AN EFFECT OF THERMAL INJURY ON *STAPHYLOCOCCUS EPIDERMIDIS*: 16S RNA DEGRADATION RESULTING FROM THE LOSS OF CELLULAR MAGNESIUM

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ABSTRACT

AN EFFECT OF THERMAL INJURY ON *STAPHYLOCOCCUS EPIDERMIDIS*: 16S RNA DEGRADATION RESULTING FROM THE LOSS OF CELLULAR MAGNESIUM

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Exposure of *Staphylococcus epidermidis* to sublethal temperatures produced a temporary change in the salt tolerance of the organism. After sublethal heat treatment at 52 C for 15 min, 96% of the viable population was unable to reproduce on media containing 7.5% NaCl. The data reveals that thermal injury resulted in alterations in the cell membrane, which allowed leakage of 260 nm absorbing material into the heating menstruum. Polyacrylamide gel electrophoresis revealed 16S ribosomal RNA to be extensively degraded in thermally injured cells, whereas 23S and 5S RNA were unaffected. When thermally injured cells were incubated in complex media, 16S RNA was regenerated accompanied by a return of salt tolerance in a system where DNA synthesis was lacking. In the presence of high Mg^{2+} concentrations in the heating media, leakage of RNA was suppressed and the degradation of 16S RNA was not observed. Ribosomal RNA degradation is, therefore, an effect of magnesium loss and is not an effect of heat in thermally injured cells of *S. epidermidis*.

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CHAPTER I

INTRODUCTION

The response of microorganisms to environmental stress and the changes in the regulation of biosynthesis experienced by cells after exposure to stress are gradually being subjected to systematic evaluation. Among the stressing agents employed such as chemicals (Lawton and Nelson, 1955 and Jacobs and Harris, 1960), heat (Rosenthal et al., 1972 and Tomlins and Ordal, 1971) and chilling and freezing (Sinskey and Silverman, 1970 and Ray and Speck, 1972), heat treatment is the best understood at the molecular level (Iandolo, 1973). Exposure of bacteria, Staphylococcus aureus in particular, to thermal stress results in significant macromolecular alterations reflected by degradation of RNA (Iandolo and Ordal, 1966; Sogin and Ordal, 1967) and ribosomes (Rosenthal et al., 1972 & Hurst and Hughes, 1978), membrane damage (Hurst et al. 1975) and changes in enzymatic activity (Pariza and Iandolo, 1969). Thermally-induced chromosomal damage has been reported in Salmonella typhimurium (Gomez et al., 1976). Although severe, these metabolic alterations are not lethal and cells in this condition may be revived under proper conditions of incubation (Iandolo and Ordal, 1966 & Gomez et al., 1976). During this incubation, termed recovery, the ribosomes and ribosomal RNA are regenerated (Iandolo, 1973 & Parita and Iandolo, 1969) and membrane damage is repaired (Hurst et al.,

1975).

Injury has been defined as the loss of ability of living cells to form visible colonies under some conditions (Hurst, 1977). Consequently, a medium which is suitable for an uninjured organism may become inadequate for an injured one. In cells of Staphylococcus and Salmonella, there exists heat-induced osmotolerance differences between normal and injured cells reflected by an increase in salt sensitivity resulting from mild heat treatment (Iandolo and Ordal, 1966 & Tomlins and Ordal, 1971). Cells of S. aureus sublethally heated at 55 C showed a significant increase in salt sensitivity (Iandolo and Ordal, 1966), with maximum sensitivity occuring at about 5% NaCl concentration (Iandolo, 1973). During sublethal heating of this microorganism there was no reported loss in viability since growth in an optimal (complex) medium remained constant. Because salt tolerance is a characteristic of S. aureus, Iandolo and Ordal (1966) suggested that the high salt medium only counted uninjured organisms while in the complex medium injured and uninjured cells were being enumerated. Consequently, thermal injury of S. aureus leads to unaltered growth in an optimal medium lacking salt accompanied by a significant increase in salt sensitivity in an optimal medium containing 7.5% NaCl.

When thermally injured cells of S. aureus were incubated at 37 C in optimal medium, complete recovery of salt tolerance within a four hour period was observed (Iandolo and Ordal, 1966; Pariza and Iandolo, 1969; Sogin and Ordal, 1967).

In cells of S. typhimurium there exists a heat-induced inability to grow on complex medium agar which is restored during the recovery period (Gomez et al., 1976). It is significant to note that the recovery period of S. aureus, S. typhimurium and other microorganisms is accompanied by a complete lack of cell division. As defined by Harris (1963), "it is the period of 'getting back' organisms from their (sublethal) environment." Subsequent to the recovery period cells of S. aureus divide logarithmically and grow equally well on optimal medium with or without 7.5% NaCl (Iandolo and Ordal, 1966). Furthermore, it was found by these investigators that recoverd cells of S. aureus achieved a total viable population that was comparable to the unheated control.

Heat-induced alterations in the macromolecular makeup of thermally injured microorganisms appear to be fairly complex. Iandolo and Ordal (1966) observed thermally induced changes in the cell membrane of S. aureus which allowed leakage of soluble cellular components into the heating menstruum. They observed a substantial increase of 260 nm absorbing material extruded from the cell which suggested loss of the nucleotide pools and RNA degradative products from the cell. Potassium and the free amino acid pool were also observed to be leaked from thermally injured cells of S. aureus. The inability of heat injured cells of S. aureus to control retention of soluble pools is reflected by the 95% decrease in the ability of these cells to concentrate arginine (Iandolo, 1973) and aspartate resulting from a significant loss of oleate

from the membrane (Hurst et al., 1974).

The site of the thermally induced nucleic acid lesion was found to be the 16S component of ribosomal RNA. In extracts from heated cells of S. aureus and S. typhimurium 16S rRNA was highly degraded (1^{st} order kinetics) and only traces remained on polyacrylamide gels (Rosenthal and Iandolo, 1970 & Tomlins and Ordal, 1971) and methylated albumin kieselguhr (MAK) columns (Sogin and Ordal, 1967). However, these investigators observed that the primary structure of 23S RNA was unaffected by heat treatment. The temperature used for injury was in the melting range for rRNA and Iandolo (1973) reported secondary structural differences in normal and heated 23S RNA. Heated 23S RNA was hydrolyzed to a greater extent by RNase than normal 23S RNA, reflected by the extent of free amino groups titrated with [^{14}C]-formaldehyde. The author concluded that although the primary structure of 23S RNA was unaffected by heat treatment, modifications in the helical configuration of the molecule occured.

Changes in the helical configuration of ribosomal RNA could lead to alteration of ribosomal subunits. When ribosomal subunits extracted from heat injured cells of S. aureus were centrifuged in a sucrose gradient, heat-induced destruction of the 30S subunit with little alteration in the 50S subunit was observed (Rosenthal et al., 1972 & Hurst, 1977). Iandolo (1973) showed that there was 90% destruction of the 30S subunit in 7.25 min. of heating and only 10% decrease in the 50S subunit after 10 min. Similar results

were reported for S. typhimurium (Tomlins and Ordal, 1971). Iandolo (1973) reported that 100% of the cells were salt sensitive when 82% of the 30S subunit had been destroyed. It is significant to note that when 70S ribosomes, subunits or naked RNA were heated in buffers containing magnesium, solubilization of nucleic acids was not observed (Iandolo, 1973). This data suggests that heat injury was not directly responsible for in vivo RNA destruction. Iandolo (1973) further showed that there was nondiscriminate in vitro destruction of both 16S and 23S RNA by RNase at sublethal temperatures. Since 23S RNA is not degraded in vivo at sublethal temperatures, unlike 16S RNA, the presence of RNase specifically associated with the 30S subunit (of which 16S RNA is associated) in vivo remains as an attractive hypothesis, although the presence of the enzyme in Escherichia coli ribosomes is thought to be due to adsorption from the periplasmic space during isolation.

Little work has been done on S. aureus dealing with heat-induced changes in DNA. In S. typhimurium, on the other hand, Gomez et al. (1976) reported that mild heat treatment induces primary lesions in DNA or its metabolic and catabolic enzyme systems. When such cells were incubated in complex media, the primary lesions were converted to in vivo DNA breaks or alkali-labile bonds yielding loss in viability.

Bluhm and Ordal (1969) reported that heating S. aureus at 55 C yielded 75% decrease in fructose diphosphate aldolase activity and 51% decrease in lactate dehydrogenase activity, compared to the unheated control, yielding a decrease in

glucose catabolism and energy yielding reactions. However, they reported that hexokinase and other enzymes of the glycolytic pathway were not affected by thermal injury. However, Tomlins and Ordal (1971) reported unaffected activities of glycolytic and TCA cycle enzymes in thermally injured cells.

An attractive feature of the phenomenon of thermal injury is the ability of the stressed microbes to undergo recovery and subsequent achieval of viability comparable to unheated cells. In S. aureus this recovery is manifested in the return of salt tolerance (Iandolo and Ordal, 1966). Recovery in S. typhimurium occurs when cells regain their tolerance to Eosin Methylene Blue-NaCl medium (Tomlins and Ordal, 1971) and regain their ability to grow on complex media (Gomez et al., 1976). An important feature of the recovery period is an extended lag phase lasting approximately four hours (Iandolo and Ordal, 1966 & Tomlins and Ordal, 1971). Previous authors (Jackson and Woodbine, 1963 & Kaufman et al., 1959) observed the extended lag phase to be a cultural response to thermal injury. The temporal coincidence of the extended lag phase and recovery of salt tolerance was found to be reflective of physiological changes during repair (Iandolo and Ordal, 1966 & Rosenthal et al., 1972; Hurst et al., 1975; Tomlins and Ordal, 1971). Iandolo and Ordal (1966) reported that recovering cells of S. aureus reform amino acid pools prior to repair of salt tolerance. The rapidity of the pool concentrating ability resumed by these cells was reported to be high, suggesting biosynthetic activity taking place. The introduction of labelled amino

acids into the recovery medium revealed that the uptake of the label into the soluble pool reached a maximum in 30 min. for S. aureus (Iandolo and Ordal, 1966). These investigators further showed that RNA was rapidly synthesized during recovery after the amino acid pool reached a maximum.

The reconcentration of the soluble pools during recovery of S. aureus was found to be mediated through energy from the oxidation of an energy source such as glucose (Iandolo and Ordal, 1966). For complete recovery of salt tolerance, S. aureus requires an energy source, a mixture of amino acids providing the organic carbon and nitrogen skeletons for RNA formation, and phosphate (Pariza and Iandolo, 1969). In contrast, it has been reported that cells of S. aureus and S. typhimurium are able to recover salt tolerance in minimal no-growth media (Hurst et al., 1975 & Gomez et al., 1976). It can be concluded that membrane damage is probably slight since reformation of the pools in S. aureus is rapid and occurs before salt sensitivity returns.

Recovering cells of S. aureus synthesize proteins only after RNA synthesis reached a maximum level (Rosenthal et al., 1972 & Pariza and Iandolo, 1969). This was shown to occur after approximately 90 min. of incubation in the recovery medium (Rosenthal et al., 1972 & Iandolo, 1973). The delay in protein synthesis can be attributed to the heat-induced breakdown of the 70S ribosome. Sogin and Ordal (1967) reported regeneration of ribosomes during early

stages of recovery established by the appearance of a large portion of newly synthesized RNA in the ribosomes. Since regeneration of the 70S ribosome in S. aureus occured in the absence of protein synthesis, the possibility of a salvage mechanism for ribosomal proteins was explored by various authors. Rosenthal et al. (1972) showed that both ribosomal subunits reassembled during recovery and the original ribosomal proteins present before heat injury are conserved and recycled. The authors reported that 100% utilization of original proteins did not take place due to resumption of protein synthesis where new ribosomal proteins diluted out the system.

The addition of chloramphenicol, an inhibitor of protein synthesis, to the recovery medium did not inhibit the formation of ribosomal subunits during the recovery of S. aureus (Rosenthal et al., 1972). However, Yoshida and Osawa (1968) reported that in normal E. coli cells chloramphenicol particles are formed as a result of nonspecific binding of intracellular protein to nascent RNA. Rosenthal et al. (1972) did not detect the presence of immature chloramphenicol particles during recovery and concluded that thermally shocked cells of S. aureus possessed a concentrated pool of basic ribosomal proteins due to 30S subunit degradation, which have a higher binding efficiency to RNA than nonribosomal proteins. They further suggested that ribosome regeneration depended on the fact that the original undamaged 50S subunits were"turned over" during re-

covery to liberate 50S protein which then associated to form new 50S particles, similar to the mechanism of *in vitro* assembly of *E. coli* subunits. This theory was substantiated by the limiting rate at which new RNA appeared in the 50S peak and once assembly had begun, the association of RNA and proteins occured rapidly without precursor formation during recovery. In contrast, heat injured cells of *S. typhimurium* showed little recycling of old ribosomal proteins during recovery and therefore required synthesis of ribosomal proteins for reconstitution of the ribosome, preceeded by the formation of ribosomal precursors (Tomlins and Ordal, 1971).

The use of antibiotics during the recovery period of thermally injured microorganisms served to determine what aspect of macromolecular synthesis was necessary in the repair process. In thermally injured cells of *S. aureus* inhibitors of protein synthesis such as chloramphenicol and 5-methyltryptophan did not inhibit recovery (Iandolo and Ordal, 1966; Rosenthal *et al.*, 1972; Pariza and Iandolo, 1969 and Sogin and Ordal, 1967). Penicillin (Iandolo and Ordal, 1966 & Hurst *et al.*, 1975), cycloserine and 2,4-dinitrophenol (Iandolo and Ordal, 1966), inhibitors of cell wall synthesis and ATP formation, also did not inhibit the recovery of *S. aureus*. However, when Actinomycin D was added to the recovery medium the return of salt tolerance of thermally injured cells of *S. aureus* was not observed (Iandolo and Ordal, 1966; Hurst and Hughes, 1978 and Sogin

and Ordal, 1967). This established that RNA synthesis is essential for the recovery of salt tolerance in thermally injured cells of S. aureus whereas inhibition of protein, cell wall or ATP synthesis does not suppress recovery. In contrast, the recovery process for S. typhimurium was found to be dependant on RNA, ATP and protein synthesis in a system where DNA synthesis was not essential and indeed lacking (Tomlins and Ordal, 1971). However, these investigators observed recovery of thermally injured S. typhimurium in the presence of 5-methyltryptophan but not in the presence of chloramphenicol, both inhibitors of protein synthesis. They concluded that the synthesis of a functional protein, either containing negligible amounts of tryptophan or incorporating 5-methyltryptophan into the molecule without loss of function during recovery, was in some way involved in ribosome formation. Such a functional protein could be a nuclease, methylase, or ribosomal protein which could be thermolabile and inactivated during heat. The synthesis and activity of this functional protein is the rate limiting step in the recovery process of S. typhimurium but is apparently not essential for the recovery of S. aureus.

Because of the many changes resulting from sublethal heating, it is difficult to determine which is the first or 'critical site' of injury. It has been suggested by Hurst et al. (1975) for S. aureus and supported by this presentation for S. epidermidis that the heat-induced loss of cellular magnesium plays a critical role in the observed effects

of thermal injury. Hurst et al. (1975) observed a 40% loss of cellular magnesium and a 65% loss of ester bound D-alanine of teichoic acid, without the loss of cell wall ribitol teichoic acid or membrane glycerol teichoic acid, resulting from sublethal heat treatment of S. aureus. D-alanine was found to be rapidly synthesized during recovery and cellular magnesium returned to normal after one hour incubation in recovery medium (Hurst et al., 1975). It is significant to note that these investigators reported that the restoration of cellular magnesium concentration occured at only 3×10^{-6} M magnesium concentration in the recovery medium and was not effected by the presence of EDTA. However, alanyl amino groups in teichoic acid were reported to reduce the binding strength of Mg^{2+} by teichoic acid in S. aureus (Hurst et al., 1975). Therefore, the observed heat-induced loss of D-alanine ester residues of teichoic acids in S. aureus (Hurst et al., 1975) would attribute to very firm binding of Mg^{2+} to teichoic acids during the recovery period. Since teichoic acids were reported to participate in the uptake and localization of metal ions (Hepinstall, 1970), magnesium can be quickly and more efficiently reconcentrated during the recovery of thermally injured S. aureus. Since magnesium is necessary for the integrity of ribosomes and ribosomal RNA, loss of magnesium from thermally injured cells of S. aureus could attribute to the observed heat-induced alterations of the ribosomes and rRNA.

It is evident that the phenomenon of thermal injury provides insight into important biological functions within

the bacterial cell. The prime advantage of the system is the existence of homogeneous populations of cells present in biosynthetic register during the entire recovery period. The recovery of salt tolerance in the absence of cell division has proven to be a valuable tool for further study of the microbial response to stress; specifically, RNA synthesis and ribosome biogenesis. Furthermore, as revealed by the foregoing introduction, the response to heat stress and subsequent recovery is variant among microorganisms and therefore provides insight into the intricate processes of the cell. It is, therefore, the purpose of this presentation to reveal the effects of thermal injury on macromolecular alterations in S. epidermidis, a microorganism which has been neglected in the investigations of thermal injury. Since rRNA (more specifically the 16S component) is degraded in thermally injured Staphylococcus, an attempt was made to suppress the selective degradation of 16S rRNA and leakage of RNA from the cell. This effect was achieved by the addition of high concentrations of magnesium in the heating buffer. This leads to the conclusion that degradation of ribosomes in thermally injured Staphylococcus results from instability of 16S RNA resulting from heat-induced loss of cellular magnesium.

CHAPTER II

MATERIALS AND METHODS

Bacteria and Culture Conditions

Staphylococcus epidermidis, a skin isolate, was kindly provided by Leonard Perry. Stock cultures were maintained at 5 C on Trypticase Soy Agar (TSA) slants. For growth studies the bacterium was cultured in Trypticase Soy Broth (TSB) and grown on a rotary shaker at 37 C for 18-24 hr. At this time the cultures contained approximately 8 x 10^8 viable bacteria/ml.

Sublethal Injury Procedure

The technique for injury was essentially as described by Iandolo and Ordal (1966). All manipulations were performed using standard sterile technique. Forty ml. of an overnight culture was removed and the cells were harvested by centrifugation and washed in sterile 0.1 M sodium phosphate buffer (PPB). The pellet was then resuspended in 5 ml. PPB by vortexing and added to 45 ml. PPB previously equilibrated at 52 C. To minimize settling, the suspension was swirled for 10 sec. at 5 min. intervals. Samples (1 ml.) were removed at 5 min. intervals for 15 min. and serially diluted in PPB. Samples were plated, in triplicate, on TSA and Trypticase Soy Salt Agar (TSSA) containing 7.5% NaCl using the pour plate technique. The plates were incubated at 37 C for 72 hr. and colonies were then counted. The extent of the injured

population was determined as the difference in the cell numbers on TSA and TSSA.

Recovery Procedure

After thermal injury the cell suspension was cooled in ice. To determine the effect of thermal injury on the recovery process a 5 ml. aliquot of the cooled suspension was centrifuged and the pellet was suspended in 5 ml. TSB. This cell suspension was added to 45 ml. TSB and incubated with shaking at 37 C. To observe recovery in a medium minus nutrients, PPB was substituted for TSB. Samples (1 ml.) were removed from the recovery medium at intervals and samples were plated, in triplicate, on TSA and TSSA. The plates were incubated at 37 C for 72 hr. and then were counted.

When recovery was followed spectrophotometrically, samples from the recovery medium were removed at intervals and A_{550} was measured in a Coleman 124 Double Beam Spectrophotometer.

Injured cells were subjected to various temperature shifts to observe the effect on the recovery process. The culture was incubated at 20 C and after 2 hr. one-half of the culture was shifted to 37 C while the other half was maintained at 20 C. Samples (1 ml.) were removed at intervals, serially diluted, and plated on TSA and TSSA for counting.

Characterization of Extracellular 260 nm. Absorbing Material

To distinguish between the DNA and RNA content of the 260 nm. absorbing material in the heating media of thermally

injured cells, the diphenylamine reaction (Burton, 1956) and the orcinol reaction (Lin and Schjeide, 1969) were performed on aliquots from the clarified heating media. Cells from an overnight culture were thermally injured in PPB (pH 7.2) at 52 C for 10 min. The heated cell suspension was then cooled and cetrifuged at 2000 x g. The supernatant was further clarified by passage through a 0.45μ filter unit. For the diphenylamine reaction, two 3 ml aliquots were removed and placed in seperate tubes. A 3 ml sample of 5% trichloroacetic acid (TCA) was placed in a seperate tube serving as the blank. Six ml of diphenylamine reagent (1.59 g diphenylamine purchased from Eastman Kodak Co. in 1.5 ml concentrated H_2SO_4 and 100 ml of acetic acid) was added to each tube. The tubes were then heated at 95 C for 30 min, cooled in ice, and absorbance values were read at 650 nm in a Coleman 124 Double Beam Spectrophotometer.

A 5 ml sample from the clarified heating media was used for the orcinol reaction. Since this assay is positive in the presence of ribose, contaminating carbohydrates were removed with Dowex-1 resin as suggested by Smillie and Krotkov (1960). The sample was placed on a column of Dowex 1 x 8, chloride form, 200 to 400 mesh, purchased from Sigma Chemical Co. The column was washed with 10 mM NaCl, and the nucleotides were eluted with an HCl-NaCl solution (20 ml of 10 N HCl + 5.6 g of NaCl in 240 ml of water). Two 3 ml aliquots of the eluted RNA were placed in seperate tubes. A 3 ml sample of 5%TCA was placed in a seperate tube serving

as the blank. Five ml of orcinol reagent (100 ml of concentrated HCl containing 0.15 g $CuCl_2$ and 2 ml of 95% ethanol containing 1 g orcinol purchased from Fisher Scientific Co.) was added to each tube. The tubes were heated at 95 C for 30 min, cooled in ice, and absorbance values were read at 660 nm. Standard RNA curves were kindly provided by Dr. Anthony E. Sobota.

Preparation of Polyacrylamide Gels

The monomers were recrystallized in the following manner. A 70 g sample of acrylamide, purchased from Eastman Kodak Co., was dissolved in 1 liter of chloroform at 50 C and the solution was filtered. The filtrate was cooled slowly to -20 C and the crystals were recovered by filtration in a chilled filter funnel. The crystals were washed briefly with cold chloroform and dried first in air and finally in vacuo.

Bisacrylamide, purchased from Eastman Kodak Co., was dissolved in acetone (approx. 12 g/liter at 40-50 C) and filtered hot. The solution was slowly cooled to -20 C and the crystals were recovered and washed with cold acetone by filtration.

Polyacrylamide gels were prepared according to Loening (1967) with slight modification. For the preparation of dilute gels in which the final acrylamide concentration was between 2% and 5% (w/v), the bisacrylamide concentration used was 5% that of the acrylamide. For gel concentrations between 5% and 8% (w/v), the bisacrylamide concentration was 2.5% that of the acrylamide.

Stock solutions of acrylamide and bisacrylamide were prepared by dissolving 15 g of recrystallized acrylamide and 0.75 g of recrystallized bisacrylamide (0.375 g for 7.5% gels) in a total volume of 100 ml of distilled water. Gel buffer (5 x concentrated electrophoresis buffer) consisted of Tris (0.18 M), monobasic sodium phosphate (0.15 M), and disodium EDTA (5 mM). For 2.6% acrylamide gels, stock acrylamide solution (5 ml) was mixed with gel buffer (5.8 ml) and water (17.8 ml). Gels of higher concentration (7.5%) were prepared by mixing 5 ml stock acrylamide solution with 2 ml gel buffer and 2.7 ml of water. The mixture was degassed in a round bottom flask in vacuo for 15-30 sec at room temperature. NNN^1N^1 Tetramethylethylenediamine (o.025 ml), purchased from Eastman Kodak Co., and freshly dissolved 10% (w/v) ammonium persulphate (0.25 ml) were then added. The solution was mixed, without excessive aeration, and 2 ml aliquots were rapidly pipetted into 6mm x 80mm plexiglass tubes containing plastic rings to prevent the soft gels from sliding out. Water was immediately layered over the solution with a hypodermic syringe to ensure a flat gel surface.

Procedure for Electrophoresis

After insertion of the gel tubes, the buffer compartments were filled with electrophoresis buffer (gel buffer diluted 1:5 with water) containing 0.2% sodium lauryl sulphate (SLS) purchased specially pure from BDH Chemicals. The gels were pre-run at room temperature for 30 min at 5 ma/gel tube to remove ultraviolet absorbing materials along with im-

purities in the gels and to allow SLS to enter the gels. Nucleic acid samples containing approximately 25-50 μg in 5% sucrose, dissolved in electrophoresis buffer, were applied per gel and the samples were electrophoresed at 5 ma/gel for 2-3 hr. At the completion of the run the gels were gently blown out of the tubes with a rubber teat, soaked in distilled water for 30 min., and then scanned at 265 nm by using a Joyce Loebl Polyfrac ultraviolet scanner.

Extraction Technique

Undegraded nucleic acids were extracted by a combined pyrocarbonate-phenol-SLS method (Solymosy et al., 1968 & Pigott and Midgley, 1968). A 200 ml sample from an 18 hr. culture was centrifuged at 2000 x g for 10 min. at 2 C. The resulting pellet, after washing in 0.05 M Tris-HCl buffer (pH 7.2), was resuspended in 3 ml 0.05 M Tris buffer (pH 7.6) containing 5 mM $MgCl_2$ (TM buffer) and 3% SLS. Diethyl-oxidiformate (0.4 ml), purchased from Eastman Kodak Co., was added as a nuclease inhibitor. Homogenization was then carried out in an ice cold mortar and pestle containing 5 g 150-200μ glass beads for 5 min. The sample was then incubated at 37 C for 5 min. after which time the mixture, including beads, was placed into a centrifuge tube and vortexed vigorously for 3 min. The sample was centrifuged at 15000 g in the cold for 10 min. and the supernatant removed. An equal volume of phenol in 0.1 M Tris-HCl buffer (pH 7.2) containing 0.1% 8-hydroxyquinoline was added and the solution stirred 10 min in ice. The aqueous phase was removed after

centrifugation at 10000 x g and adjusted to 0.15 M NaCl with ice cold 2 M NaCl solution. Phenol (0.5 vol) was then added and the solution stirred 10 min in ice. The aqueous phase was retrieved by centrifugation and the nucleic acids were precipitated with 2 volumes of ice cold 95% ethanol and stored at -16 C over a 3-24 hr period. The nucleic acids were collected by centrifugation at 10000 x g in the cold, dissolved in 1 ml of 0.15 M sodium acetate buffer (pH 6.0) containing 0.5% SLS, and reprecipitated with 2 vol ice cold 95% ethanol.

Labelling Technique

Approximately 40 ml of an overnight culture was centrifuged at 2000 x g. The resulting pellet, after a PPB wash, was suspended in PPB equilibrated at 52 C (total vol--40 ml) and incubated for 10 min. A 1 ml sample was removed, cooled in ice, and centrifuged. The pellet was resuspended in 5 ml TSB containing 5 μc ^{32}P-orthophosphate. Recovery was carried out on a rotary shaker (70 oscillations/min) at 37 C. A control was established with uninjured cells. Incorporation of ^{32}P into DNA and RNA during the recovery period was determined by the filter paper disk technique described by Sobota (1976).

Samples (50 μl) were recovered at intervals during the recovery period and deposited in individual wells of a microtitration plate (Catalog Number 14-245-3), purchased from Fisher Scientific Company, containing 50 μl of 0.6 N KOH per well. The plate was then incubated at 37 C for 4 hr to

hydrolyze the RNA. A second set of samples was prepared in the following manner. Whatman 3 mm filter paper disks (Fisher Scientific Co.), 2.3 cm in diameter, were labelled T (total nucleic acid) using a lead pencil and 50 μl of sample was added to each filter. The disks were then placed in ice cold 10% TCA and allowed to incubate a minimum of 30 min in the cold.

Filter paper disks were then prepared for the samples in the microtitration plates. Half of these were labelled DR (DNA + residue) and half were labelled R (residue). At the end of the incubation period the disks (folded into quarters) were dipped individually into the wells of the microtitration plate to absorb the sample. They were then placed in cold 10% TCA and incubated for 30 min. The disks labelled R were then removed from the 10% TCA and placed into 5% TCA (5 ml/disk) at 90 C for 30 min to hydrolyze the DNA. After the incubation the 5% TCA was removed and replaced 2x with cold 10% TCA.

The accumulated disks were then processed through a wash sequence. Each wash consisted of a 5 min incubation with gentle swirling at the beginning and termination of the incubation. The wash sequence consisted of two washes with ice cold 10% TCA, three washes with 95% ethanol saturated with sodium acetate, three washes with ethanol-diethyl ether (3:1 v/v) and one wash with diethyl ether. The disks were allowed to air dry and then were placed in vials with 20 ml scintillation fluid containing per liter of toluene: 5 g

of 2,5-Diphenyloxazole (PPO) and 0.3 g of 1,4-Bis(2-(5-Phenyloxazolyl))Benzene (dimethyl POPOP) purchased from Fisher Scientific Company. The vials were then counted to 5% error in a Beckman LS-133 liquid scintillation spectrometer. The activity of RNA was obtained by subtracting the counts per minute of the DR sample from the counts per minute of the T sample. The DNA activity was obtained by subtracting the counts per minute of the R sample from the counts per minute of the DR sample.

Effects of Magnesium on Injury

Cells from an overnight culture (50 ml) were harvested at 2000 x g for 10 min. The resulting pellet, after washing in 50 mM Tris-HCl buffer (pH 7.2), was resuspended in a minimal amount of Tris buffer and added to 50 mM Tris (pH 7.2) or 50 mM Tris (pH 7.2) containing 5×10^{-2} M $MgCl_2$ previously equilibrated at 52 C, yielding a volume of 50 ml. Injury was carried out for 15 min at 52 C. Samples (3 ml) were removed at intervals and delivered into a pistonless syringe, attached to a 0.45μ Swinnex-25 filter unit, and injected into 10 x 75mm culture tubes. This sampling process was less than 30 seconds. Distilled water (1 ml) was added to each culture tube. The absorbance of the filtrates was measured at 260 nm and 280 nm in a Coleman 124 Double Beam Spectrophotometer.

In assaying the effects of magnesium on rRNA degradation during injury, 0.1 M $MgCl_2$ was added to the injury buffer (50 mM Tris, pH 7.2). After injury, nucleic acids

were extracted and subjected to electrophoresis as described above.

To support the observation that the degradative effect of thermal injury on 16S RNA is caused by an extensive heat-induced loss of cellular magnesium rather than directly to heat per se, the following experiment was performed. Nucleic acids were extracted from unheated cells of S. epidermidis and heated in vitro in magnesium conserving buffer (50 mM Tris containing 0.05 M $MgCl_2$ and 0.1% SLS) and buffer lacking magnesium (50 mM Tris containing 0.1% SLS). After 10 min at 52 C, the nucleic acids were reprecipitated with ethanol and electrophoresed on polyacrylamide gels.

CHAPTER III

RESULTS

An increase in salt sensitivity of S. epidermidis was found when the heated cells were plated on TSA and TSSA (Fig. 1). The total viable population represented by the TSA counts was essentially unchanged after 15 min. of heating. When the same suspensions of heat treated cells were plated on TSSA differing results were obtained (Fig. 1). It is evident that there was a steady decrease in salt tolerance over the 20 min. incubation period. After 20 min. of thermal injury more than 96% of the cells were no longer able to grow in the presence of 7.5% NaCl.

When the thermally injured cells were suspended in TSB and incubated at 37 C there was a gradual return of salt tolerance (Fig. 2). Initially 98% of the cells were incapable of growth on TSSA, but were still viable, as shown by the counts on TSA. Over a 2-4 hr. period the cells recovered their tolerance to salt. The TSA count revealed an extended lag period, as compared to the unheated control, which lasted until salt tolerance was regained. The 2 hr. duration of the lag phase was substantiated by measuring growth spectrophotometrically during recovery (Fig. 3). Upon emergence from the lag phase the heat treated cells grew equally well on TSA and TSSA and at a normal rate (Fig. 2).

To determine whether a recovery medium minus nutrients could support the retrieval of complete salt tolerance, thermally injured cells were suspended in sodium phosphate buffer and incubated at 37 C (Fig. 2). It is evident that under these conditions the cells did not regain their tolerance to salt. A substantial decrease in the TSA and TSSA counts were observed over a 6 hr. period.

Recovery in TSB was assayed under various temperature shifts (Fig. 4). After 6 hours at 20 C there was no appreciable increase in salt tolerance as revealed by counts on TSSA. When the culture was shifted to 37 C after 2 hours, there was an increase in viability (TSA counts) accompanied by an immediate increase in salt tolerance (TSSA counts). Four hours after shifting the culture to 37 C, more than 50% of the viable population were able to meet the salt challenge as compared to 8% of the viable population growing at 20 C. Under these experimental conditions, however, there was no extended lag phase during the return of salt tolerance.

An assay of the effects of thermal injury on molecular alterations of RNA was performed using polyacrylamide gel eletrophoresis. In this system electrophoretic mobility is inversely proportional to the molecular weight. Electrophoresis of nucleic acids extracted from uninjured cells of S. epidermidis on 2.6% polyacrylamide gels reveals undegraded 23S and 16S rRNA components (Fig. 5a). In

contrast, nucleic acid preparations from thermally injured cells revealed that the 16S rRNA was extensively degraded and only traces remained (Fig. 6a). Furthermore, the extent of 16S degradation is temperature dependant. The 23S RNA and 16S RNA peaks were cut out of the gel profiles and weighed individually to yield the percent degradation of 16S RNA of heated cells compared to the unheated control. Cells thermally injured at 50 C showed 51.5% degradation of 16S RNA; thermal injury at 52 C showed 68.8% degradation of 16S RNA and thermal injury at 55 C showed 86.4% degradation of 16S RNA, where 23S rRNA was unaffected and behaved normally in the electrophoretic field. Electrophoresis of nucleic acids extracted from heated cells on 7.5% polyacrylamide gels reveals a broadening of the 4S peak (Fig. 6b) as compared to the uninjured control (Fig. 5b).

The break down products of the 16S rRNA appear to be leaked out into the heating media. The composition of the heating menstruum after thermal injury revealed a 280:260 ratio of 0.55, which implied that the solution was primarily nucleic acid (Fig. 7). The diphenylamine test of a sample from the heating media was negative. However, the orcinol reaction revealed a 62% increase in the leakage of RNA after 10 min. of heating.

Since thermally injured cells of S. epidermidis regain their tolerance to salt under proper conditions, it was of interest to see if 16S rRNA was regenerated during the recovery period. Polyacrylamide gel electro-

phoresis of nucleic acids extracted from heated cells recovering in TSB for 2 hours reveals regeneration of the 16S rRNA component (Fig 8). It is important to note that 16S regeneration occurred in the absence of cell division.

The release of 260 nm absorbing material from heated cells was dependent on the composition of the heating menstruum. Heating at 52 C in 50 m$\underline{M}$ Tris buffer (pH 7.2) released more RNA from the cell than heating in Tris buffer (pH 7.2) containing 0.05 $\underline{M}$ $MgCl_2$ (Fig. 10).

When nucleic acids were extracted from cells heated at 52 C in Tris buffer (pH 7.2) and subjected to polyacrylamide gel electrophoresis, a profile similar to Figure 6a was attained. In contrast, nucleic acid preparations from cells heated at 52 C in Tris buffer (pH 7.2) containing Mg^{2+} revealed no degradation of 16S rRNA on 2.6% polyacrylamide gels (Fig. 9). Also, there was no degradation observed in the soluble RNA (sRNA) region of the gel when Mg^{2+} was present in the heating menstruum.

Labelling thermally injured cells with ^{32}P during the recovery period revealed incorporation of the label into RNA after 2 hr. of incubation at 37 C (Fig. 11). In contrast, unheated cells incorporated the label into RNA after a 15 min. lag (Fig. 11, inset). It was also found (data not shown) that ^{32}P was incorporated into the DNA of unheated cells after a kinetic delay of 0.5 hr. However, no DNA was synthesized in thermally injured cells during the recovery period.

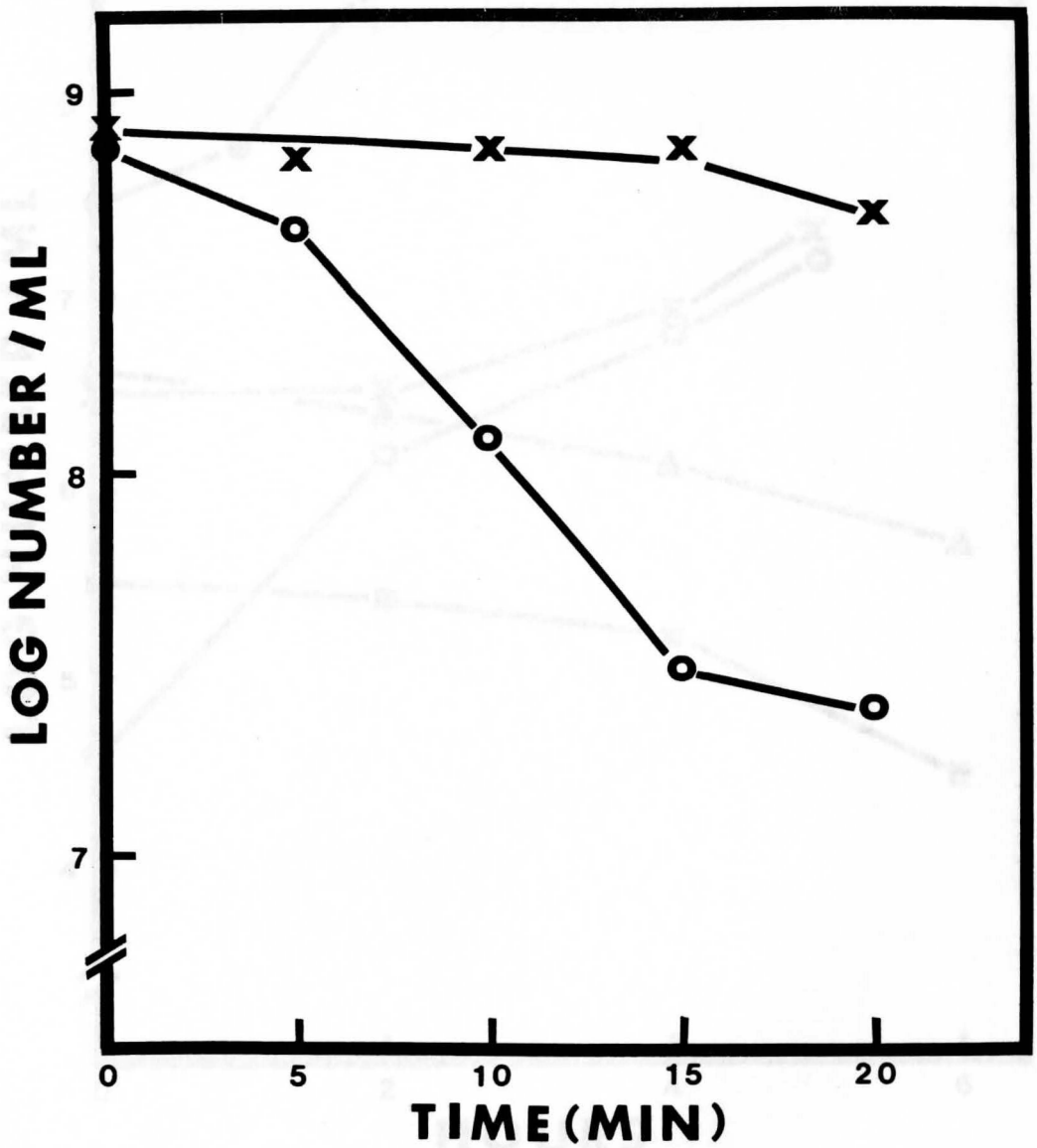

Fig. 1.--Effect of 7.5% NaCl on the survival of S. epidermidis heated for varying lengths of time. Cells were heated in PPB at 52 C. Samples were removed at intervals and plated on TSA (**X**) representing viability and TSSA (**O**) to determine salt sensitivity.

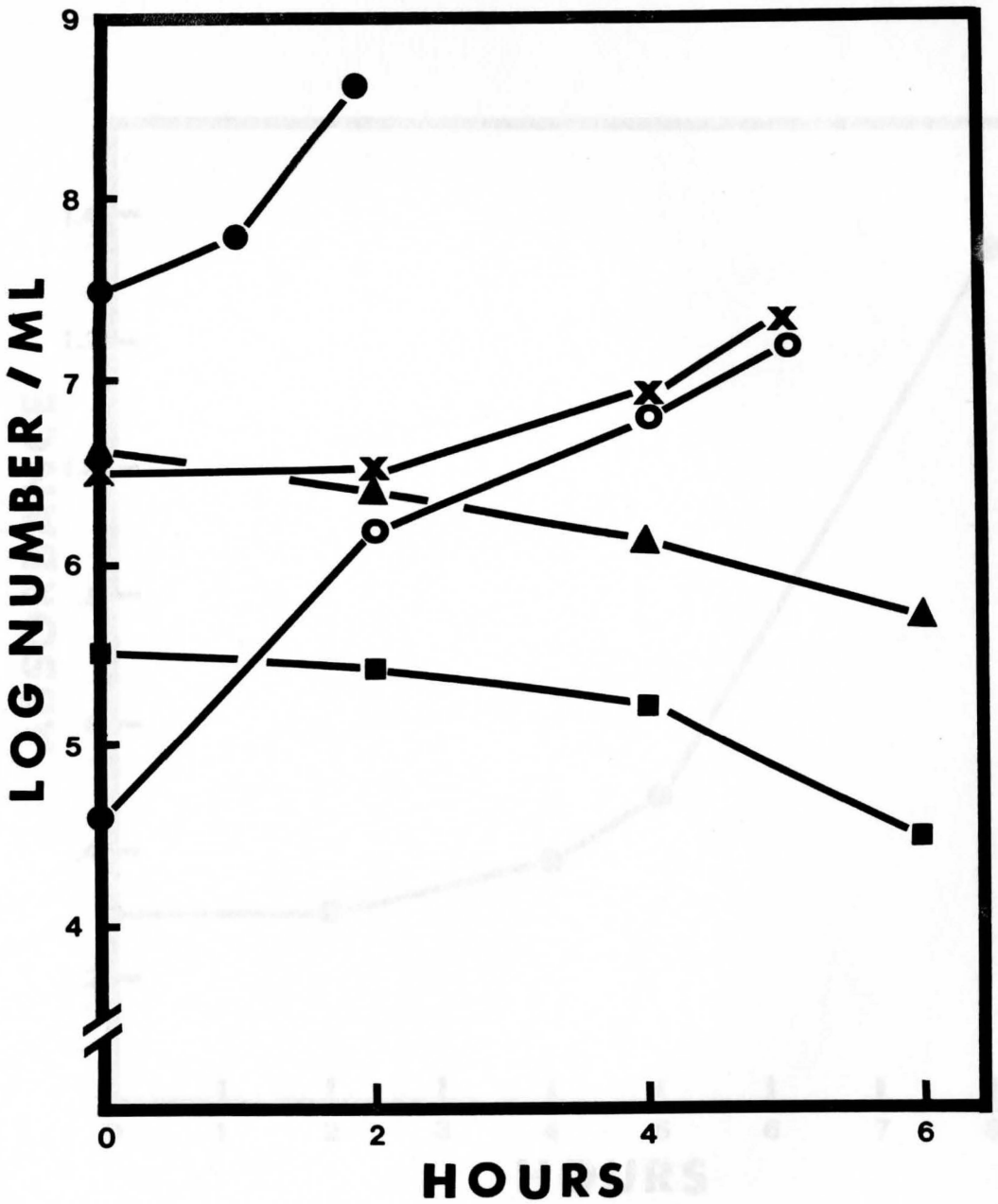

Fig. 2.--Recovery of S. epidermidis after sublethal heat treatment. Thermally injured cells were resuspended in TSB or PPB and incubated at 37 C. Samples were removed at intervals and plated on TSA and TSSA. The unheated control (●) represents average counts on TSA and TSSA. Symbols: x and o , counts on TSA and TSSA, respectively, with TSB as the recovery media; ▲ and ■, counts on TSA and TSSA, respectively, with phosphate buffer as the recovery media.

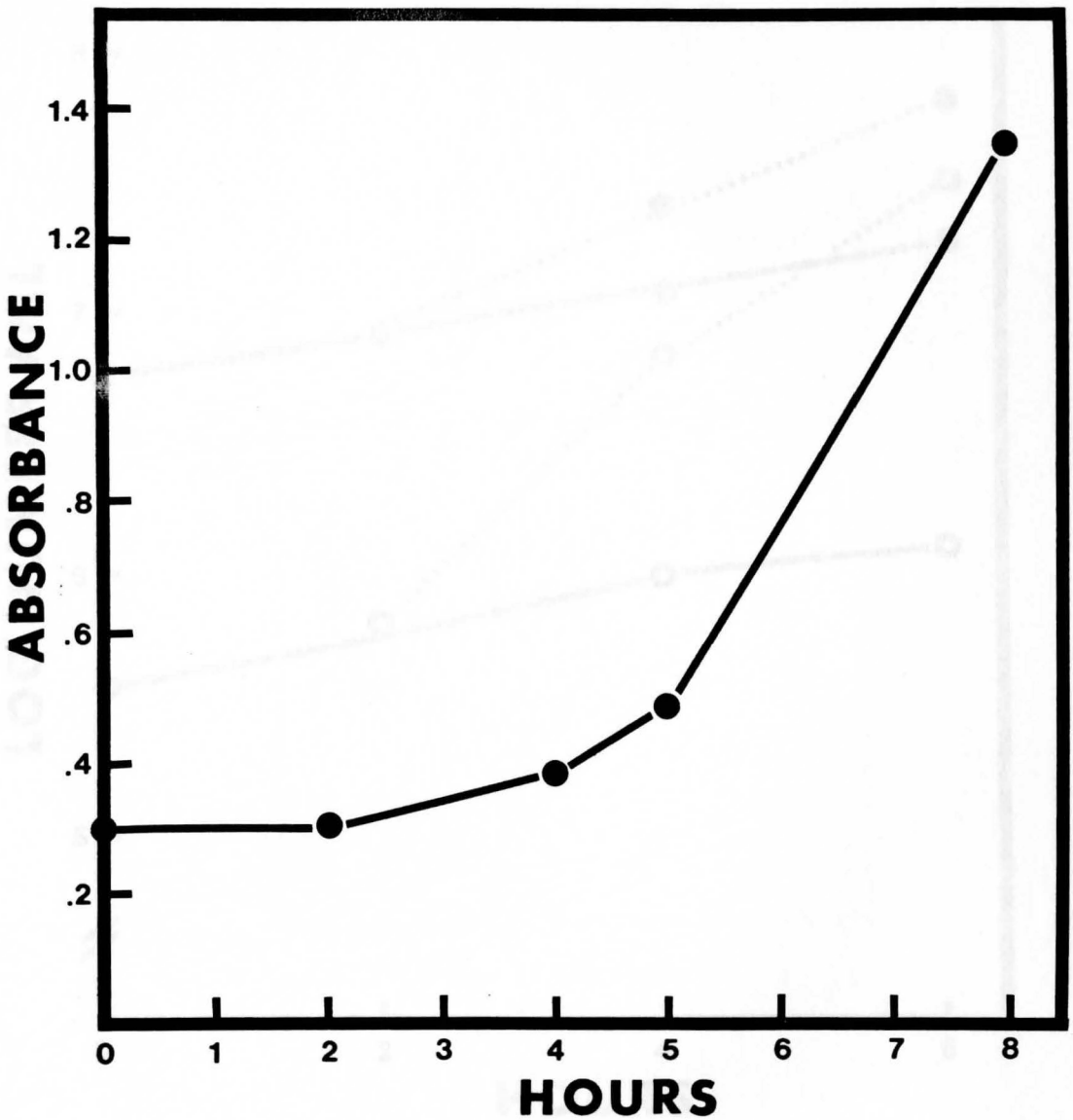

Fig. 3.--Absorbance measurements of S. epidermidis during the recovery period. Heat treated cells were incubated in TSB at 37 C. Samples were removed at intervals and growth was measured spectrophotometrically.

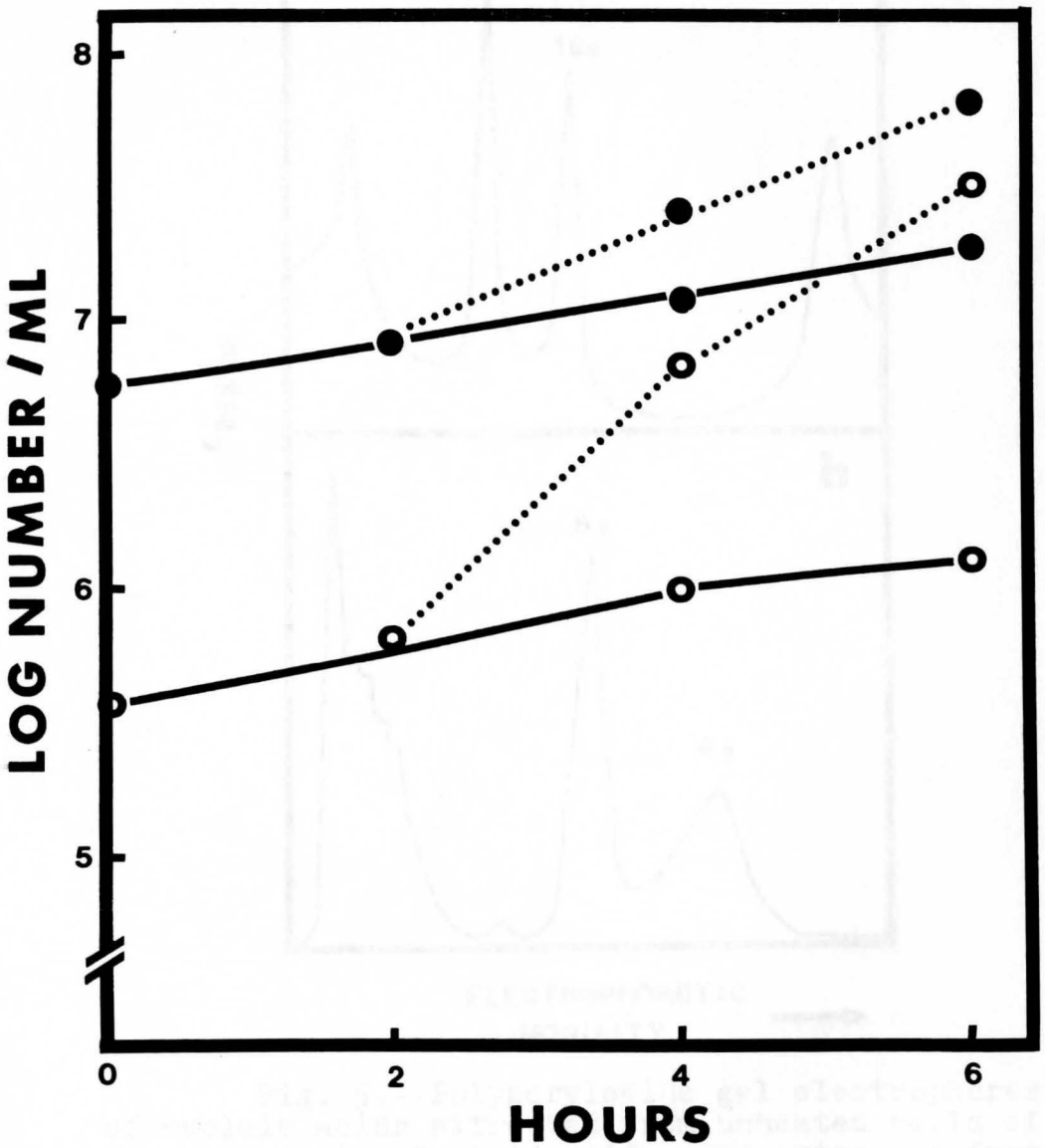

Fig. 4.--Effect of temperature-shifts on the recovery of S. epidermidis. Thermally injured cells were resuspended in TSB and incubated at 20 C. After 2 hr., half of the culture was shifted to 37 C while the other half was maintained at 20 C. Samples were removed at intervals and plated on TSA (●) and TSSA (o). The dotted line represents growth at 37 C.

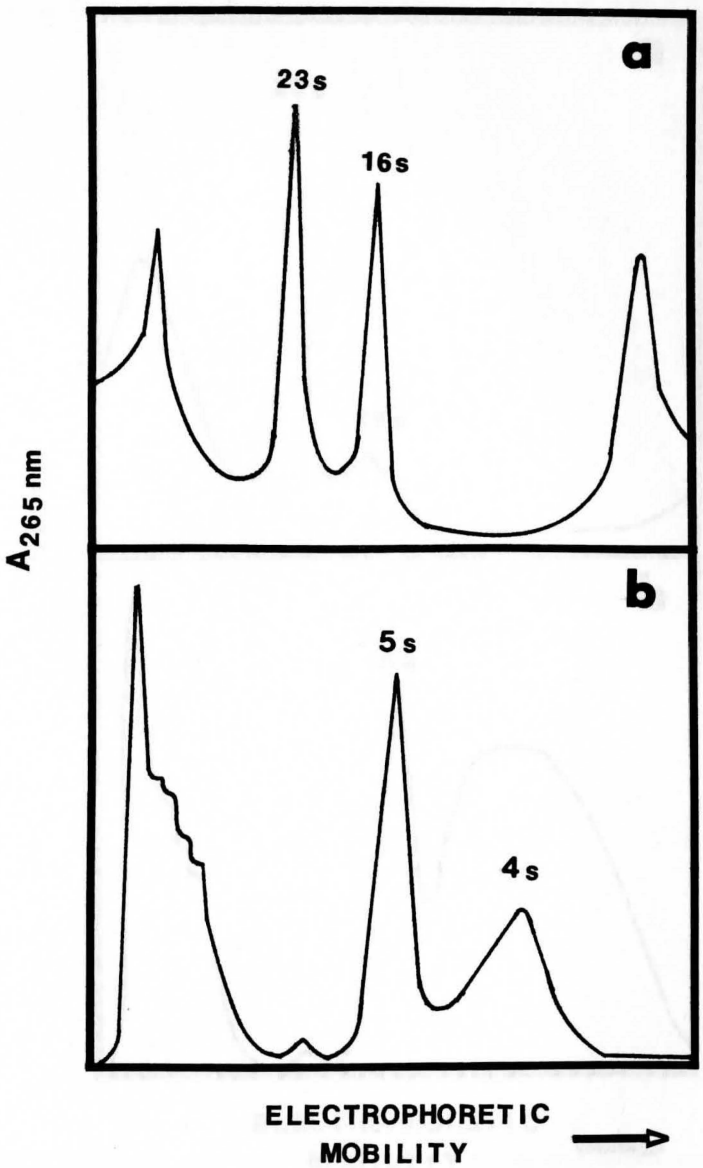

Fig. 5.--Polyacrylamide gel electrophoresis of nucleic acids extracted from unheated cells of S. epidermidis. Nucleic acids were extracted from an overnight culture by a pyrocarbonate-phenol-SLS method and electrophoresed on (a) 2.6% gels and (b) 7.5% gels for 2 hr. at 5 ma/gel.

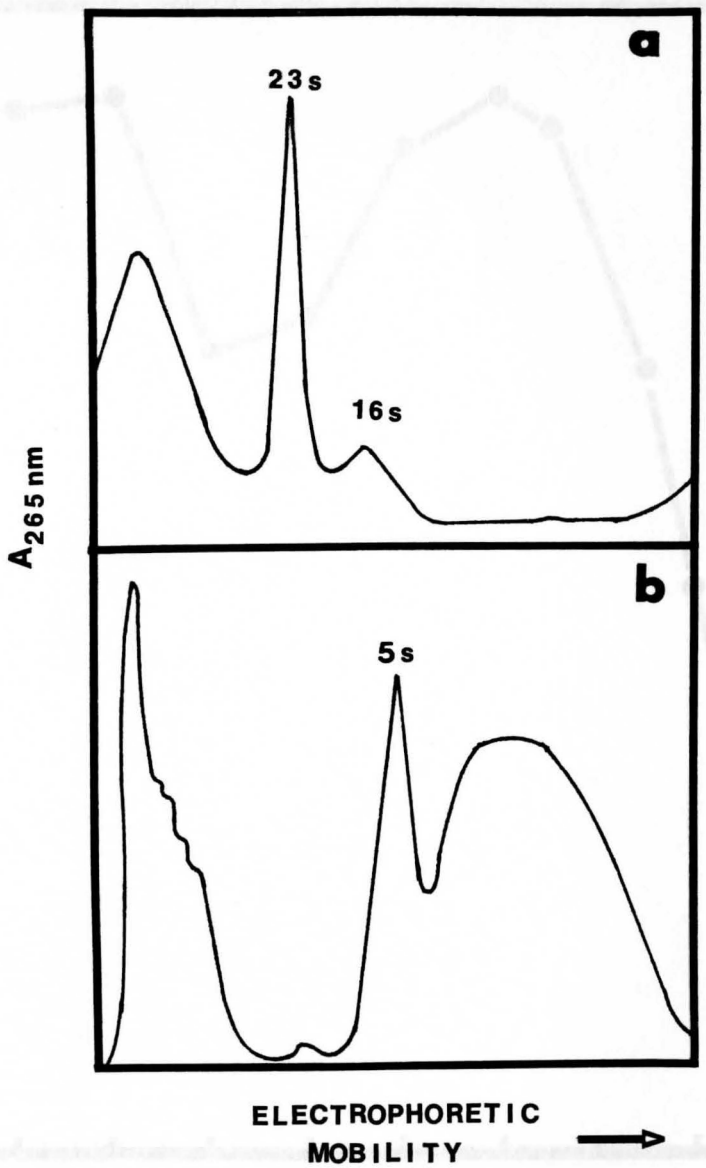

Fig. 6.--Polyacrylamide gel electrophoresis of nucleic acids extracted from thermally injured cells of S. epidermidis. Cells from an overnight culture (200 ml) were thermally injured in 15 ml. PPB at 52 C for 10 min. Nucleic acids were extracted as in Fig. 5 and electrophoresed on (a) 2.6% gels and (b) 7.5% gels for 3 hr. at 5 ma/gel.

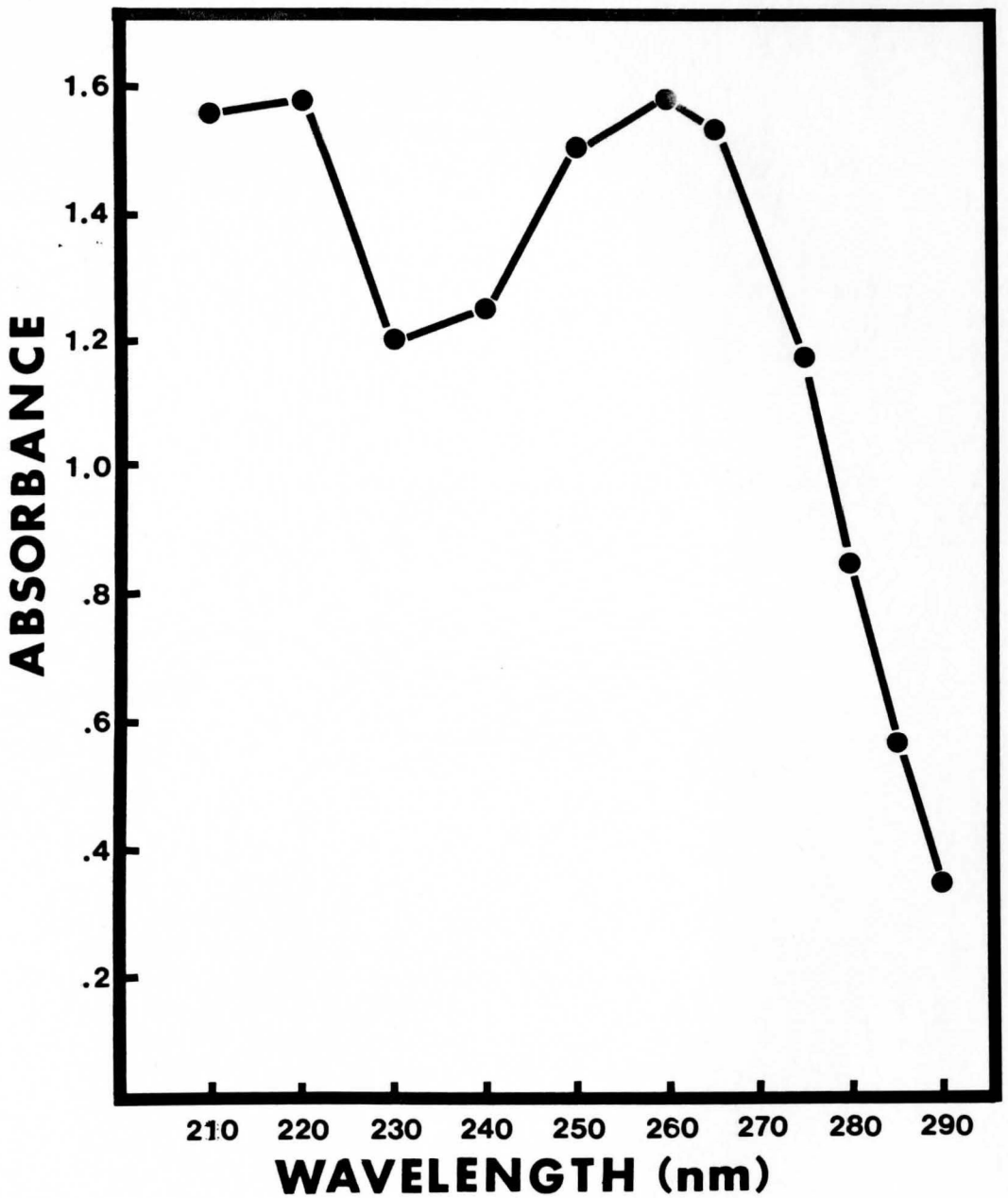

Fig. 7.--Ultraviolet spectrum of the heating menstruum after thermal injury of S. epidermidis. Cells from an overnight culture (200 ml) were heated in 15 ml. PPB at 52 C for 15 min. The media was then clarified by passage through a .45μ millipore filter. The cell-free filtrate was then analyzed spectrophotometrically for 260 nm absorbing material.

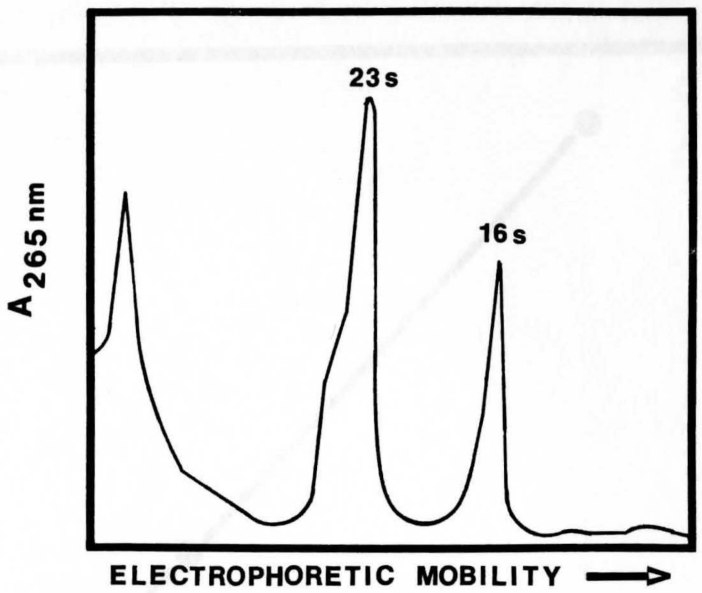
23s
16s
$A_{265\,nm}$
ELECTROPHORETIC MOBILITY

Fig. 8

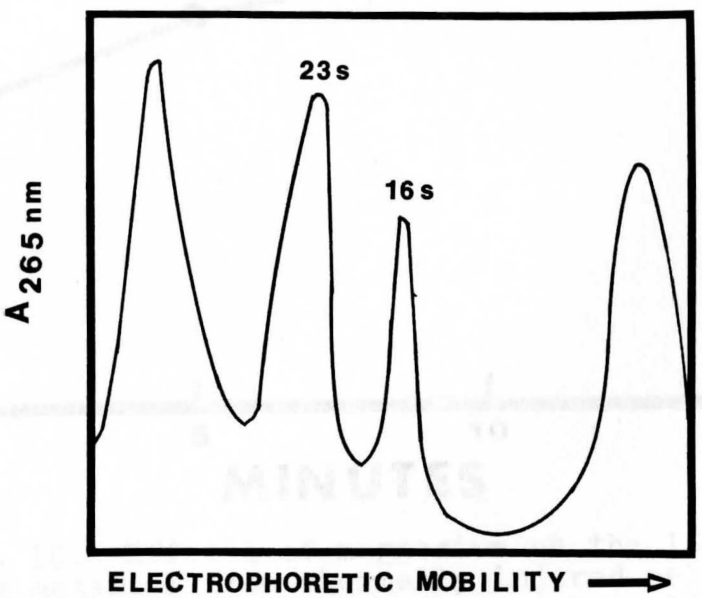
23s
16s
$A_{265\,nm}$
ELECTROPHORETIC MOBILITY

Fig. 9

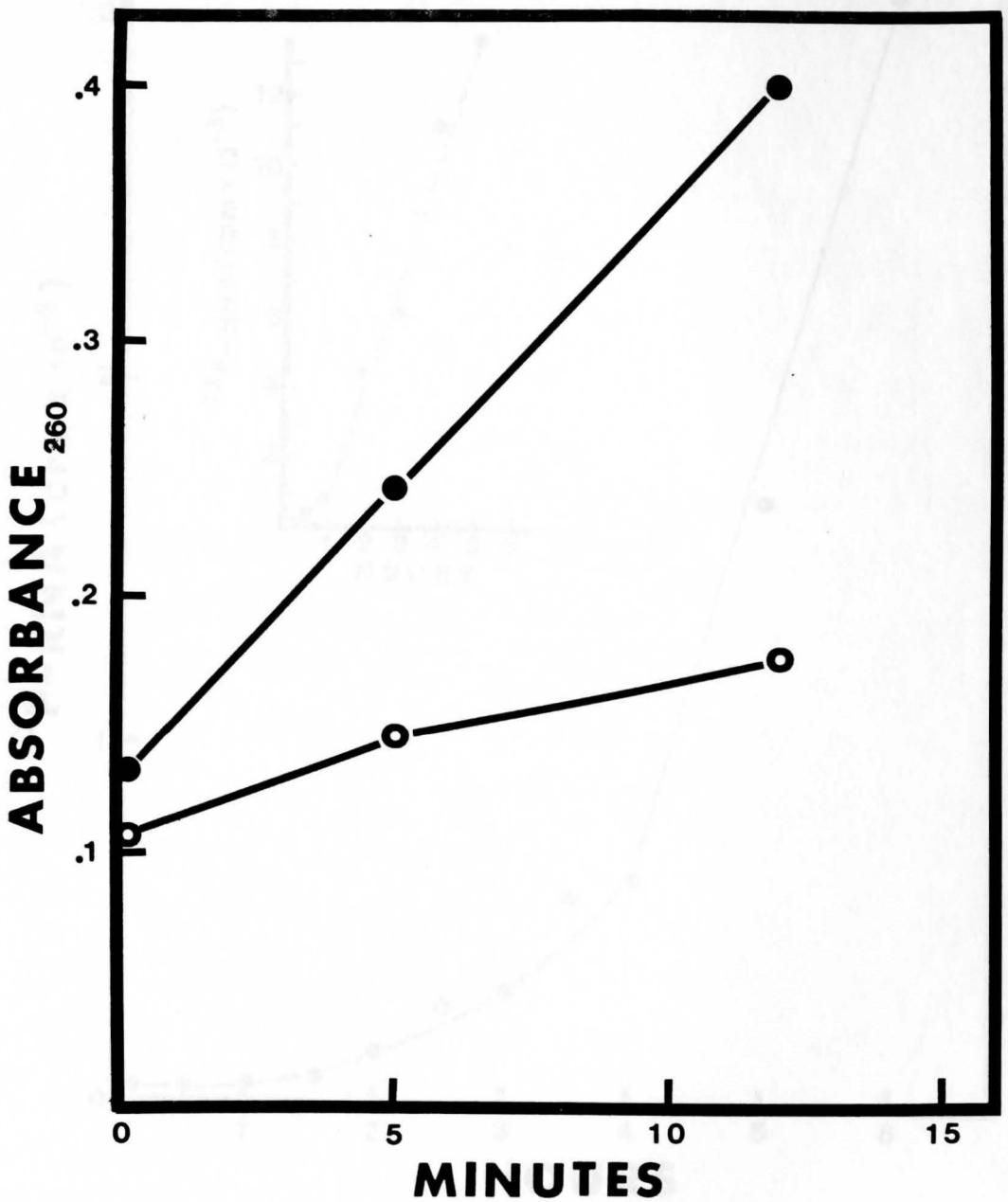

Fig. 10.--Effects of magnesium on the loss of 260-nm absorbing material from thermally injured cells. Cells from an overnight culture (50 ml.) were thermally injured in 50mM Tris buffer (●) or 50mM Tris buffer containing .05M $MgCl_2$ (o). Samples were removed at intervals and filtered. The absorbance of the filtrates was measured at 260 nm.

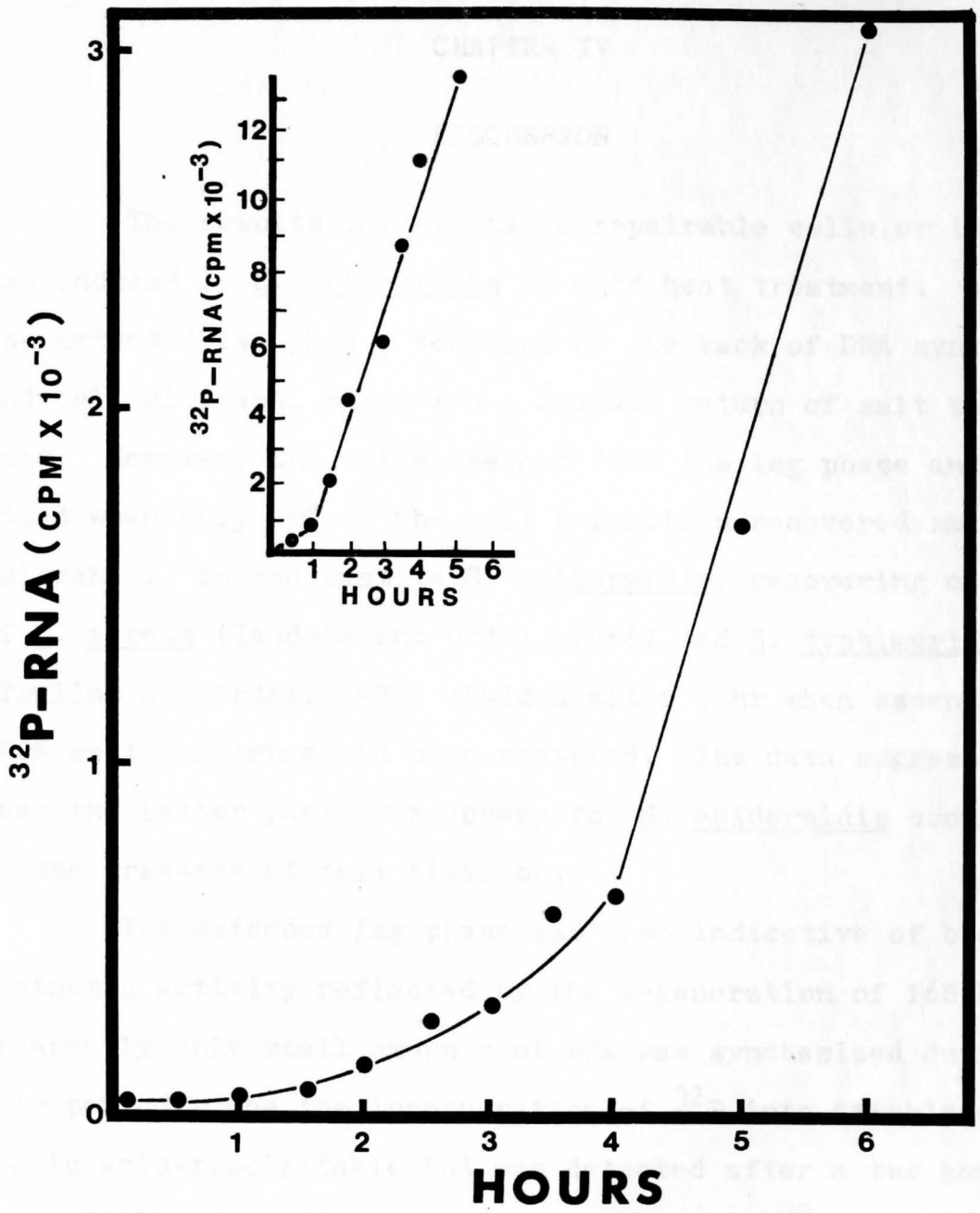

Fig. 11.--Incorporation of ^{32}P into the RNA of thermally injured cells of S. epidermidis during the recovery process. Thermally injured cells were incubated at 37 C in TSB containing 1 μc/ml ^{32}P. Samples were removed at intervals and assayed for radioactivity. Inset: incorporation of ^{32}P into the RNA of unheated control cells.

CHAPTER IV

DISCUSSION

The results reveal that a repairable cellular injury was induced in S. epidermidis by mild heat treatment. During the extended lag phase, verified by the lack of DNA synthesis and cell division, there was a gradual return of salt tolerance. However, the cells emerged from the lag phase and divided when only 45% of the cell population recovered salt tolerance. In contrast to S. epidermidis, recovering cells of S. aureus (Iandolo and Ordal, 1966) and S. typhimurium (Tomlins and Ordal, 1971) divided after 4 hr when essentially 100% salt tolerance had been restored. The data suggests that the latter part of recovery for S. epidermidis occurs in the presence of cell division.

The extended lag phase was also indicative of biosynthetic activity reflected by the regeneration of 16S RNA. Apparently only small amounts of RNA was synthesized during this period since the incorporation of ^{32}P into trichloroacetic acid-precipitable RNA was detected after a two hour lag. Similar results were obtained when the ^{32}P activity in the recovery medium was increased to 5 μc/ml of TSB (data not shown). It is significant to note that unheated control cells incorporated the label into RNA after a 15 min lag. It is apparent that the low levels of RNA synthesis during the first two hours of recovery was directed toward the reconsti-

tution of 16S RNA and was not detectable by labelling with ^{32}P.

The degradation of 16S RNA in thermally injured cells of S. epidermidis did not appear to be the primary cause of increased salt sensitivity. When these cells were allowed to recover in TSB, 55% of the cell population remained sensitive to salt when complete regeneration of 16S RNA had occured. Lee and Goepfert (1975) manipulated the conditions of heat injury in S. typhimurium so that RNA breakdown did not occur, yet significant proportions of the cell population were injured and killed. Complete recovery of salt tolerance in S. epidermidis could therefore depend on the physical annealment of the membrane, restoring control of sodium ion permeation. Consequently, removal of excess sodium from the cells, rather than regeneration of 16S RNA, could be rate limiting in the recovery process of this microorganism.

Degradative metabolism was observed when thermally injured cells of S. epidermidis were incubated at 37 C in phosphate buffer. Similar results were reported for S. aureus which required a medium containing glucose, amino acids, and phosphate for the recovery of salt tolerance (Iandolo and Ordal, 1966 & Erwin and Haight, 1973). Stiles and Witter (1965), on the other hand, reported limited recovery of salt tolerance in S. aureus in the presence of only glucose in 5% concentration. However, these investigators added glucose directly to the heating menstruum which contained considerable amounts of the amino acid pool and other soluble com-

ponents. Consequently, these cells were able to reconcentrate lost cellular components from the heating menstruum and partially recover tolerance to salt. The results reveal that S. epidermidis requires nutrients for the recovery of salt tolerance. Furthermore, the temperature-shift studies reveal that a 37 C environment, reflecting the functioning of synthetic pathways, is also essential for the recovery of this microorganism.

Another essential factor involved in the recovery period is the reconcentration of magnesium ions (Hurst et al. 1975 & Hoover and Gray, 1977). Since the results in this presentation reveal that heat-induced Mg^{2+} loss is responsible for the degradation of 16S RNA, it is essential that magnesium ions are reconcentrated during the recovery period to maintain stability in the regenerated 16S RNA molecule. Magnesium loss from thermally injured cells, resulting in the appearance of 260 nm absorbing material in the heating media, could result from heat-induced loss of lipoteichoic acid since it has been reported that this membrane constituent has importance in Mg^{2+} binding and in providing a controlled cationic environment for cellular constituents in Staphylococcus (Hepinstall, 1970 & Archibald, 1972) and Bacillus (Hughes et al., 1973). The loss of either cell wall teichoic acid or lipoteichoic acid was not observed during sublethal heating of S. aureus (Hurst et al., 1975). However, these investigators observed a 40% to 50% heat-induced loss of polar membrane lipids, which bind Mg^{2+} jointly with

teichoic acids. Since S. epidermidis has a membrane make-up similar to S. aureus (Archibald, 1972), heat-induced loss of phospholipids from S. epidermidis could explain the loss of cellular magnesium in heated cells.

The enhanced ability of thermally injured cells of S. aureus to reconcentrate magnesium during the recovery period was attributed to the heat-induced loss of D-alanine from teichoic acid (Hurst et al., 1975). In the absence of D-alanine, Mg^{2+} is more firmly bound to teichoic acid allowing an enhanced uptake of this ion during the initial stage of recovery. Hurst et al. (1975) reported that there was a decreased binding efficiency of Mg^{2+} as D-alanine was rapidly synthesized and reincorporated into teichoic acids in repaired cells of S. aureus. The heat-induced loss of D-alanine from teichoic acid appears to have a positive effect on recovering cells, manifested by an enhanced uptake of Mg^{2+} resulting in the stability of regenerated 16S RNA. The observed increase in salt sensitivity in thermally injured cells of S. epidermidis could be attributed to adverse effects of the binding of sodium ion to teichoic acids yielding cell death. In recovery, however, Na^{+} must compete with Mg^{2+} and newly synthesized D-alanine. Under these conditions, sodium could merely have inhibitory effects in a system where tolerance to salt is gradually restored.

Loss of 260 nm absorbing material from cells of Staphylococcus aureus is a typical response to thermal injury (Iandolo and Ordal, 1966). When heated cells of

S. epidermidis leaked 260 nm absorbing material, experiments revealed the primary nucleic acid lesion to be 16S rRNA. Polyacrylamide gel electrophoresis of nucleic acids extracted from heated cells also revealed extensive broadening, or degradation, in the sRNA region. Further fractionation of sRNA from this region on 7.5% polyacrylamide gels revealed a normal electrophoretic appearance of the 5S peak whereas the 4S peak showed extensive broadening. Sogin and Ordal (1967) observed an enlarged sRNA peak and the appearance of degradation products of RNA in the void region of MAK columns for thermally injured S. aureus. The results reveal that 5S RNA and 23S RNA are not degraded during thermal injury. It is interesting that the 50S ribosomal subunit, of which the thermostabile 23S RNA and 5S RNA are associated, is more resistant to degradation than the 30S ribosomal subunit which contains the thermolabile 16S RNA.

The broadening of the 4S peak could be attributed to the appearance of degradative products of 16S RNA as low molecular weight polynucleotides in this region, or the degradation of 4S RNA resulting from thermal injury. Kaplan and Apirion (1975) observed that ribosome degradation in stressed E. coli cells is triggered by the action of an endoribonuclease that attacks rRNA yielding degradative products of sizes 4S and smaller. Santer and Santer (1973) reported that when 30S subunits of E. coli were treated with T_1 RNase, products of 120, 66 and 26 nucleotides long were obtained along with smaller molecular weight material. Assuming ribonuclease activity (resulting from Mg^{2+} loss) is ultimately

responsible for the degradation of 16S RNA in thermally injured cells of S. epidermidis, the results found by these investigators support the contention that heat-induced degradation of 16S RNA yields the appearance of degradative products in the 4S region of the gel.

Other nucleases in S. epidermidis could be responsible for further degradation of the initial products of endoribonuclease attack on 16S RNA, accounting for the leakage of 260 nm absorbing material from the cell. Kaplan and Apirion (1974) reported that the products (4S and smaller) of endonuclease attack on rRNA in stressed E. coli were further degraded to acid-soluble material by RNase I, RNase II, and polynucleotide phosphorylase. In E. coli, RNase I is an endonuclease which yields 3'-mono and oligonucleotides (Spahr and Hollingworth, 1961), RNase II is an exonuclease degrading RNA from the 3' end yielding 5'-nucleoside monophosphates (Nossal and Singer, 1968) and polynucleotide phosphorylase is an exonuclease attacking RNA from the 3' end yielding nucleoside diphosphates (Klee and Singer, 1968). Cohen and Kaplan (1977) reported that RNase I and polyribonucleotide phosphorylase participated in the final steps of RNA solubility in starved E. coli cells. Since RNase I of E. coli has been found in the cell wall membrane fraction of S. aureus (Okabayasi and Mizuno, 1974), a similar enzyme (resulting from the heat-induced loss of cellular magnesium) mechanism of RNA degradation in stressed cells of S. epidermidis might account for the appearance of 260 nm absorbing material in

the heating media.

The addition of magnesium to the heating buffer decreases the loss of RNA from heat treated cells of S. epidermidis. Similar results were reported for S. aureus (Hurst and Hughes, 1978). Cells of S. epidermidis thermally injured in the presence of high concentrations of $MgCl_2$ did not reveal degradation of 16S RNA electrophoresed on polyacrylamide gels. Furthermore, the sRNA region in the gel was comparable to the unheated control. These results confirm that the degradative effect of thermal injury on 16S RNA is not due directly to heat per se, but is caused by an extensive heat-induced loss of cellular magnesium. To further support this observation, nucleic acids were extracted from normal cells of S. epidermidis and heated in vitro in magnesium conserving buffer and buffer lacking magnesium for 10 min at 53 C followed by reprecipitation and electrophoresis as described in the Materials and Methods. There was no degradation in either 23S RNA or 16S RNA and the sRNA region appeared normal under both experimental conditions. It is interesting to note that the RNA precipitated from the magnesium buffer was less readily solubilized than the RNA precipitated from the heating buffer lacking magnesium. This can be attributed to the more compact configuration that magnesium induces on rRNA.

The heat induced loss of magnesium could lead to the activation of a ribonuclease responsible for degradation of 16S RNA. The existence of thermostabile nucleases attaining

optimal activity at around 50 C have been reported in stressed E. coli cells (Cohen and Kaplan, 1977). Mg^{++}-inhibited ribonucleases have been reported in S. typhimurium (Datta and Burma, 1972), and E. coli (Weiss and Tal, 1973). Weiss and Tal (1973) observed an in vivo thermal activation of E. coli ribosomes at 53 C. If these cells were incubated in the absence of Mg^{++}, complete degradation of the 30S subunit occurred. The authors attributed these results to a heat-activated ribonuclease. Hurst and Hughes (1978) reported similar results for S. aureus but argued against the action of a nuclease due to the rapid rate of 30S degradation.

Pinder and Gratzer (1972) reported that digestion of E. coli RNA by pancreatic nuclease proceeded more slowly in the presence of Mg^{++}. Removal of Mg^{++} resulted in the release of ribosomal proteins from the RNA moiety, exposing a number of labile points open to ribonuclease attack. A similar mechanism could be responsible for the rapid degradation of the 30S ribosomal subunit observed by Hurst and Hughes (1978) for thermally injured S. aureus. The observed stabilizing effect that Mg^{++} exerts on 16S RNA in thermally injured cells of S. epidermidis could be attributed to stabilized RNA-protein interactions in the ribosome.

The selective degradation of the 16S RNA could also be attributed to the association of a RNase specifically associated with the 30S ribosomal subunit. Santer and

Santer (1973) reported that the most accessible part of the 16S RNA in E. coli, the region near the 3'OH end of the chain, was fairly exposed on the surface of the 30S subunit. Datta and Burma (1972) suggested that this exposed region of 16S RNA was responsible for the binding of RNase 1 to the 30S subunit in S. typhimurium. Santer and Santer (1973) and Rinke et al (1972) found that this RNase-accessible area of 16S RNA in 30S subunits of E. coli represented 100 to 150 nucleotides near the 3'OH end of the chain and were not involved in strong interactions either with protein or other regions of the RNA. Therefore, the broadening of the 4S peak in 7.5% polyacrylamide gels of nucleic acids extracted from heated cells of S. epidermidis could be explained by the excision of a polynucleotide of such length (approximately 100 nucleotides long). When ribosomes of E. coli were loaded with a large excess of RNase in vitro, increased binding of the enzyme to only the 30S particle was observed (New and Heppel, 1964). Although 23S rRNA also has several exposed regions in the 50S subunit (Datta and Burma, 1972) it is unknown why RNase is not found associated with the 50S ribosomal subunit.

The results clearly show that thermal injury of S. epidermidis results in the selective degradation of 16S RNA, which appears in the heating menstruum as 260 nm absorbing material. Since bare ribosomal ribonucleic acid from this organism did not show in vitro degradation at 53 C, destruction of 16S RNA is attributed to the action

of a ribonuclease. Thermal stability of 16S RNA can be induced in this microorganism by loading the heating media with high concentrations of $MgCl_2$. Therefore, 16S RNA degradation in thermally injured cells is the consequence of Mg^{++} loss and not of heating. The exact mechanism of destruction of the 30S subunit and 16S RNA has not yet been elucidated. However, it can be speculated from the results that the heat-induced loss of Mg^{++} from thermally injured cells of S. epidermidis causes the loss of RNA bound ribosomal proteins yielding exposed regions of 16S RNA available to attack by a thermally activated ribonuclease. Furthermore, an exposed region of 16S RNA (approximately 100 nucleotides in length) in the 30S subunit of this microorganism, as observed in E. coli, could account for the appearance of degradative products in the 4S region of polyacrylamide gels.

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