal heat treatment, inoculated into nutrient broth at 37 C, and observed a decrease in viable numbers. The fall was followed by a lag phase of growth. They showed the same doubling time was reached as in unheated organisms upon reaching the logarithmic phase of growth. Explanations for the lag were given as 1) low survival level and 2) injury of the cells in such a way as to make subsequent growth take longer.

Lawton and Nelson (1955), working with a pseudomonas species partially killed by heat, showed that survivors had a greatly increased lag phase when holding at 5 or 10 C. A slightly increased lag phase was noted when holding was at 25 C.

Hershey (1939) demonstrated that the latent period increased as the heat treatment increased. He concluded that the extended latency of E. coli is a direct effect of injury and not merely the result of selection of resistant individuals inherently slower to develop, since delayed

multiplication was noticeable after exposure to temperatures at which no death occurred.

The viability of damaged organisms, as noted by Harris (1963), is often extremely sensitive to environmental changes which do not influence undamaged cells. It is pointed out that the shapes of survival curves seem to depend on the recovery environment but that the curves cannot reveal information about the method of damage.

The inoculation of very small numbers of heated E. coli strain B/r in 0.1% solution of Krebs cycle metabolites showed a decrease in viable number (Chambers et. al., 1957). Contrary to this data Heinmets et. al., (1954), working with the same bacterial strain, demonstrated that the heat treated organisms produced higher counts on minimal media if they were previously incubated in 0.2% solutions of various Krebs cycle metabolites.

Two methods, one based on the calculation of most probable number of survivors and the other on the use of the Millipore filter, have been used by Hurwitz et.al., (1957) to test the hypothesis of chemical reactivation. Experiments showed that chemical reactivation as described by Heinmets (1954) does not occur. Results were in accord with Garvie (1955) who contended that apparent reactivation arises from proliferation of survivors in the metabolite solutions used for reactivation.

The assumption that conditions satisfactory for the growth of unheated organisms are equally satisfactory for

the growth of bacteria which have been subjected to heat treatment of sublethal intensity is questioned by Nelson (1943). He states that heated bacteria are more demanding in their requirements of media for growth and that dormancy of heat treated organisms can be reduced or eliminated by the provision of suitable recovery media.

Straka and Stokes (1959) demonstrated that cold injury is manifested by an increase in nutritional requirements. Injured Pseudomonas ovalis would not grow on a simple glucose-salts agar medium but could develop on a rich, complex medium, trypticase soy agar.

Iandolo et. al., (1964) had previously reported that there was an inhibition when using 8% NaCl in the recovery medium regardless of the pH or incubation temperature for heated S. aureus cells. Iandolo and Ordal (1966) reported that exposure of S. aureus M31 to sublethal temperatures produced a temporary change in salt tolerance and growth. A 15-min exposure at 55 C left only 1% of the viable population able to reproduce on media containing 7.5% NaCl. The data demonstrated thermal injury was partly due to changes in cell membrane, allowing soluble cellular components to be released into the menstruum. Exposure longer than 15 min did not indicate the induction of additional salt sensitivity. An extended lag phase observed upon heating was attributed to readjustment in the form of cell repair. Sogin and Ordal (1967) observed an extended lag phase and loss of the ability to reproduce on an agar containing 7.5% NaCl after

subjecting S. aureus MF-31 to 55 C for 15 min. Chapman (1945) states that the addition of a concentration of 7.5% NaCl to a solid medium inhibits the growth of most bacteria other than staphylococci. Stiles and Witter (1965) described the heat resistance of S. aureus MF-31 by determining the D and z values. D values were decreased when trypticase soy agar containing 7.5% (w/v) NaCl was used as a plating medium for surviving cells. No substantial difference in the influence of temperature on salt tolerance was indicated by similar z values for organisms plated in media of low or high NaCl content.

Using Plate Count Agar and Staphylococcus No. 110 (S-110) medium, Busta and Jezeski (1963) studied the survival of heated S. aureus 196-E. Lower thermal death times were found to be related to the NaCl content of the S-110 medium, because use of S-110 agar containing lesser concentrations of NaCl resulted in growth of larger numbers of heat-shocked S. aureus 196-E.

Postgate and Hunter (1963) and Strange and Shon (1963) studied the effect of Mg^{++} on growth of bacteria. Strange and Shon (1963) observed that Mg^{++} (0.01 to 5 mM) and, to a lesser extent, Mn^{++} (0.5 mM) or Co^{++} (5 mM) decreased the death rate of heat treated A. aerogenes.

A study was carried out by Heather and Vanderzant (1957) to determine the effect of time and incubation temperature, and of pH of the medium on heated cultures of Pseudomonas fluorescens, Ps. fragi, and Pseudomonas.

putrefaciens. The heated psychrophiles did not grow as well during the early phase of incubation as did the unheated ones and their lag was greater at 5, 32, and 35 than at 25 C. Longer incubation times were required at lower incubation temperatures to get maximum plate counts. Lawton and Nelson (1954) observed the same phenomena at 5 and 10 C when using partially inactivated psychrophilic bacteria.

Straka and Stokes (1957) reported that rapid and extensive destruction of bacteria occurs in commonly used diluting fluids such as tap, distilled, and phosphate water, and saline. As much as 40 to 60% of the bacterial population may die in distilled water within 20 min and over 90% in one hr. It is apparent that improper handling of heated cultures after heat treatment can lead to errors in the quantitative determination of bacterial numbers.

Causes for death of heated organisms have been studied through the use of various metabolic inhibitors. Iandolo and Ordal (1966) state that penicillin, cycloserine, 2,4-dinitrophenol, and chloramphenicol did not have an effect on the repair of thermally injured S. aureus M-31. Actinomycin D, however, completely suppressed recovery, implying RNA synthesis was involved. Morse and Carter (1949) state that nucleic acids are synthesized during the lag period, before actual cell multiplication. Stiles and Witter (1965) determined that penicillin, vanomycin, chloramphenicol, and ethylenediaminetetraacetic acid had no

effect on the recovery of salt tolerance in heat-injured S. aureus MF-131. Recent investigations by Friedman et. al., (1967) provide evidence for the heat stability of Bacillus stearothermophilus ribosomes. It was concluded by the authors that it is not clear whether heat stability is inherent in the structure of the ribosomal RNA, or ribosomal protein, or rather a consequence of an additional component which is tightly bound to the ribosomes. Analysis of the RNA components of heated S. aureus MF-31 on Methyl Albumin Kieselguhr (MAK) columns and sucrose gradients has shown that the ribosomes and ribosomal RNA were almost completely broken down (Sogin and Ordal, 1967).

Other hypotheses for death of organisms above maximal growth temperatures include inactivation of the enzyme and enzyme-forming system(s) (Edwards and Rettger, 1937; Nashif and Nelson, 1953; and Upadhyay and Stokes, 1963), accelerated use of the intracellular amino acid pool (Hagen and Rose, 1963), disruption of intracellular organization (Ingraham and Baily, 1959), changes in the extent of cellular lipid saturation (Kates and Hagen, 1964), and leakage of ribonucleotides, ammonia, ninhydrin-positive material, potassium, and phosphate (Stokes, 1963).

METHODS AND MATERIALS

Isolation and Classification of Microorganisms

Several unidentified bacterial isolates from raw and partially processed meat samples of commercial origin were supplied by Dr. R. A. Greenberg of Swift and Company, Chicago, Illinois. Three isolates were selected at random and identified according to the classification scheme given by Gibbs and Skinner (1966).

The organisms were grown in Bacto All Purpose plus Tween broth (APT), a Difco formulation of the medium described by Evans and Niven (1951). Cells from 24-hr cultures were used for all the physiological and biochemical tests.

Carbohydrates used for fermentation diagnosis included lactose, arabinose, trehalose, melibiose, melezitose, mannitol, sorbitol, and glycerol. The final concentration of each was 0.5%. Ability to grow at 10, 45 and 50 C and to grow in APT containing 0, 7.5, and 10% sodium chloride (NaCl) was determined. Hemolytic ability, using pour plates of 5% (v/v) sheep blood in nutrient agar, was examined after 24 hr and 48 hr at 25 C. Assessment of the type of hemolysis was according to Wilson and Miles (1964). Tests were carried out for the ability of organisms to reduce colorless 2,3,4-triphenyltetrazolium chloride to a red formazan, potassium tellurite (0.04%, w/v) in APT agar, and methylene

blue in milk. Gelatin (12%, w/v) stabs incubated at 21 and 35 C were examined for liquefaction and failure to re-solidify, respectively. The ability of each isolate to hydrolize starch, produce ammonia from arginine, produce acetoin (Voges-Proskauer reaction), and grow at pH 9.6 was determined.

The isolates were designated as S21, S23, and S28; S21 and S23 were identified as Streptococcus faecalis var. liquefaciens and S28 was determined to be Streptococcus faecalis. All three strains were frozen in APT broth and stored at -20 C for later use as stock cultures.

Survivor Curve Determinations

Cultures were grown in APT broth 24 hr at 30 C. One ml of the culture was inoculated into 16 x 150 mm screw-cap test tubes containing 15 ml of sterile APT broth pre-heated to 60 C in a Bronwill Heater-Stirrer water bath (Bronwill Scientific Division, Will Corporation, Rochester, New York). After appropriate times of heat exposure, tubes were removed from the water bath and plunged into an ice bath for immediate cooling. Proper dilutions were made in sterile deionized water and the heat treated organisms were plated in duplicate or triplicate in APT agar using the pour-plate technique. Each experiment was performed in triplicate. Incubation was at 30 C and counts were made 48 to 72 hr after pouring. Survivor curves were drawn plotting the log of survivors against time of heating.

To determine the effect of incubation temperature on the recovery of S. faecalis after exposure to sublethal heating, organisms heated for various lengths of time were incubated at temperatures ranging from 7.3 to 45 C. Counts were made from 24 hr to 27 days after pouring.

Procedure for the Determination of Heat Destruction
as Affected by Age of the Cell

Cultures were grown in APT broth for 10 hr at 30 C and then transferred to fresh APT broth and allowed to incubate at 30 C for 9 hr. From the 9-hr culture, 0.1 ml was inoculated into each of ten 13 x 100 mm screw-cap test tubes containing 5 ml of fresh APT broth. Absorbancy values at 625 m μ were read on a Bausch and Lomb Spectronic 20 spectrophotometer at half-hr intervals. Tubes of average absorbancy were selected at various times during the growth cycle and heat resistance at 60 C were analyzed according to the procedure above. Cultures of average absorbancies were also selected at various times during the growth cycle but not subjected to heat in an attempt to determine the total viable count at the selected times. Again, APT was used as the recovery medium and counts were made after 48 to 72 hr of incubation at 30 C.

Salt-Containing Recovery Media

Salt-containing recovery media were prepared by adding various amounts of NaCl, potassium chloride (KCl), magnesium chloride (MgCl₂), or KCl and MgCl₂ to APT agar.

Except for the concentrations of these salts in the recovery media, the procedure for determining the effect of salts on the recovery of heat treated S. faecalis was the same as that for establishing survivor curves.

Ribonucleic Acid Analyses

A procedure for the isolation of ribonucleic acid (RNA) as described by Clark (1964) was modified slightly to determine the RNA content of the three strains of heated and unheated S. faecalis. Cultures of S. faecalis were grown in APT broth for 23 to 24 hr at 30 C. One-thousand milliliters of the culture was centrifuged at 5000 RPM (Sorvall Superspeed, Model RC-2, Ivan Sorvall, Inc., Norwalk, Connecticut) for 20 min. The supernatant was removed after centrifugation and the cells were resuspended in 100 ml distilled water. Ten ml of the cell suspension were dried at 140 C for 4 hr to determine the dry weight per ml of the cell concentrate. Ten ml of cell concentrate were added to each of six 250-ml screw-cap Erlenmeyer flasks containing 90 ml of APT broth preheated at $60 \pm .25$ C in a Metabolyte Water Bath Shaker (New Brunswick Scientific Co., New Brunswick, New Jersey) and the remainder of the cell concentrate was appropriately diluted in cool APT broth and used as the sample representing zero heating time. Speed of the shaker was controlled at 100 RPM to avoid settling of the suspension. Since heat resistance of the three streptococci used was not the same, appropriate heating times were chosen for each strain to

reduce the viable population of that particular strain by approximately the same percentage (see Figure 1). S23 was heated 12 and 20 min, S21 was heated 20 and 40 min, and S28 was heated 40 and 70 min. Once the required heating times were reached, three flasks (300 ml or one sample) of the suspension were immediately cooled. From this point on in the analyses samples were handled separately but identically. Reference will be made to only one sample.

The cells of the sample were pooled by centrifugation at 5000 RPM for 20 min, washed twice with distilled water, and mechanically ruptured by means of a Bronwill Disintegrator (Type 2876, Bronwill Scientific Inc., Rochester, New York). Larger cellular components were separated from the supernatant by centrifugation at 7000 RPM for 20 min. Glass beads used for disrupting cells were washed twice, saving the supernatant each time. An equal volume of 88% phenol was added to the chilled supernatant and the mixture was stirred at room temperature for 30 min. The emulsion was then cooled in an ice bath for 5 min before breaking by centrifugation at 4500 RPM for 15 min at 0 to 5 C. The upper aqueous layer of supernatant and most of any intermediate layer containing denatured protein was decanted from the brown phenol phase. Removal of the denatured protein from the aqueous phase was accomplished by centrifugation at 7000 RPM for 5 min at 0 to 5 C. A volume of 20% (w/v) potassium acetate (pH 5.0), 1/10 the volume of the aqueous fraction, was added to the aqueous fraction followed by

precipitation of RNA with the addition of two volumes of cold absolute ethanol. The suspension was chilled in an ice bath 5 min before collecting the precipitate by centrifugation at 2500 RPM for 10 min at 0 to 5 C. The precipitate was washed once each with ethanol:water (3:1), absolute ethanol, and anhydrous ether and then air dried.

The dried precipitate was dissolved in 10 ml of 0.5 N potassium hydroxide (KOH) and hydrolysis was allowed to proceed at room temperature for 24 to 48 hr. Sufficient 20% (v/v) hydrogen perchlorate (HClO_4) was added to the chilled hydrolyzed solution to reduce the pH to about 2 followed by removal of any precipitated potassium perchlorate (KClO_4), DNA, and/or protein by centrifugation at 2500 RPM for 10 min. The pH of the supernatant was adjusted to 3.5 with 1.0 N KOH. Any additional precipitate was removed by centrifugation at 2500 RPM for 10 min.

A modification of the Ashwell (1957) procedure for colorimetric analysis of sugars was used to determine the RNA content of heated and unheated S. faecalis. The acidified hydrolyzed sample of RNA was diluted 1:10 with deionized water and suitable aliquots were placed in 16 x 150 mm screw-cap test tubes. Similarly, aliquots of a 1.0×10^{-4} M nucleic acid standard (Ribonucleic Acid, Nutritional Biochemicals Corporation, Cleveland, Ohio) were placed in test tubes. To each tube enough deionized water was added to bring the total volume to 3 ml followed by the addition of 6 ml orcinol acid reagent (0.1% FeCl_3 in concentrated HCl)

and 0.4 ml of 6% (w/v) alcoholic orcinol. The solutions were boiled for 20 min in a water bath, cooled, and the absorbancies at 660 m μ were measured using a Bausch and Lomb Spectronic 20 spectrophotometer.

RESULTS AND DISCUSSION

Classification

The three bacterial isolates supplied by Dr. R. A. Greenberg were identified according to the physiological and biochemical tests described by Gibbs and Skinner (1966). Results of these tests are summarized in Table 1. Identical results were obtained in all tests except for the ability of the organisms to liquefy gelatin at 21 C and to ferment glycerol anaerobically. One strain, S28, was unable to liquefy gelatin and was identified as Streptococcus faecalis. The S21 strain fermented four out of six tubes anaerobically using glycerol as a source of carbohydrate but all other test results agreed with those obtained from the S23 strain. These two strains were classified as Streptococcus faecalis var liquefaciens.

Survivor Curves

The heat resistances of the three streptococcus isolates were found to differ greatly. S. faecalis var liquefaciens (S28) was most resistant to thermal exposure at 60 C, being inactivated after 100 min, while the two S. faecalis strains, S23 and S21, were found to be thermally inactivated after 50 and 60 min exposure, respectively.

"Shoulders" occurred on the initial part of each

Table 1. Physiological and biochemical tests for the identification of three bacterial isolates.

	Organism		
	S21	S23	S28
Growth at 10 C	+	+	+
Growth at 45 C	+	+	+
Growth at 50 C	-	-	-
Survive 60 C/30 min	+	+	+
Growth in 0% NaCl	+	+	+
Growth in 7.5% NaCl	+	+	+
Growth in 10% NaCl	-	-	-
Growth at pH 9.6	+	+	+
Tetrazolium Reduction	+	+	+
0.04% K-Tellurite Tolerance	+	+	+
0.1% Methylene Blue	+	+	+
NH ₃ from Arginine	+	+	+
β -Hemolysis	-	-	-
Gelatin Liquefaction (21 C)	+	+	-
Starch Hydrolysis	-	-	-
Voges-Proskauer	-	-	-
Methyl Red	+	+	+
<u>Carbohydrate Fermentations</u>			
Lactose	+	+	+
Arabinose	-	-	-
Trehalose	+	+	+
Melibiose	-	-	-
Melezitose	+	+	+
Mannitol	+	+	+
Sorbitol	+	+	+
Glycerol (anaerobic)	+	+	+

survivor curve, with those of S21 and S23 being more pronounced than S28. This phenomenon may be due to each of the organisms having multiple sites vulnerable to inactivation. At low doses of heat, the probability that all the sites will be inactivated is negligible so that the survivor curve remains flat instead of falling. White (1963), working with S. faecalis, and Jordan and Jacobs (1947), working with E. coli, observed the same type of survivor curves. The possibility of protection from thermal inactivation by clumping also exists.

Effect of Incubation Temperature

Figures 2, 3, and 4 reveal the sensitive nature of heat-damaged S. faecalis. The same total counts were observed from unheated cells when incubation temperatures ranged from 7.3 to 45 C. It should be noted, however, that longer incubation periods were required at 7.3, 10, and 15.6 C than at 21, 25, 30, and 37 C in order to account for the total number of viable cells. The shortest incubation period required for total outgrowth of viable organisms was at 45 C. In all cases the incubation time required for maximum recovery was longer for heated than for unheated organisms, regardless of incubation temperature. This same extended latency phenomenon was observed by Hershey (1939). Optimum temperatures for maximum recovery of heated cells ranged from 21 to 37 C. A decrease or increase in incubation temperature below 21 C or above 37 C, respectively showed a decrease in the total recovery of heated S. faecalis. No

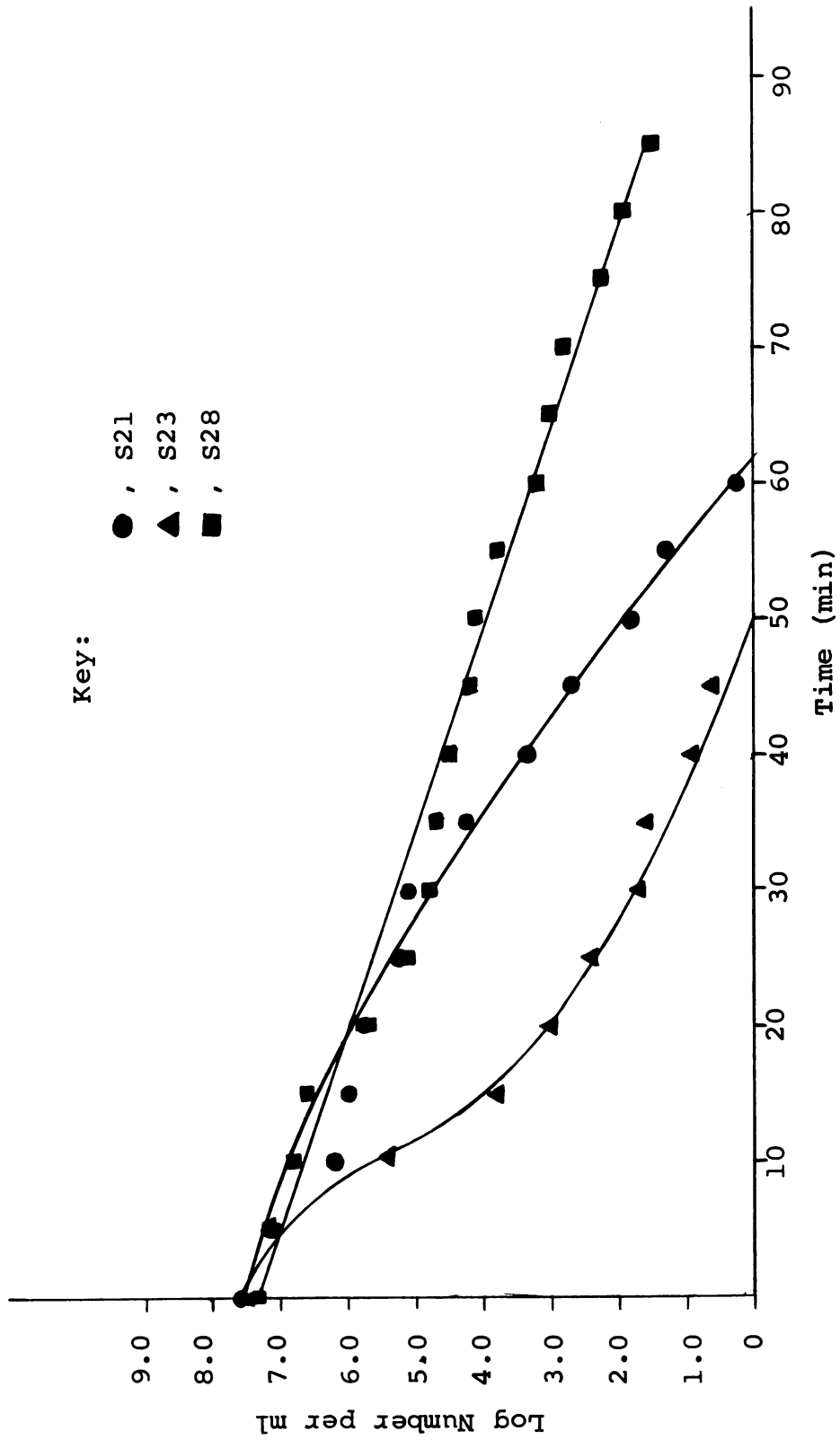


Figure 1. Survivor curves for heated Streptococcus faecalis strains at 60 C.

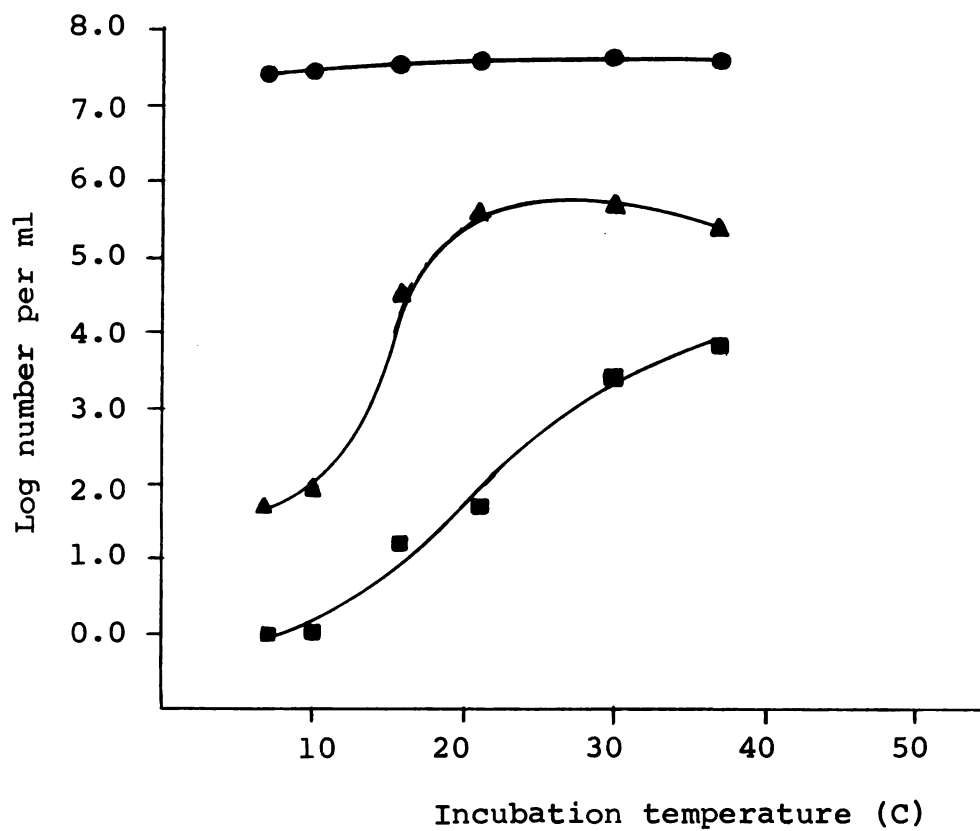


Figure 2. Effect of incubation temperature on the recovery of unheated and heated Streptococcus faecalis (S21).

Key:

- , 0 min at 60 C
- ▲ , 20 min at 60 C
- , 40 min at 60 C

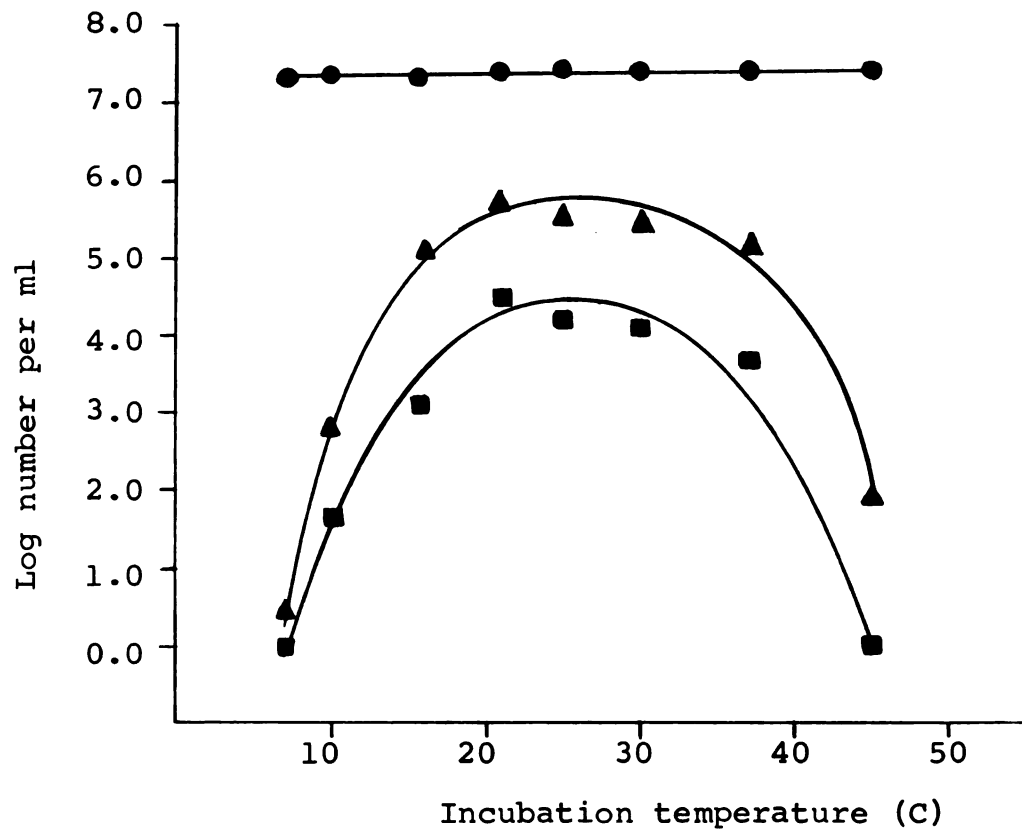


Figure 3. Effect of incubation temperature on the recovery of unheated and heated Streptococcus faecalis (S23).

Key:

- , 0 min at 60 C
- ▲ , 12 min at 60 C
- , 20 min at 60 C

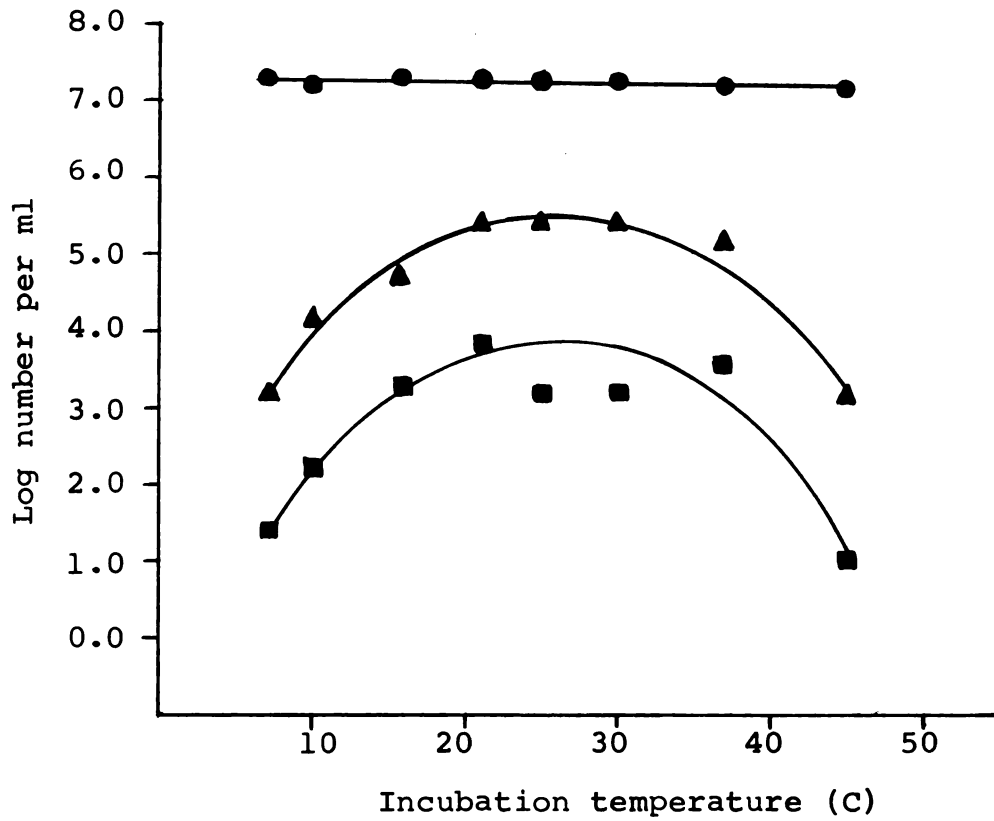


Figure 4. Effect of incubation temperature on the recovery of unheated and heated Streptococcus faecalis (S28).

Key:

- , 0 min at 60 C
- ▲ , 40 min at 60 C
- , 70 min at 60 C

survival was observed in the case of S23 (Figure 3) when the organisms were heated 20 min at 60 C and incubated at 7.3 and 45 C. This is a reduction in total recovery of over 4 log cycles from the total recovery of organisms subjected to the same amount of heat but incubated at 21, 25, or 30 C. The sensitivity of heated S. faecalis is clearly evident when incubation temperatures during recovery are varied from the optimum.

Effect of Age

The effect of age on thermal resistance of S. faecalis is shown in Figures 5, 6, and 7. The growth curves were measured over a 23-hr period in which the total number of viable organisms increased by about 1.5 log cycles. Analysis of heat resistance of organisms in different phases of the growth cycle shows that sensitivity to heat changes with age of the culture. Slight resistance is observed during the initial lag phase but decreases to zero resistance during the initial and middle logarithmic growth phases. Late logarithmic growth phase shows an increase in heat resistance. Maximum resistance is not reached until the organisms are in a stationary phase of growth. The pronounced effect of age on thermal resistance of S. faecalis was also noted by White (1953). She observed that resistance increased by transferring to fresh medium but decreased during the lag phase and did not increase until the rate of growth became slow.

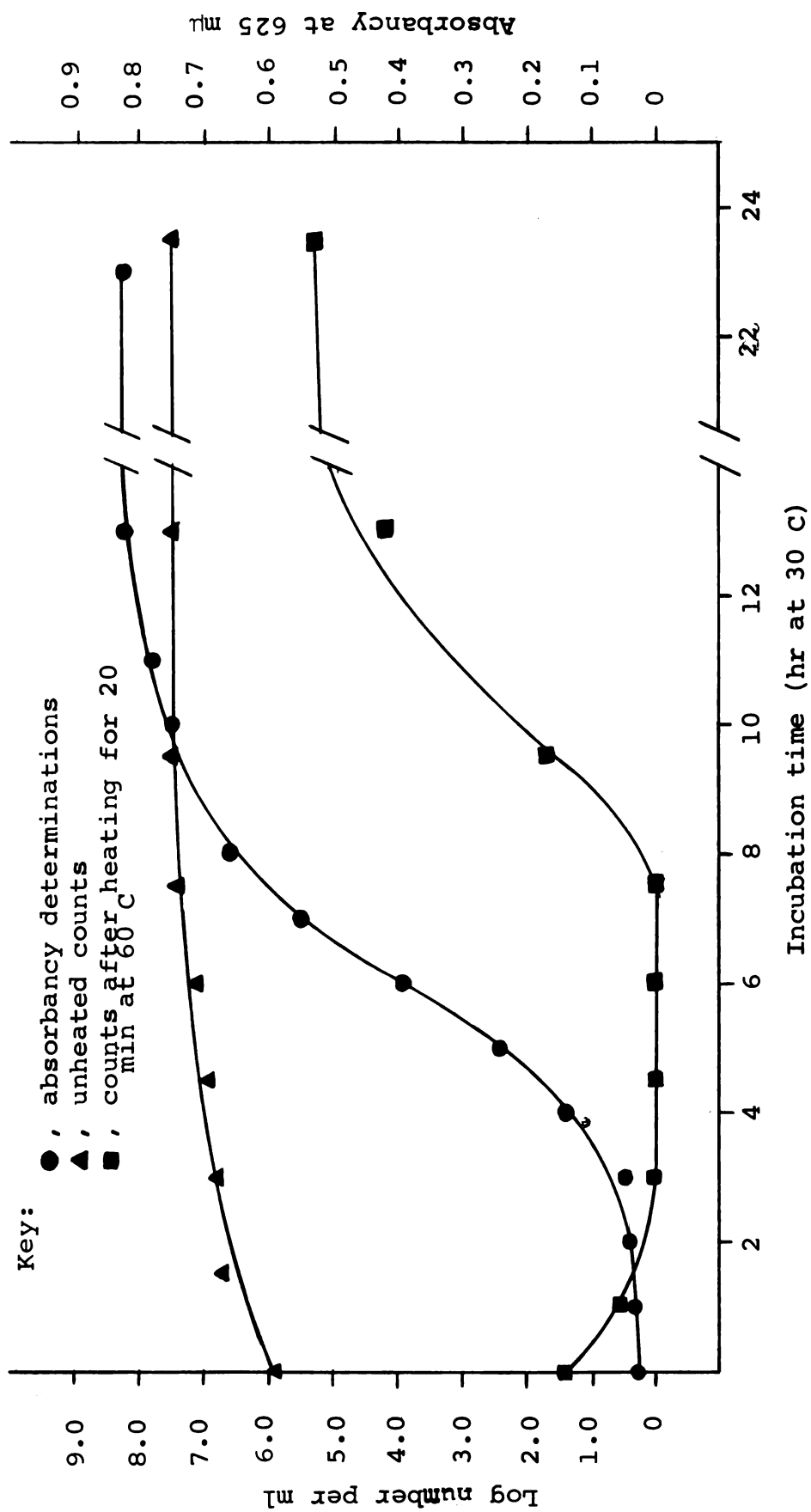


Figure 5. Effect of age of Streptococcus faecalis (S21) on thermal resistance.

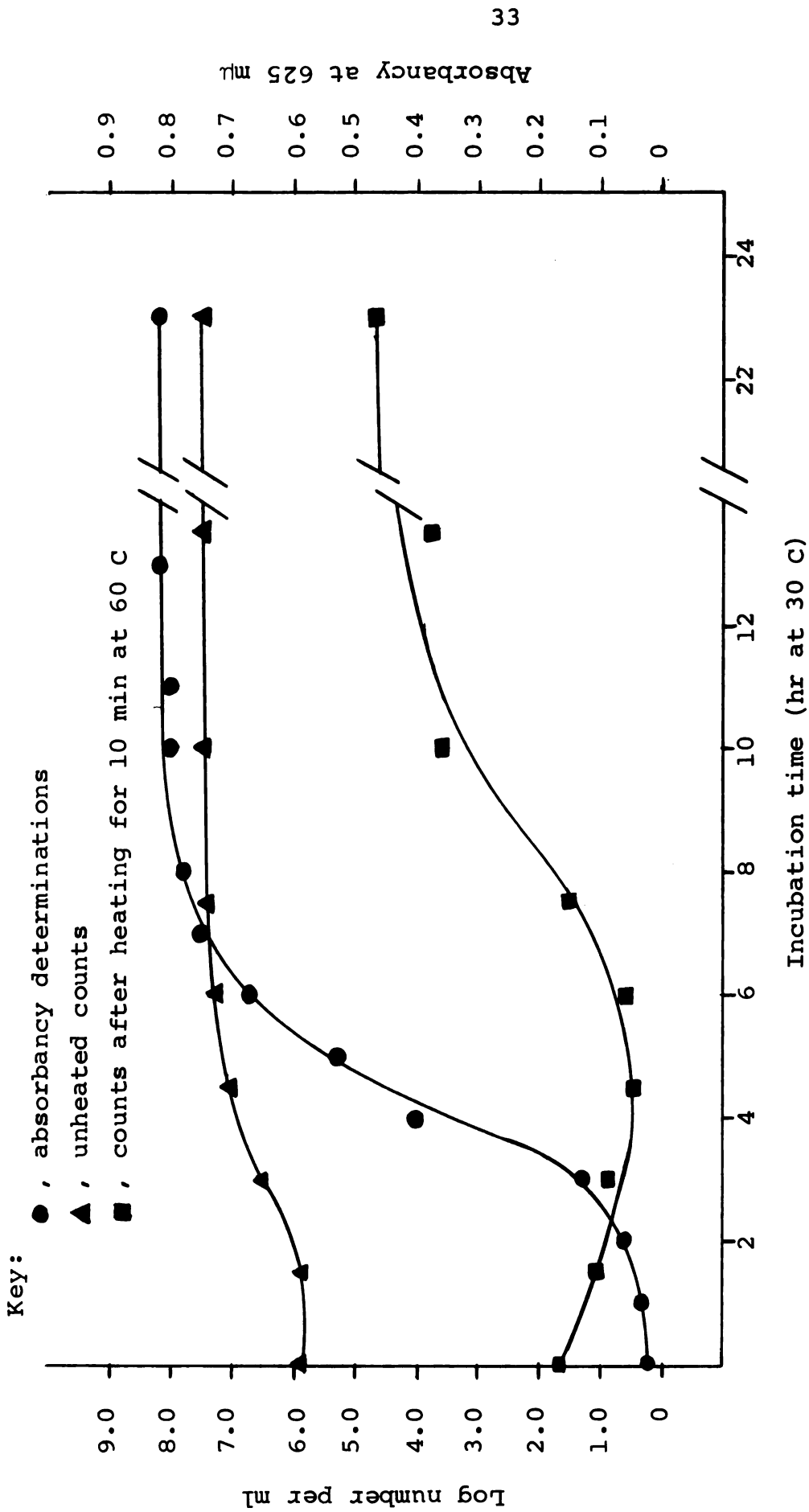


Figure 6. Effect of age of Streptococcus faecalis (S23) on thermal resistance.

Key:

● , absorbancy determinations

▲ , unheated counts

■ , counts after heating for 40 min at 60 C

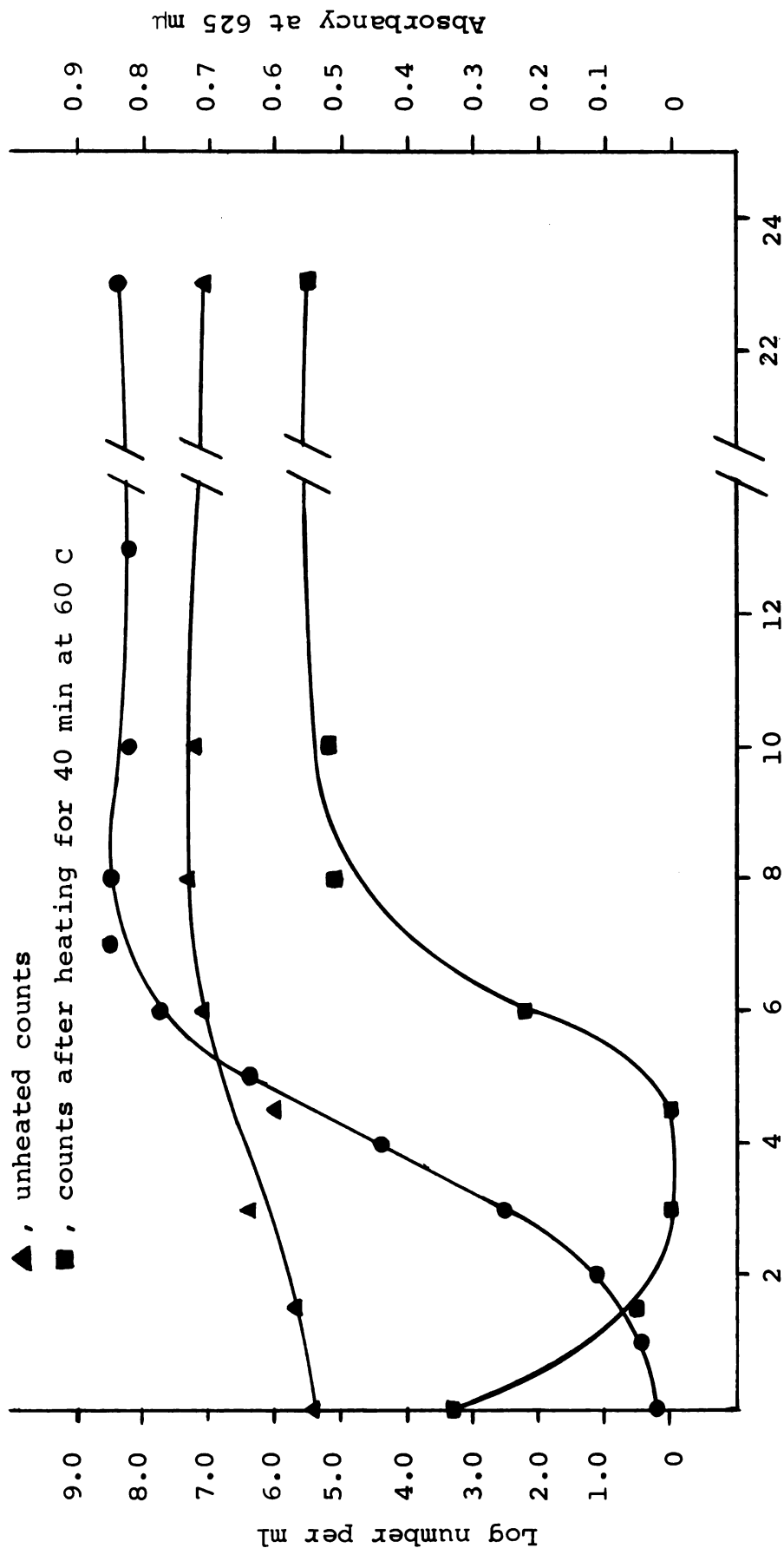


Figure 7. Effect of age of Streptococcus faecalis (S28) on thermal resistance.

Changes in heat resistance cannot be explained entirely by the protective effect of high populations (Watkins and Winslow, 1932; Strange and Shon, 1964) since the population increase with time is not directly proportional to the change in resistance. Other physical and/or biochemical changes must occur within the cell or medium to account for the variation in resistance to heat throughout the growth cycle.

Effect of Salt

The effect of adding Na^+ , K^+ , and Mg^{++} to APT recovery medium on the survival of heated and unheated S. faecalis is shown in Figures 8 through 19. There was little difference between the total viable counts of unheated organisms when concentrations of up to 1.39 m Na^+ , 1.34 m K^+ , 1.29 m Mg^{++} , or 1.34 m K^+ plus 0.089 m Mg^{++} were present in the recovery media. Concentrations above 0.27 m Na^+ decreased the total recovery of all three strains of heated S. faecalis. At concentrations greater than 0.14m K^+ or 0.089 m Mg^{++} the decrease in total recovery was more pronounced than with similar concentrations of Na^+ . This is in agreement with Strange and Shon (1963) who found that K^+ concentrations above 0.1 M were more lethal than equivalent concentrations of Na^+ when heating Aerobacter aerogenes at 47 C under aerobic conditions. There was a slight difference in total counts of heated organisms recovered on medium containing only K^+ and a medium containing the same concentration of K^+ plus 0.089 m Mg^{++} . Differences were more

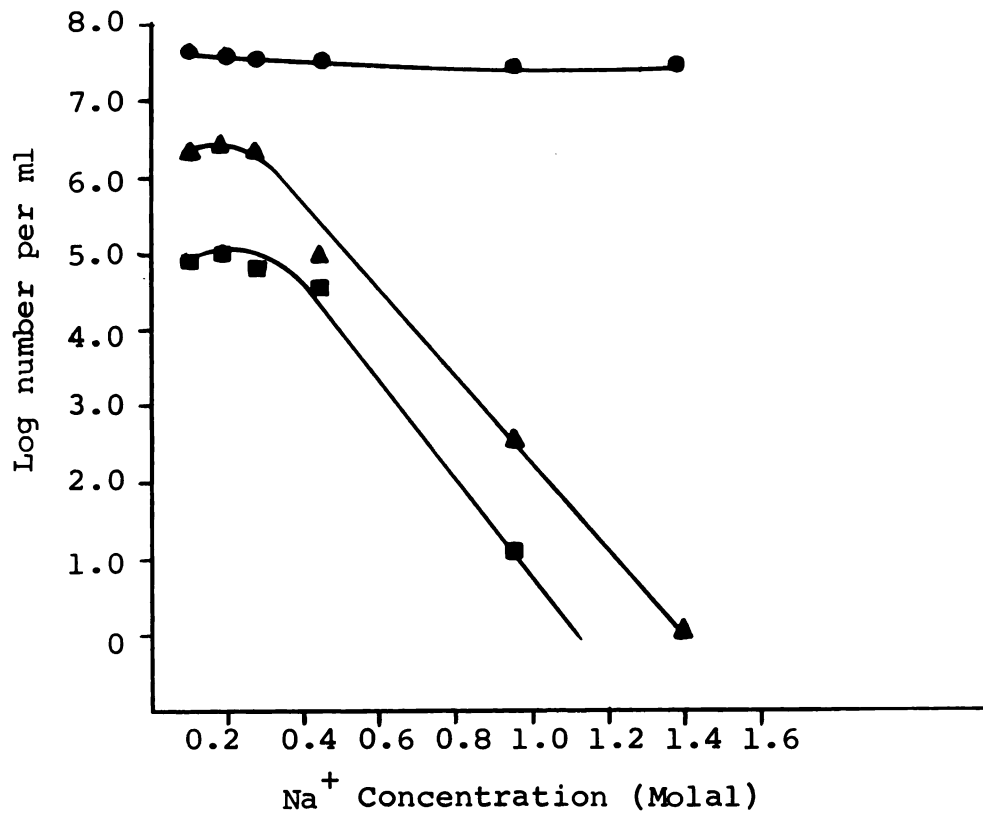


Figure 8. Effect of adding Na⁺ to APT recovery medium on the survival of Streptococcus faecalis (S21) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 20 min at 60 C
- , 40 min at 60 C

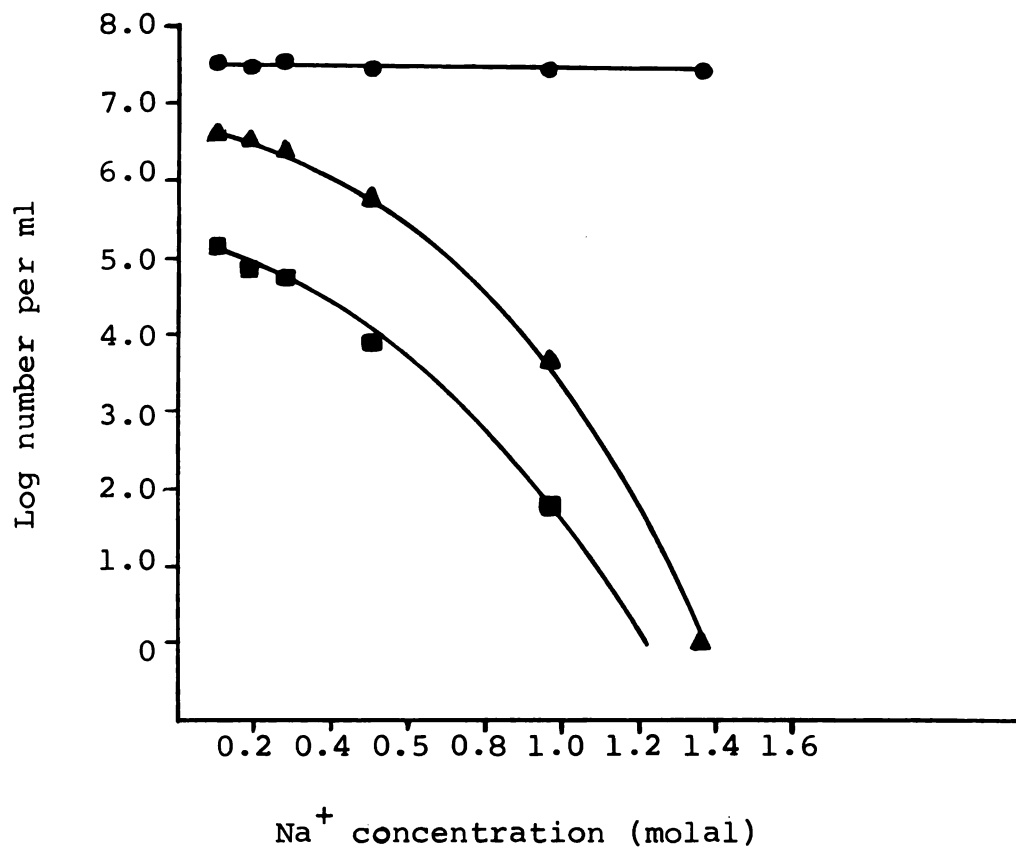


Figure 9. Effect of adding Na⁺ to APT recovery medium on the survival of Streptococcus faecalis (S23) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 12 min at 60 C
- , 20 min at 60 C

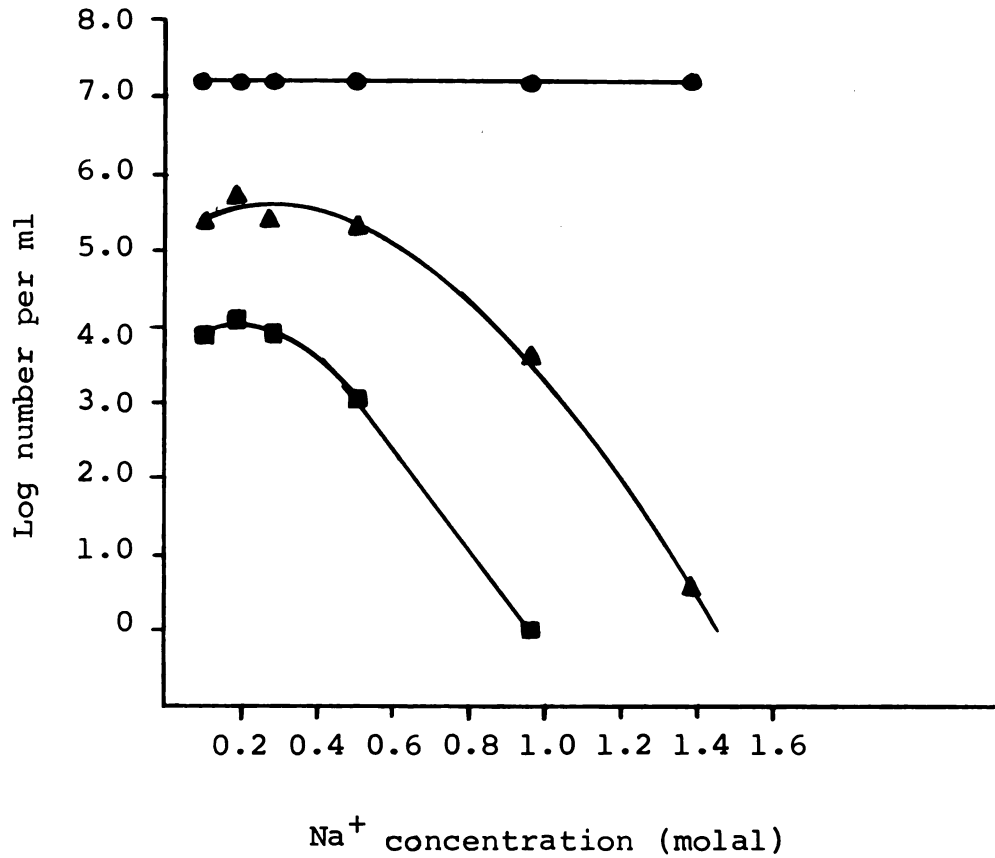


Figure 10. Effect of adding Na⁺ to APT recovery medium on the survival of Streptococcus faecalis (S28) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 40 min at 60 C
- , 70 min at 60 C

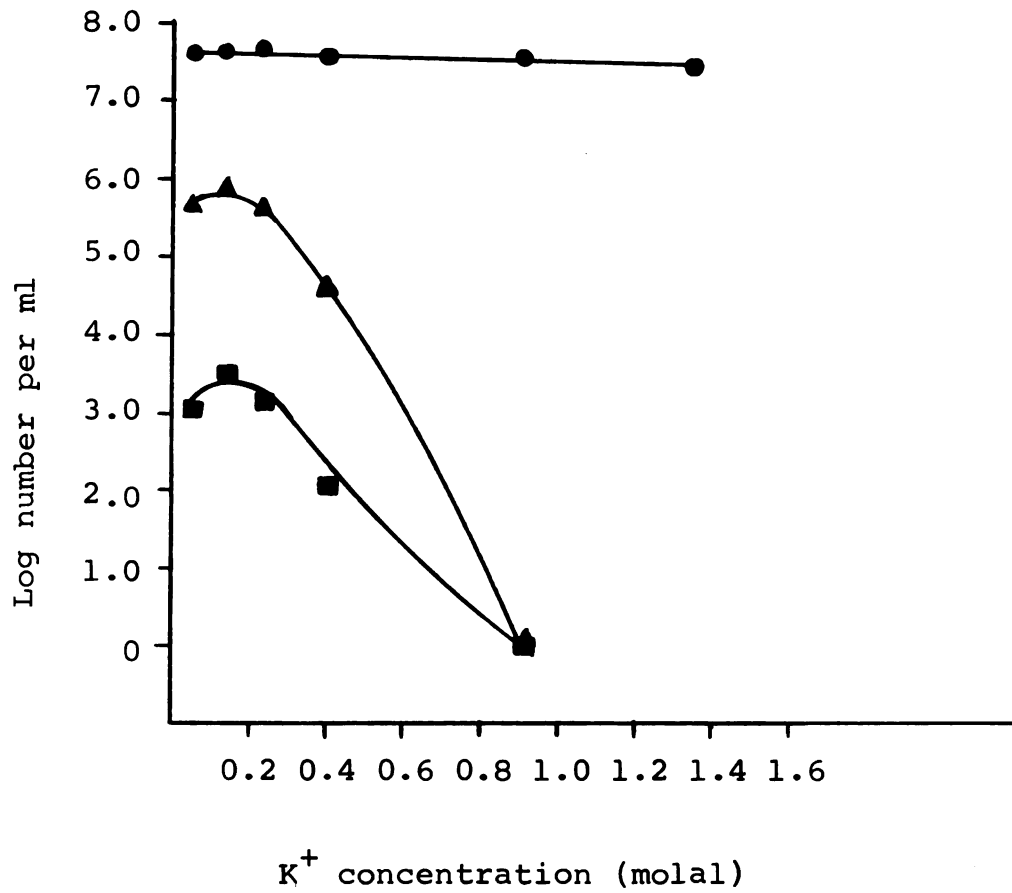


Figure 11. Effect of adding K⁺ to APT recovery medium on the survival of Streptococcus faecalis (S21) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 20 min at 60 C
- , 40 min at 60 C

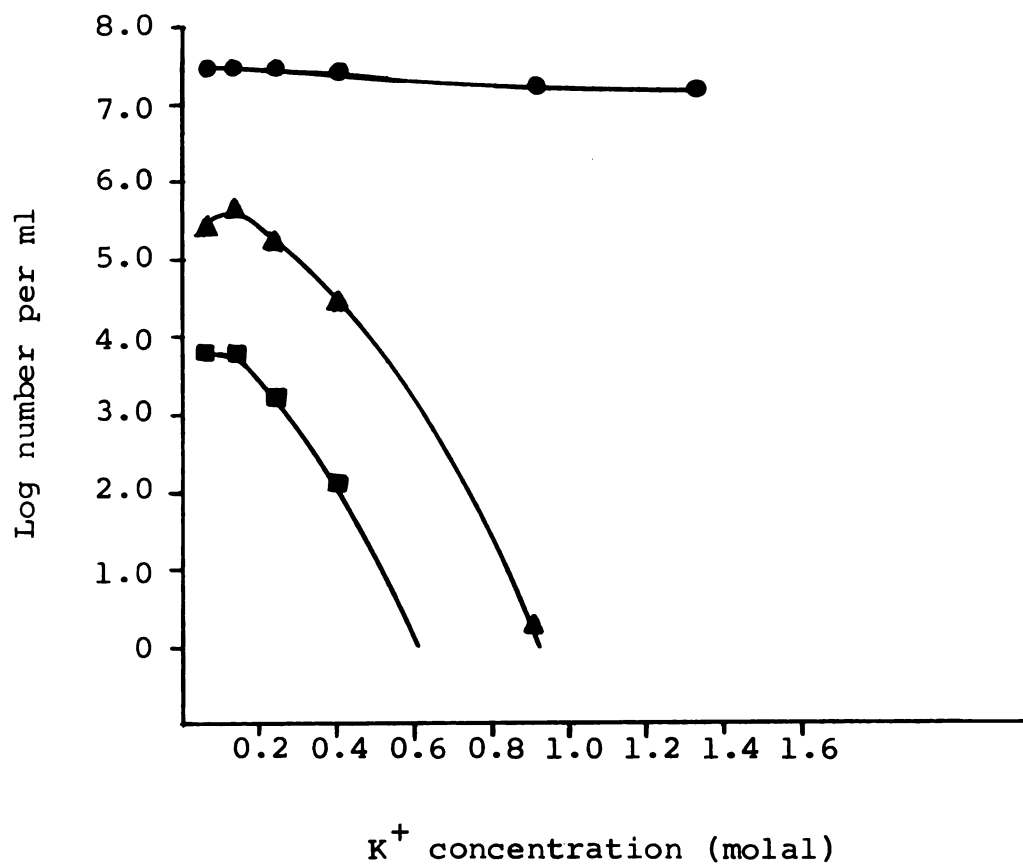


Figure 12. Effect of adding K⁺ to APT recovery medium on the survival of Streptococcus faecalis (S23) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 12 min at 60 C
- , 20 min at 60 C

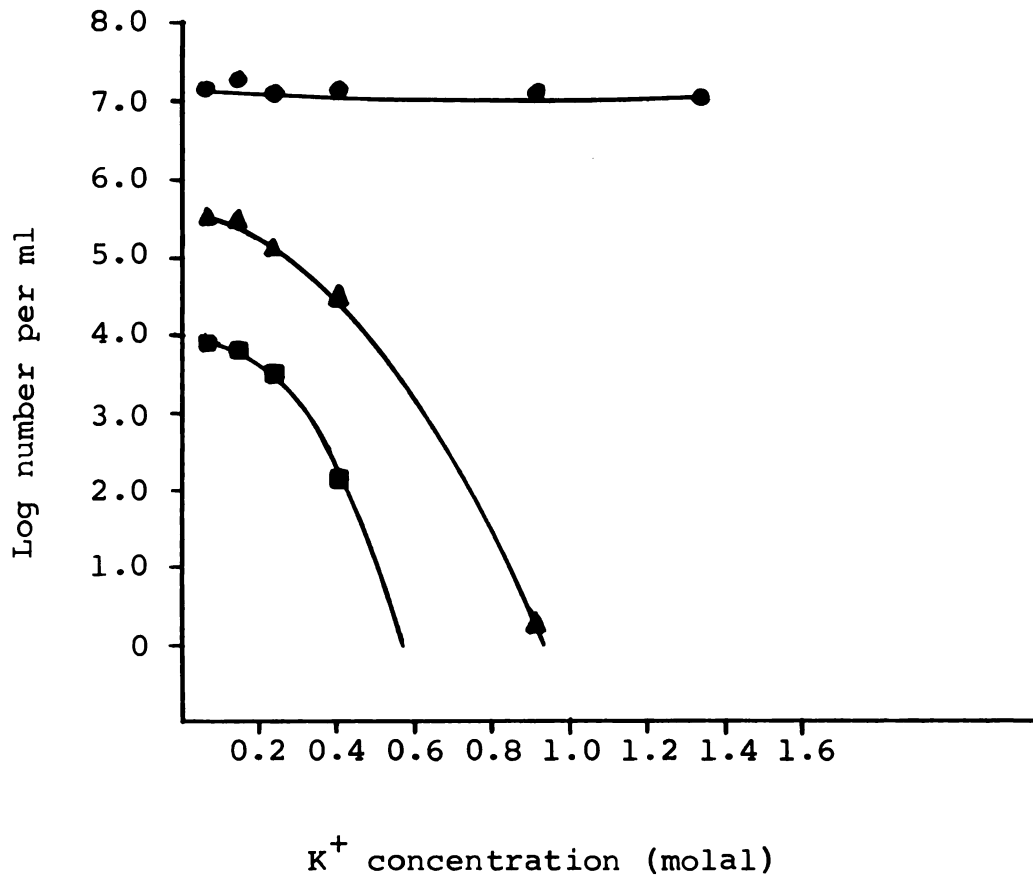


Figure 13. Effect of adding K^+ to APT recovery medium on the survival of Streptococcus faecalis (S28) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 40 min at 60 C
- , 70 min at 60 C

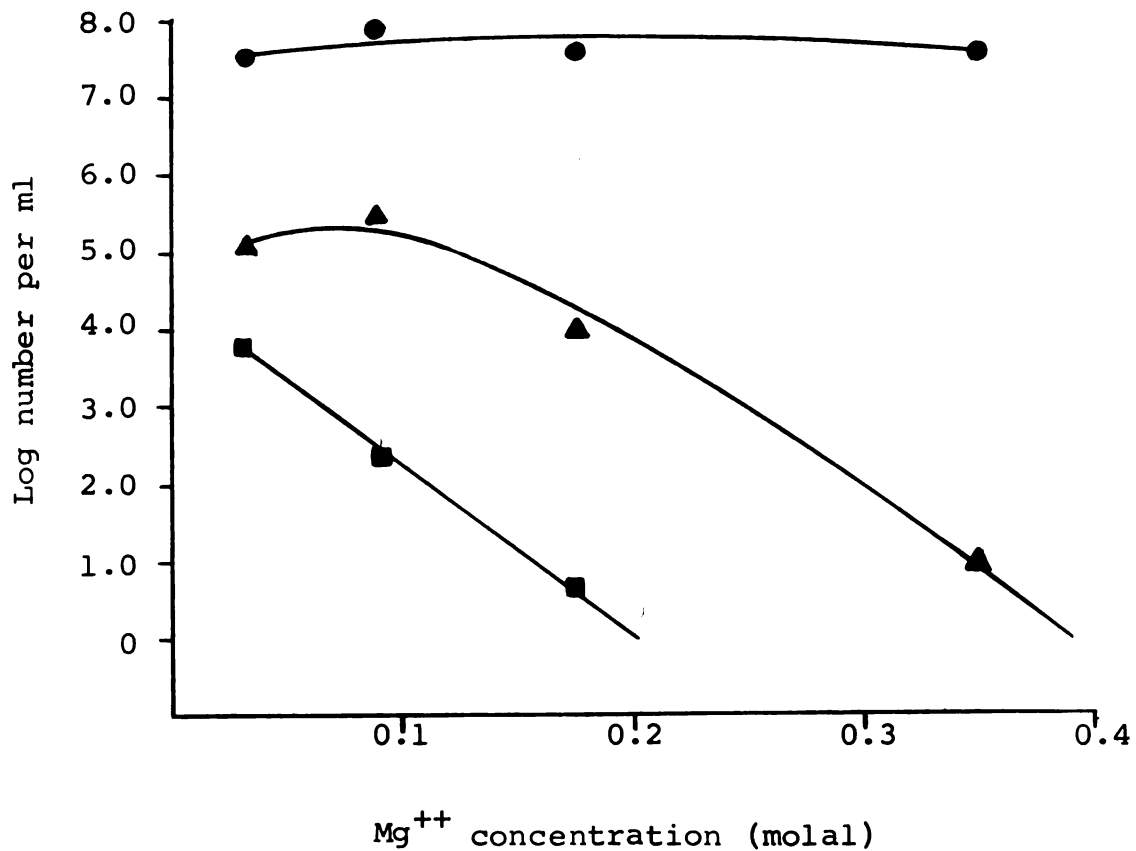


Figure 14. Effect of adding Mg^{++} to APT recovery medium on the survival of Streptococcus faecalis (S21) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 20 min at 60 C
- , 40 min at 60 C

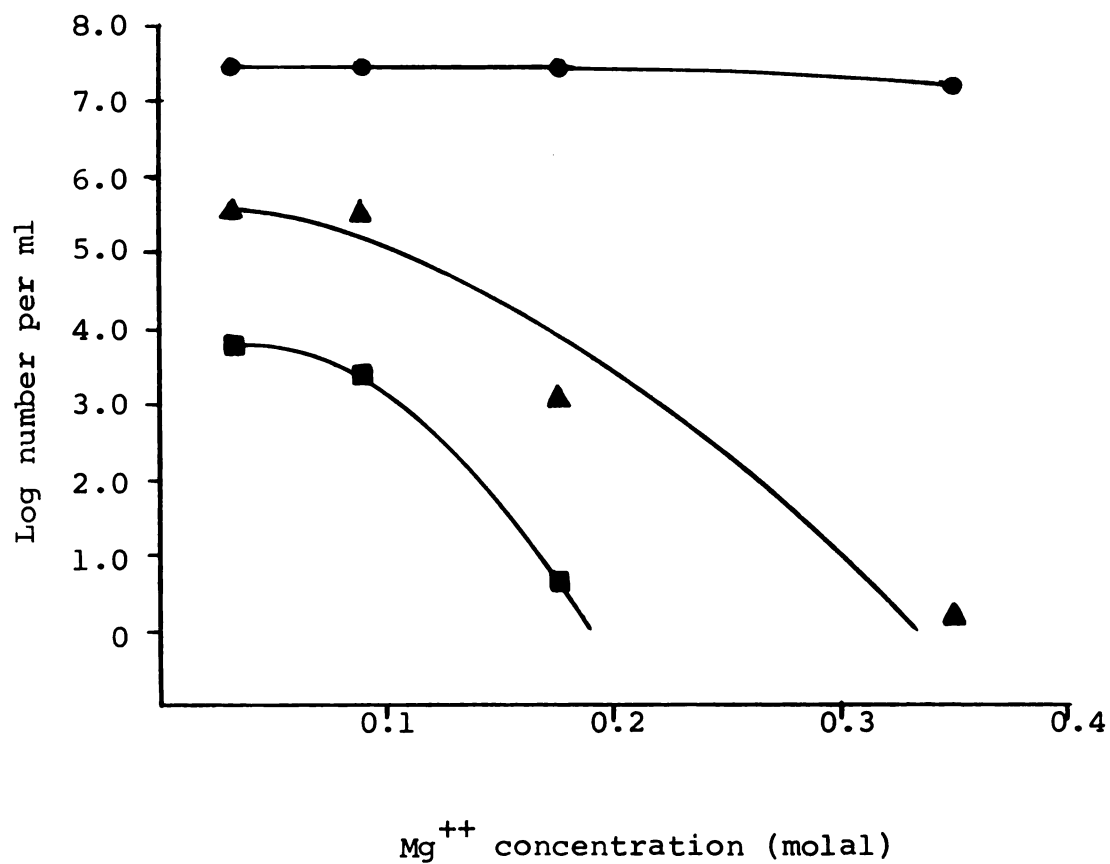


Figure 15. Effect of adding Mg^{++} to APT recovery medium on the survival of Streptococcus faecalis (S23) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 12 min at 60 C
- , 20 min at 60 C

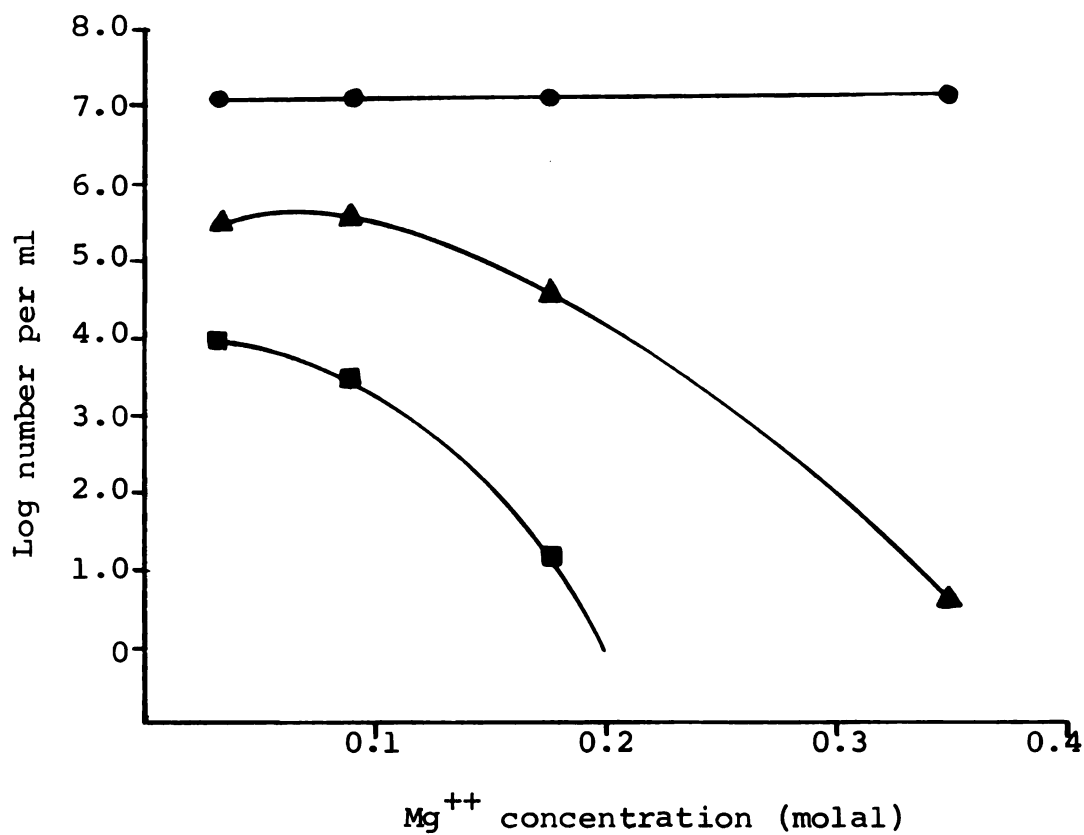


Figure 16. Effect of adding Mg^{++} to APT recovery medium on the survival of Streptococcus faecalis (S28) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 40 min at 60 C
- , 70 min at 60 C

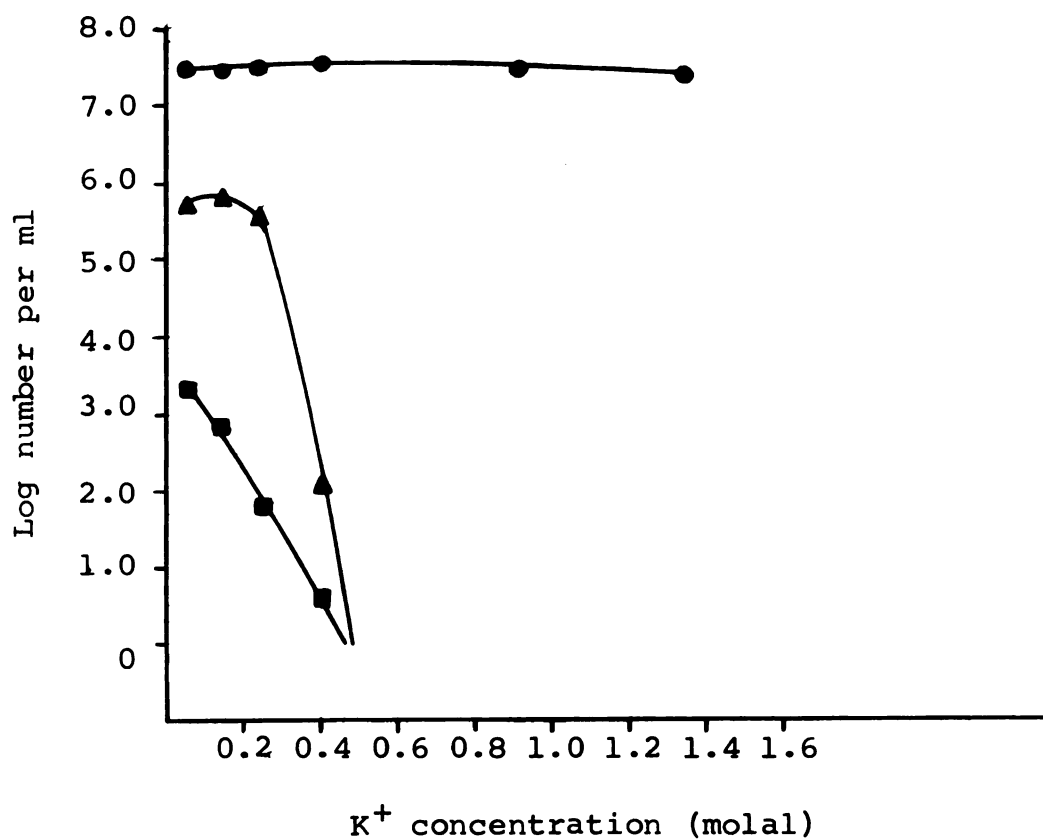


Figure 17. Effect of adding K^+ and 0.089 m Mg^{++} to APT recovery medium on the survival of *Streptococcus faecalis* (S21) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 20 min at 60 C
- , 40 min at 60 C

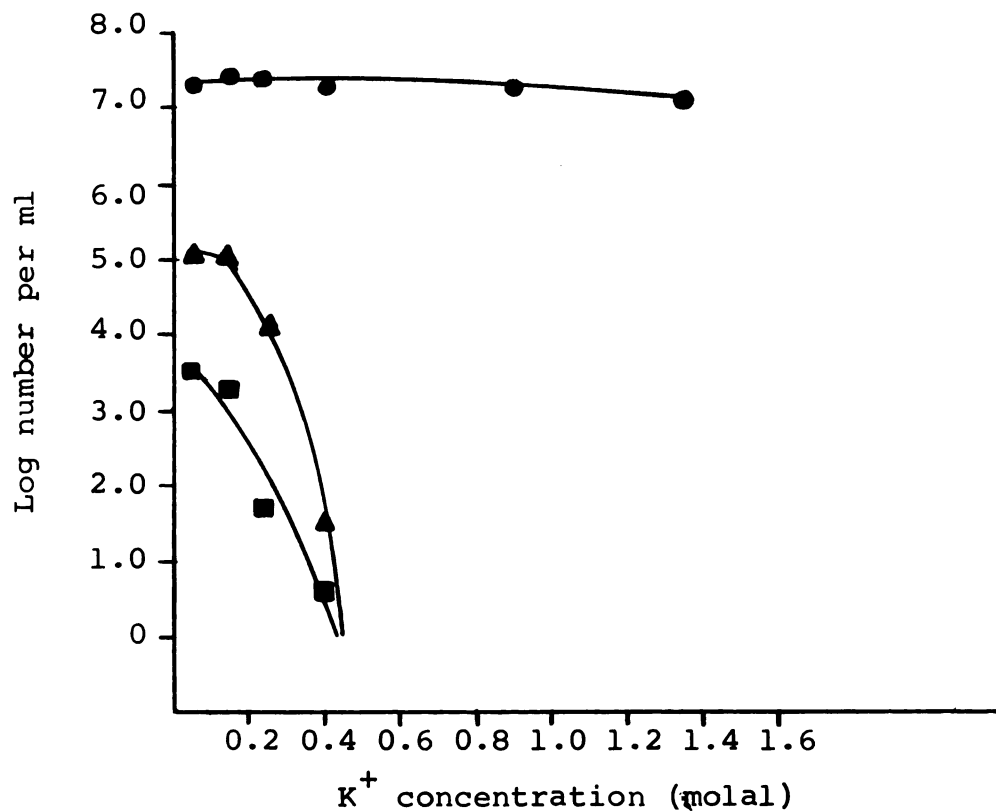


Figure 18. Effect of adding K^+ and 0.089 m Mg^{++} APT recovery medium on the survival of *Streptococcus faecalis* (S23) heated for varying lengths of time.

Key:

● , 0 min at 60 C

▲ , 12 min at 60 C

■ , 20 min at 60 C

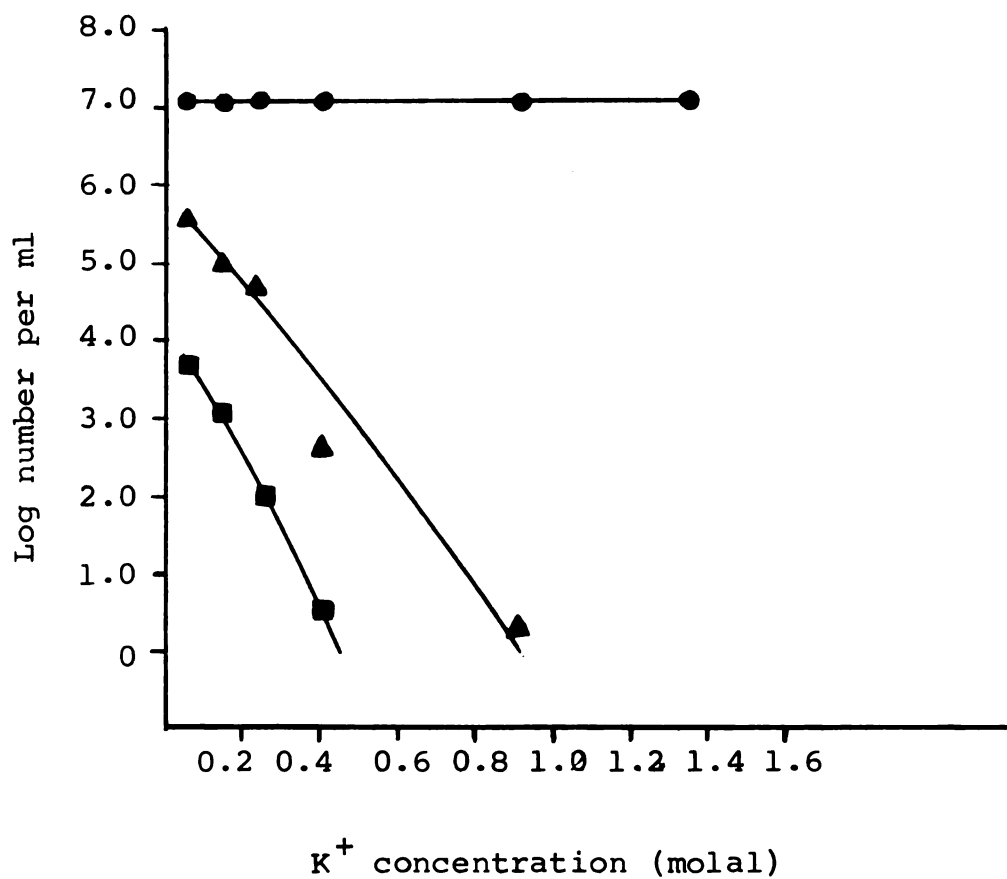


Figure 19. Effect of adding K^+ and $0.089\text{ m } Mg^{++}$ to APT recovery medium on the survival of Streptococcus faecalis (S28) heated for varying lengths of time.

Key:

- , 0 min at 60 C
- ▲ , 40 min at 60 C
- , 70 min at 60 C

evident at longer heating times. When heated S. faecalis were plated in recovery media containing 0.089 m Mg^{++} and concentrations greater than 0.14 m K^+ , recovery was decreased.

Generally, an increase in the Na^+ , K^+ , Mg^{++} , or K^+ and Mg^{++} concentration in the recovery media for heated S. faecalis caused a decrease in the total number of damaged cells which survived. Whether the inability to repair which is brought about by these cations is due to unfavorable osmotic conditions, disruption of the transport mechanism for transfer of metabolites across the cell membrane in or out of the cell, coagulation of cell protein, or some other physical or chemical phenomenon is not known.

Ribonucleic Acid Analyses

The ribonucleic acid (RNA) content of heated and unheated S. faecalis cells was determined and results are presented in Table 2. Analyses showed that there is reduction in RNA content upon heating, but that the percent reduction of cellular RNA is not directly proportional to the percent reduction of viable cells. For example, in the case of S21, a 99% reduction in viable cells shows a 31% reduction in cellular RNA. This would indicate that not all of the non-viable cells have disrupted sufficiently to allow extrusion of RNA into the menstruum. In fact, comparison of heated and unheated cells using phase contrast microscopy revealed little difference in gross appearance. An estimation of the

amount of RNA lost from the intact heated cells through leakage was not made. Part of the decrease in RNA content of heated S. faecalis is probably due to both cell wall breakage and leakage through the cell wall.

Table 2. Percent ribonucleic acid (RNA) in Streptococcus faecalis cells (dry weight basis) before and after heating at 60 C.

	Heat sufficient to reduce the viable population approximately:		
	0.0%	99.0%	99.99%
% RNA (S21)	10.41	7.13	5.76
% RNA (S23)	11.97	4.84	3.44
% RNA (S28)	11.61	4.92	2.78

SUMMARY AND CONCLUSIONS

Experiments were carried out to determine: 1) what effect the physiological condition of Streptococcus faecalis has on sensitivity to heat; 2) how the nature and extent of the heating process effects viability of S. faecalis; and 3) the effect of various environmental conditions on the recovery of heated S. faecalis.

Classification of three enterococcus isolates obtained from commercial meat samples was carried out. Two of the isolates were identified as S. faecalis var liquefaciens and the third was identified as S. faecalis. Codes to designate the three strains were assigned as S21, S23, and S28, respectively.

The thermal resistance at 60 C of the three strains was determined. Twenty-four hour cultures were heated in fresh All Purpose plus Tween (APT) broth preheated to 60 C. At various times throughout the heating procedure cells were removed and plated in APT agar. Counts were made after 48 to 72 hr incubation at 30 C and survivor curves were plotted. Strain S28 was most resistant to heat whereas S21 was less resistant and S23 was least resistant. Shoulders observed during the initial part of each survivor curve may be attributed to more than one site of inactivation existing within each cell, a minimum number of which have to be acted upon to cause death of the cell.

To assess the effect of incubation temperature on the recovery of heated S. faecalis, organisms were subjected to heat for various lengths of time, plated on APT agar, and incubated at 7.3, 10, 15.6, 21, 25, 30, 37, and 45 C. Unheated cultures grew equally well at all temperatures whereas heated cultures required incubation temperatures of 21, 25, 30, or 37 C to obtain full recovery.

The effect of age on thermal resistance of S. faecalis was determined by subjecting cells in different phases of the growth cycle to heat. Resistance was low during the initial lag phase, failing to zero throughout the initial and middle logarithmic growth phases. Heat resistance was not observed again until the late logarithmic phase and normal resistance was not observed until the culture had reached maturity during the stationary phase of growth. These data would suggest that great physical or chemical changes are occurring within the growing cells which are reflected as variations in the resistance to heat.

The effect of adding Na^+ , K^+ , and Mg^{++} to the APT recovery medium on the survival of heated and unheated S. faecalis was determined. Unheated organisms grew equally well when media containing concentrations of up to 1.39 m Na^+ , 1.34 m K^+ , 1.29 m Mg^{++} , or 1.34 m K^+ plus 0.089 m Mg^{++} were used for propagation. Concentrations above 0.27 m Na^+ , 0.14 m K^+ , or 0.089 m Mg^{++} decreased the recovery of heated S. faecalis. The time required for growth of heated organisms on a medium containing higher concentrations of Na^+ , K^+ , or

Mg⁺⁺ was longer than for lower concentrations and longer than for unheated organisms plated on the medium containing all concentrations of the cations employed.

It is evident that physical and/or chemical changes are occurring in the cell upon heating. Repair of the damage incurred requires not only time but more favorable environmental conditions than those required for the growth of unheated S. faecalis.

A modified procedure from Clark (1964) was used to isolate ribonucleic acid (RNA) from heated and unheated S. faecalis. There was a reduction of cellular RNA upon heating, the percent reduction not being directly proportional to the percent reduction of viable cells. It is probable that loss of cellular RNA is due to both leakage from intact cells as well as rupture of cells.

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