

DEGRADATION OF 16S RNA IN THERMALLY INJURED STAPHYLOCOCCUS EPIDERMIDIS¹ANTHONY G. DiLELLA² and ANTHONY E. SOBOTA, Department of Biology, Youngstown State University, Youngstown, OH 44555

Abstract. Exposure of *Staphylococcus epidermidis* to sublethal temperatures produced a temporary change in the salt tolerance. After sublethal heat treatment at 52 °C for 20 min, 96% of the viable population was unable to reproduce on media containing 7.5% NaCl. The thermal injury results in alterations in the cell membrane allowing leakage of RNA into the heating media. Polyacrylamide gel electrophoresis revealed 16 S ribosomal RNA to be extensively degraded in thermally injured cells, whereas 23 S and 5 S RNA were unaffected. When the thermally injured cells were incubated at 37 °C in Trypticase Soy Broth, 16 S RNA was regenerated accompanied by a return of salt tolerance in the absence of DNA synthesis. In the presence of high concentrations of magnesium, cellular leakage of RNA was suppressed and the degradation of 16 S RNA was not observed in thermally injured cells.

OHIO J. SCI. 80(1): 8, 1980

The response of various microorganisms to heat stress is well documented (Hurst 1977 and Iandolo 1974). Exposure of bacteria, *Staphylococcus aureus* in particular, to thermal stress results in significant macromolecular alterations reflected by degradation of RNA (Iandolo and Ordal 1966 and Sogin and Ordal 1967) and ribosomes (Hurst and Hughes 1978 and Rosenthal *et al* 1972), membrane damage (Hurst *et al* 1975) and changes in enzymatic activity (Pariza and Iandolo 1969). 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). During this incubation, termed recovery, ribosomes and rRNA are regenerated (Iandolo 1974 and Pariza and Iandolo 1969) and membrane damage is repaired (Hurst *et al* 1975).

The purpose of this investigation was to reveal the effects of thermal injury on macromolecular alterations in *Staphylococcus epidermidis*. An attempt was made to suppress degradation and subsequent leakage of RNA from the cell,

since 16S RNA was found to be specifically degraded in thermally injured *S. epidermidis*. This suppression was achieved by the addition of high concentrations of magnesium in the heating buffer. Recently, Hurst and Hughes (1978) reported that ribosomal degradation in thermally injured *S. aureus* does not occur in the presence of magnesium. We suggest that degradation of ribosomes in thermally injured *Staphylococcus* results from instability of 16S RNA resulting from a heat-induced loss of cellular magnesium.

MATERIALS AND METHODS

Staphylococcus epidermidis, a skin isolate, was 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 yielding approximately 8×10^8 viable bacteria ml⁻¹.

Sublethal injury and recovery procedure. Cells from an overnight culture (40 ml) were harvested by centrifugation at 2000 g and washed in 0.1 M sodium phosphate buffer (SPB). The cell pellet was then resuspended in a minimal amount of SPB at 25 °C and then SPB, previously equilibrated at 52 °C, was added to a total volume of 50 ml. At 5 min intervals the cell suspension was swirled for 10 sec and samples (1 ml) were removed and serially diluted in SPB.

After thermal injury the cell suspension was cooled in ice. To determine the effect of ther-

¹Manuscript received 24 August 1978 and in revised form 1 November 1978 (#78-50).

²Present address: Northeastern Ohio Universities College of Medicine, Molecular Pathology Department, Rootstown, Ohio 44272.

mal injury on the recovery process, a 5 ml aliquot of the cooled suspension was centrifuged at 2000 x g and the pellet was resuspended in 50 ml TSB and incubated with shaking at 37 °C. Samples (1 ml) were removed from the recovery medium at intervals and serially diluted in SPB.

All samples were plated in triplicate on TSA and TSA containing 7.5% NaCl (TSSA) using the pour plate technique as previously described (Sobota 1976). 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 percentage of cell numbers on TSSA compared to TSA.

Preparation and Characterization of DNA and RN.A. To assay for leakage of cellular material into the heating medium, thermally injured cells were centrifuged at 2 °C and the supernatant was further clarified by passage through a 0.45 μ filter unit (Nalge, Rochester, New York). The absorbance of the filtrate was then measured at 260 nm in a Coleman 124 Double Beam Spectrophotometer. Samples of the filtrate were also assayed for DNA by the diphenylamine reaction (Burton 1956) and RNA via the orcinol reaction (Lin and Schjeide 1969) after removal of contaminating carbohydrates with Dowex-1 resin as described by Sobota (1972).

Nucleic acids were extracted by a combined pyrocarbonate SLS phenol method (Solymosy *et al* 1968 and Pigott and Midgley 1968). Cells (1 g wet weight) were washed in 50 mM Tris-HCl buffer (pH 7.2) and resuspended in 3 ml of 50 mM Tris HCl buffer (pH 7.6) containing 5 mM MgCl₂ and 3% SLS. Diethyloxidiformate (0.4 ml, Eastman Kodak Co.) was added as a nuclease inhibitor. The cell suspension was ground with 150–200 μ glass beads in a mortar and pestle at room temperature. Beads and cell debris were removed by centrifugation at 15,000 x g (4 °C) and the supernatant was adjusted to 0.4 M NaCl with ice cold 2 M NaCl solution. An equal volume of phenol in 50 mM Tris HCl buffer (pH 7.2) containing 0.1% (w/v) of 8 hydroxyquinoline was added and the solution was stirred 10 min in ice. The aqueous phase was retrieved by centrifugation at 10,000 x g (4 °C) and nucleic acids were precipitated with 2 vol of 95% ethanol (-20 °C), washed with 0.15 M sodium acetate buffer (pH 6.0) containing 0.5% SLS, and reprecipitated with ethanol at -20 °C.

Gel electrophoresis was carried out according to Loening (1967) with the exception that the electrophoresis buffer contained 36 mM Tris, 30 mM monobasic sodium phosphate, 1 mM disodium EDTA, and 0.2% SLS. Nucleic acids were dissolved in electrophoresis buffer containing 5% sucrose and approximately 25–50 μ g were applied per gel. Electrophoresis was carried out at 5 ma per 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 Loebel Poly frac ultraviolet scanner.

To measure the synthesis of RNA during the recovery period, sterile carrier free ³²P-orthophosphate (New England Nuclear, 285 Ci mg⁻¹ of ³²P) was added to the recovery medium at a concentration of 5 μ Ci ml⁻¹. Incorporation of the label into DNA and RNA was determined by the filter paper disk technique of Sobota (1976).

The effect of magnesium on injury. Cells were thermally injured in 50 mM Tris (pH 7.2) or 50 mM Tris (pH 7.2) containing 50 mM MgCl₂ at 52 °C. Samples (3 ml) were removed at intervals, passed through a 0.45 μ Swinnex 25 filter, and the absorbance of the filtrates was then measured at 260 nm.

In assaying the *in vivo* thermostability of 16S rRNA in the presence of magnesium, 0.1 M MgCl₂ was added to the injury buffer [50 mM Tris HCl (pH 7.2)] followed by nucleic acid extraction and electrophoresis as described above.

RESULTS

A decrease in salt tolerance in *S. epidermidis* was found when the heated cells were plated on TSA and TSSA (fig. 1a). The total viable population (TSA counts) remained unchanged after 20 min. of heating to 52 °C, whereas only 4% of the cell population was able to form colonies in the presence of 7.5% NaCl (TSSA counts).

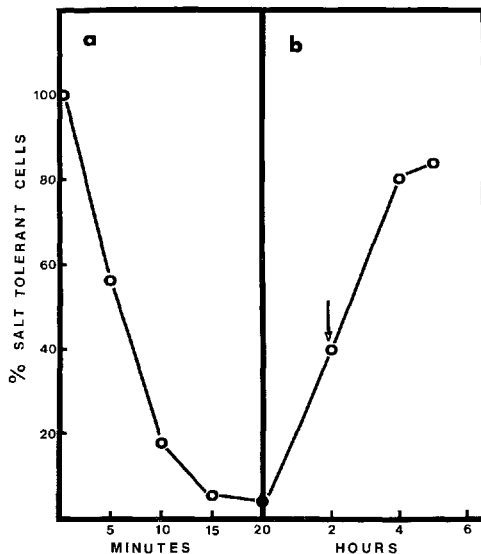


FIGURE 1. Injury-recovery curve for *S. epidermidis*. Cells were heated in SP buffer at 52 °C and then incubated in TSB at 37 °C. Samples were removed at intervals and plated on TSA and TSSA. The arrow indicates the time at which cell division occurred during recovery.

When the thermally injured cells were resuspended in TSB and incubated at 37 °C, there was a gradual return of salt tolerance (fig. 1b). Initially 98% of the cells were incapable of growth on TSSA, but were still viable as revealed by TSA counts. At 4 hr 80% of the cell population recovered its tolerance to salt. The TSA count during the recovery period revealed an extended lag phase, as compared to an unheated control, which lasted until 40% of the cell population was able to grow on salt. At this time (2 hr), recovering cells began to divide. The 2 hr duration of the lag phase was verified by spectrophotometric measurements and radiotracer studies revealing the lack of DNA synthesis.

Electrophoresis of nucleic acids extracted from uninjured cells of *S. epidermidis* on 2.6% polyacrylamide gels showed a normal pattern (fig. 2a). In contrast, nucleic acid preparations from thermally injured cells revealed that the 16S rRNA was extensively degraded and only traces remained (fig. 2b), whereas 23S rRNA was unaffected. Electrophoresis of nucleic acids from heated cells on 7.5% polyacrylamide gels gave a thermostable 5S rRNA accompanied by products of degradation in the 4S region of the gel (fig. 2c, d).

Cell-free filtrates of the heating medium after thermal injury gave a 280:260 nm ratio of 0.55, suggesting that the solution was primarily nucleic acid. The di-

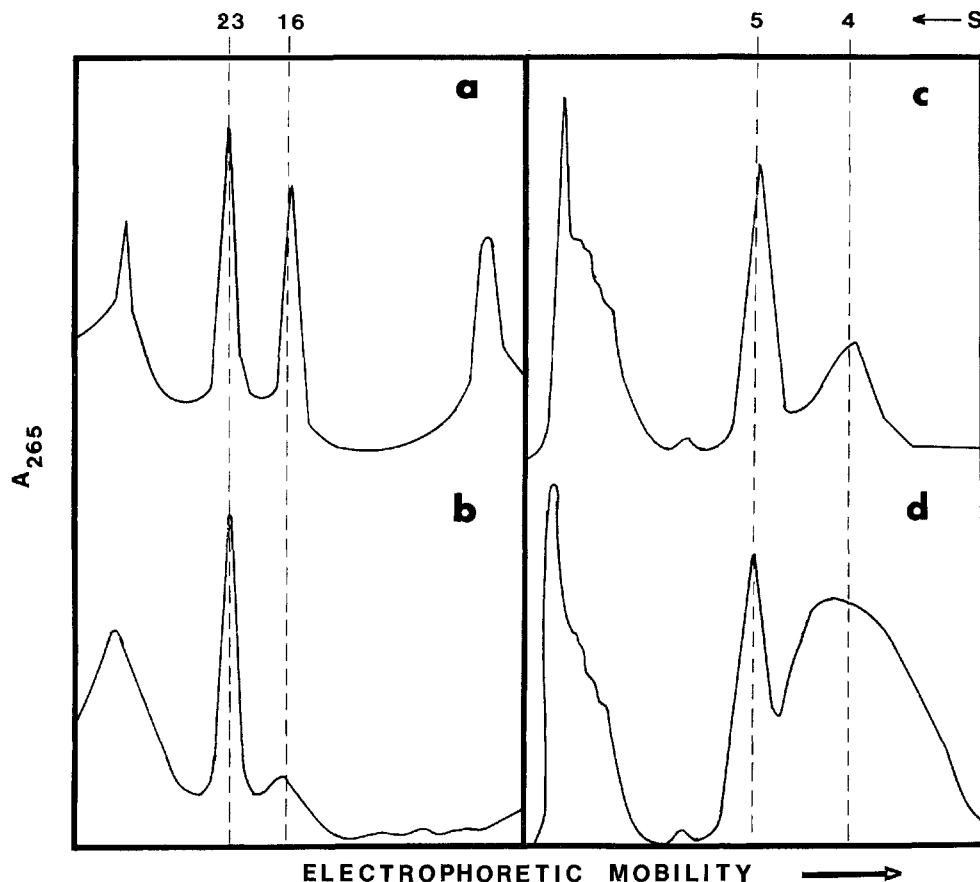


FIGURE 2. Polyacrylamide gel electrophoresis of nucleic acids extracted from *S. epidermidis* where (a) and (c) indicate nucleic acids extracted from unheated cells and electrophoresed on 2.6% and 7.5% polyacrylamide, respectively, and (b) and (d) indicate nucleic acids extracted from heat injured cells and electrophoresed on 2.6% and 7.5% polyacrylamide, respectively. Electrophoresis was carried out at 5 ma per gel for 2 hr.

phenylamine test of a sample from the heating medium was negative; however, the orcinol reaction revealed a 62% increase in the cellular leakage of RNA after 10 min of heating.

Since thermally injured cells of *S. epidermidis* regain their tolerance to salt under proper conditions of incubation, it was of interest to see if 16S RNA was regenerated during the recovery period. Polyacrylamide (2.6%) gel electrophoresis of nucleic acids extracted from injured cells recovering in TSB for 2 hr revealed regeneration of the 16S RNA component, and the electrophoretic pattern was similar to Figure 2a. It is significant to note that 16S RNA regeneration occurred in the absence of cell division.

The release of degradation products of nucleic acids from heated cells was dependent on the composition of the heating medium. Heating cells at 52 °C in buffer lacking Mg^{2+} resulted in the release of more cellular RNA than heating in buffer containing Mg^{2+} (fig. 3). When nucleic acids were extracted from cells

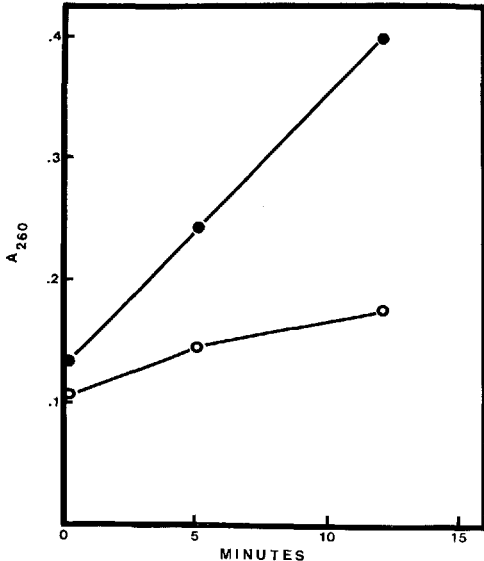


FIGURE 3. Effects of magnesium on the leakage of RNA from thermally injured cells. Cells from an overnight culture (50 ml) were thermally injured in 50 mM Tris HCl buffer (pH 7.2, ●) or 50 mM Tris HCl buffer (pH 7.2) containing 50 mM $MgCl_2$ (○). Samples were removed at intervals and filtered. The absorbance of the filtrates was measured at 260 nm.

heated at 52 °C in Tris buffer (pH 7.2) and subjected to polyacrylamide gel electrophoresis, a profile similar to figure 2b was observed. In contrast, nucleic acid preparations from cells heated at 52 °C in Tris buffer (pH 7.2) containing 0.1 M Mg^{2+} showed no degradation of 16S RNA on polyacrylamide gels (similar to fig. 2a) accompanied by stable soluble RNA (5S and 4S).

Labelling thermally injured cells with ^{32}P during the recovery period revealed incorporation of the label into RNA at significantly low levels during the first two hours as compared to the unheated control. Thereafter, the label was incorporated linearly into RNA with respect to time. It was also found that DNA synthesis was lacking in thermally injured cells during the first two hours of recovery.

DISCUSSION

A repairable cellular injury was induced in *S. epidermidis* by mild heat treatment. Similar results were reported for *S. aureus* by Iandolo and Ordal (1966) and for *S. typhimurium* by Tomlins and Ordal (1971). The extended lag phase, accompanied by approximately 50% return in salt tolerance, was indicative of biosynthetic activity reflected by the regeneration of 16S rRNA. Apparently, only small amounts of RNA were synthesized during this period since the incorporation of ^{32}P into TCA-precipitable RNA was at low levels during the first 2 hr of recovery. Unheated control cells, on the other hand, incorporated the label exponentially into RNA after a 15 min lag. It is apparent from polyacrylamide gel electrophoresis that the low level of RNA synthesis during the first 2 hr of recovery was directed toward the reconstitution of 16S RNA.

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 thermally injured cells were allowed to recover in TSB, 60% of the cell population remained salt sensitive when complete regeneration of 16S RNA had occurred. Similar findings were reported for *S. typhimurium* by Lee and Goepfert (1975). Complete recovery of salt tolerance in *S.*

epidermidis could depend on the physical annealing of the membrane, thereby restoring control of sodium ion permeation. Removal of excess sodium from the cells, rather than regeneration of 16S RNA and 70S ribosomes, could be rate-limiting in the recovery process of this microorganism.

An essential factor involved in the recovery period of *S. aureus* is the re-concentration of magnesium ions (Hurst *et al* 1975 and Hoover and Gray 1977). In *S. epidermidis* we found that heat-induced magnesium loss is responsible for the degradation of 16S RNA and that re-concentration of magnesium ions during the recovery period is essential to maintain the stability of the regenerated 16S RNA molecule. Magnesium loss from thermally injured cells of *Staphylococcus* resulted in the appearance of degraded RNA in the heating media, a result which appears to be due to the loss of some membrane component necessary for providing a controlled cationic environment for cellular constituents. Hurst *et al* (1975) observed a 40–50% heat-induced loss of polar membrane lipids, which bind magnesium jointly with teichoic acid in *S. aureus*. These investigators observed an enhanced ability of thermally injured cells of *S. aureus* to re-concentrate magnesium during recovery attributed to the heat-induced loss of membrane-bound D-alanine in the absence of which magnesium is more firmly bound to teichoic acid. 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, sodium could compete with magnesium and newly synthesized D-alanine in the binding to teichoic acid. 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 *S. aureus* is a typical response to injury (Iandolo and Ordal 1966). When heated cells of *S. epidermidis* leaked 260 nm absorbing material, our experiments showed the primary nucleic acid lesion to be in 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 gave 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*. We demonstrated that 5S RNA and 23S RNA are not degraded during thermal injury. Consequently, the thermostable 23S and 5S RNA can account for the thermostability of the 50S ribosomal subunit whereas degradation of the 30S ribosomal subunit can be attributed to thermolabile 16S RNA.

Ribonuclease activity could be ultimately responsible for the degradation of 16S RNA in thermally injured cells of *S. epidermidis*. The existence of thermostable nucleases attaining optimal activity around 50 °C have been reported in stressed *E. coli* cells (Cohen and Kaplan 1977), and Mg²⁺ inhibited ribonucleases have been reported in *S. typhimurium* (Datta and Burma 1972) and in *E. coli* (Weiss and Tal 1973). Hurst and Hughes (1978), however, argued against the action of a nuclease due to the rapid rate of 30S subunit degradation in thermally injured *S. aureus*.

The specific association of a RNase with the 30S ribosomal subunit could be responsible for the selective degradation of 16S RNA in thermally injured *S. epidermidis*. Datta and Burma (1972) suggested that exposed regions of 16S RNA on the surface of the 30S subunit were responsible for the binding of RNase I in *S. typhimurium*. Although 23S rRNA has several exposed regions in the 50S subunit (Datta and Burma 1972), it is not known why RNase is not found associated with the 50S ribosomal subunit.

Our results show that thermal injury in *S. epidermidis* results in the selective degradation of 16S RNA. *In vivo* thermal stability of 16S RNA can be induced in this microorganism by loading the heating media with high concentrations of MgCl₂. The exact mechanism of destruction of the 30S subunit and 16S RNA has not yet been elucidated, but

heat-induced magnesium loss could result in the release of ribosomal proteins from the RNA moiety, exposing a number of labile points open to ribonuclease attack.

LITERATURE CITED

- Burton, K. 1956 A study of the conditions and mechanism of the diphenylamine reaction for the colorimetric estimation of deoxyribonucleic acid. *Biochem. J.* 62: 315-322.
- Cohen, L. and R. Kaplan 1977 Accumulation of nucleotides by starved *Escherichia coli* cells as a probe for the involvement of ribonucleases in ribonucleic acid degradation. *J. Bacteriol.* 129: 651-657.
- Datta, A. K. and D. P. Burma 1972 Association of ribonuclease I with ribosomes and their subunits. *J. Biol. Chem.* 247: 6795-6801.
- Hoover, D. G. and R. J. H. Gray 1977 Function of cell wall teichoic acid in thermally injured *Staphylococcus aureus*. *J. Bacteriol.* 131: 477-485.
- Hurst, A. and A. Hughes 1978 Stability of ribosomes of *Staphylococcus aureus* S6 sublethally heated in different buffers. *J. Bacteriol.* 133: 564-568.
- 1977 Bacterial injury: a review. *Canadian J. Microbiol.* 23: 936-944.
- , A. Hughes, M. Duckworth and J. Bad-diley 1975 Loss of D-Alanine during sublethal heating of *Staphylococcus aureus* S6 and magnesium binding during repair. *J. Gen. Microbiol.* 89: 277-284.
- Iandolo, J. J. 1974 Repair of stress induced macromolecular alterations in *S. aureus*. *Ann. New York Academy of Sciences* 236: 160-174.
- and Z. J. Ordal 1966 Repair of thermal injury of *Staphylococcus aureus*. *J. Bacteriol.* 91: 134-142.
- Lee, A. C. and J. M. Goepfert 1975 Influence of selected solutes on thermally induced death and injury in *Salmonella typhimurium*. *J. Milk Food Technol.* 38: 195-200.
- Lin, R. I. S. and O. A. Schjeide 1969 Micro-estimation of RNA by the cupric ion catalyzed orcinol reaction. *Anal. Biochem.* 27: 473-483.
- Loening, U. E. 1967 The Fractionation of high molecular weight ribonucleic acid by polyacrylamide gel electrophoresis. *Biochem. J.* 102: 251-257.
- Pariza, M. W. and J. J. Iandolo 1969 Coagulase production by injured *Staphylococcus aureus* MP-31 during recovery. *Appl. Microbiol.* 17: 836-838.
- Pigott, G. H. and J. E. M. Midgley 1968 Characterization of rapidly labelled ribonucleic acid in *Escherichia coli* by deoxyribonucleic acid ribonucleic acid hybridization. *Biochem. J.* 110: 251-263.
- Rosenthal, L. J., S. E. Martin, M. W. Pariza J. J. Iandolo. 1972 Ribosome synthesis in thermally shocked cells of *Staphylococcus aureus*. *J. Bacteriol.* 109: 243-249.
- Sobota, A. E. 1976 An adaptation of the filter paper disk technique for the differential estimation of DNA and RNA of rat liver. *Anal. Biochem.* 75: 478-483.
- 1976 A method for eliminating the contribution of *Agrobacterium tumefaciens* to studies of nucleic acid biosynthesis in crown gall tumors. *Plant Physiol.* 57: 415-419.
- 1972 Ribonucleic acid synthesis associated with a developmental change in the gametophyte of *Pteridium aquilinum*. *Plant Physiol.* 49: 914-918.
- Sogin, S. J. Z. J. Ordal 1967 Regeneration of ribosomes and ribosomal ribonucleic acid during repair of thermal injury to *Staphylococcus aureus*. *J. Bacteriol.* 94: 1082-1087.
- Solymosy, F., I. Fedorcsak, A. Gulyas, G. L. Forkas and L. Ehrenberg. 1968 A new method based on the use of diethyl pyrocarbonate as a nuclease inhibitor for the extraction of undegraded nucleic acid from plant tissues. *Eur. J. Biochem.* 5: 520-527.
- Tomlins, R. I. and Z. J. Ordal 1971 Precursor ribosomal ribonucleic acid and ribosome accumulation *In Vivo* during the recovery of *Salomella typhimurium* from thermal injury. *J. Bacteriol.* 107: 134-142.
- Weiss, A. and M. Tal 1973 *In Vivo* thermal stability and activation of *Escherichia coli* ribosomes. *Biochemistry* 12: 4534-4540.