Effects of Thermal Stress on Viability and Ribonucleic Acid of \textit{Aerobacter aerogenes} in Aqueous Suspension

BY R. E. STRANGE AND M. SHON

Microbiological Research Establishment, Porton, Wiltshire

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SUMMARY

The death-rate of washed \textit{Aerobacter aerogenes} in aqueous suspension at 47$^\circ$ depended on the nature of the growth medium, the composition of the liquid used to wash and resuspend the bacteria, the bacterial growth phase, the bacterial concentration in heated suspensions, the pH value, the oxygen tension and the composition of the diluent in which bacteria were heated. The relative resistance of bacteria in different growth phases differed 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. High bacterial concentration favoured survival at 47$^\circ$ under most conditions; cell-free filtrate from a heated dense suspension ($10^{10}$ bacteria/ml.) protected a sparser population of fresh bacteria ($10^2-10^6$/ml.) heated in it. Protective material in filtrate was heat-stable (100$^\circ$ for 15 min.) and diffused through cellophan. The optimum pH value for survival at 47$^\circ$ was near pH 6.5. Aerobic conditions favoured survival in distilled water but not in salt solutions or phosphate saline (pH 6-5). The effects of various concentrations of NaCl and KCl on the survival of bacteria at 47$^\circ$ under aerobic conditions were different, K$^+$ concentrations above 0.1M 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. Growth medium, Mg$^{2+}$ (0.01-5 mM) and, to a lesser extent, Mn$^{2+}$ (0.5 mM) or Co$^{2+}$ (5 mM) decreased the death-rate, whereas ethylenediamine tetraacetic acid (mM), or various sugars, increased it. Mg$^{2+}$ but not Mn$^{2+}$ reversed the lethal effect of sugars.

Generally, conditions which accelerated the death-rate of \textit{Aerobacter aerogenes} at 47$^\circ$ also increased the rate of degradation of endogenous RNA. This was accompanied by an increase in the ultraviolet absorption of cold acid-extracts of bacteria and of the suspending fluid. Bacterial protein was degraded to a smaller extent. Depletion of RNA is probably not the primary cause of death at 47$^\circ$ but the effect on bacterial metabolism of a rapid increase in endogenous pool constituents resulting from RNA degradation may contribute to the lethal effect.

INTRODUCTION

The general topic of the heat-inactivation of micro-organisms in aqueous suspension has been reviewed by Rahn (1945) and Wood (1956). Mechanisms proposed for heat destruction of bacteria include coagulation of protein, inactivation of enzymes, disruption of cellular lipids and damage to the genetic apparatus. Chick (1910) proposed protein denaturation as the cause of death during heating. Edwards & Rettger (1987) observed excellent agreement between the maximum growth
temperature of several bacilli and the minimum temperature at which some of their respiratory enzymes were inactivated. However, Rahn & Schroeder (1941) showed that under conditions where 99% of a bacterial population was killed by heat, only 14% of the peroxidase and 20% of the catalase had been inactivated; they concluded that enzyme coagulation was not the cause of death. Califano (1952) also considered that the lethal effect of high temperature on bacteria is not due to protein denaturation. He demonstrated that exposure to heat caused a separation of ribonucleic acid in soluble form from the bacteria into the suspending fluid; the temperature at which the process was initiated depended on the species of bacteria and was related to the temperature of inactivation. Wood (1956) suggested that it is possible to explain the lethal effects of high temperature on various microorganisms by assuming that heat exerts detrimental effects on the reproductive system of these organisms; but that in yeast, at least a portion of the heat damage is probably due to cytoplasmic injury. The present paper records a study of the effect of comparatively mild thermal stress on the viability and ribonucleic acid of *Aerobacter aerogenes* in aqueous suspension and includes some data obtained with *Serratia marcescens*.

**METHODS**

**Organisms.** *Aerobacter aerogenes* (NCTC 418) and a laboratory strain of *Serratia marcescens* were used.

**Media and cultural conditions.** Organisms were grown at 37° in shaken flasks (2 l.) containing 100 ml. of either the carbon-limiting mannitol + ammonia + salts medium previously described (Strange, Dark & Ness, 1961) or Douglas’s digest (TM) broth (Medical Research Council, 1931) seeded with 4 ml. of a fully grown culture (18–20 hr) in the same medium. ‘Exponential phase’ bacteria were harvested after 3 hr when 40–50% of the maximum growth had occurred. In experiments concerned with the effect of growth phase on thermal resistance, bacteria were grown in a batch culture vessel essentially as described by Elsworth, Meakin, Pirt & Capell (1956) at a continuously maintained pH value of 7.2–7.4 with adequate aeration (Strange *et al.* 1961).

**Viability determinations.** Viable counts were made using 3 or 5 Douglas’s digest broth (TM) agar plates for each determination as previously described (Strange *et al.* 1961). Direct determinations of the % viable bacteria in suspensions were made with dark ground illumination after slide culture on the rich medium (containing glycerol, ammonia, salts, Difco yeast extract, Difco casamino acids, meat digest broth and set with agar) described by Postgate, Crumpton & Hunter (1961).

**Heat treatment of bacterial suspensions.** Bacteria were separated from the culture by centrifugation, washed once or twice and resuspended to a suitable concentration in diluent. Rapid heating of bacterial suspensions was achieved by dilution (1/10–1/50) in warm diluent held in a temperature-controlled bath at 47° (± 0.05°). Washed air or nitrogen was bubbled by means of a Pasteur pipette through diluents before and after adding suspension, to obtain aerobic or anaerobic conditions. The diluents used were: distilled water; phosphate saline which contained NaCl (0.11 m) and appropriate concentrations of K₂HPO₄ + KH₂PO₄ (0.02 m·PO₄; pH 6.5); sodium phosphate (0.0875 m) buffers containing appropriate volumes of Na₂HPO₄ (5.8 g./l.) and NaH₂PO₄, 2H₂O (5.8 g./l.) solutions to give the required pH value; others are
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mentioned in Results. All diluents and diluents + additives were filtered through well-washed sterile Oxoid membrane-filter (grade A.P., Oxo Ltd.) before use.

**Materials.** Water used for media and phosphate saline was passed twice through a mixed-bed ion-exchange resin (Amberlite MB-1; from British Drug Houses, Ltd.) column before use. Water used for washing bacteria and as a diluent during heating was glass-distilled. Whenever possible, Analytical Reagent grade chemicals were used. Dehydrated firefly tails were obtained from L. Light and Co. Ltd.

**Analytical methods.** ‘Filtrate’ from a bacterial suspension (equiv. 0.5-2.5 mg. dry wt./ml.) was obtained by filtration of a heated sample through a well-washed and dried membrane filter. In some experiments, cold acid-soluble substances were then extracted from the bacteria on the filter with chilled 0.25 N-HClO₄ (10-15 ml.) and after 30 min. the extract was sucked through under reduced pressure. When assays of nucleic acids were required, bacteria separated from suspension (5 ml.) by centrifugation were extracted with 0.25 N-HClO₄ (5 ml.) for 30 min. at 0° and recovered by centrifugation; nucleic acids were then dissolved by two extractions with 0.5 N-HClO₄ for 15 min. at 70°. RNA in the combined hot acid extracts was determined with the Bial reaction (Morse & Carter, 1949) with yeast RNA as standard and DNA by Burton’s (1956) modification of the diphenylamine reaction, with thymus DNA as standard. Ninhydrin-reacting substances in filtrate from bacterial suspension were determined by the colorimetric method of Yemm & Cocking (1955) with alanine as standard. The ATP-content of cold acid extracts of bacteria was determined by a firefly luminescence technique (Strehler & Totter, 1952) as described previously (Strange & Dark, 1962); before assay, extracts were neutralized with KOH and, after standing for several hr at 0°, precipitated KClO₄ was separated by centrifugation. Magnesium was determined colorimetrically with Titan Yellow as described by Gardner (1946) except that preliminary treatment of samples with trichloroacetic acid was omitted; it is generally accepted that this method lacks precision so the results obtained were usually checked by titrating samples with ethylenediamine tetraacetic acid (EDTA) using Eriochrome Black T as indicator (Debney, 1952). Potassium was precipitated with sodium cobaltinitrite and determined colorimetrically (Barry & Rowland, 1958). Bacterial protein and dry weight were determined as previously described (Strange et al. 1961). Ultra-violet absorption (u.v.) was measured in a Unicam quartz spectrophotometer, model S.P. 500, with a 1 cm. light path.

**RESULTS**

**Determination of the viability of heat stressed bacteria**

Heating bacteria at sublethal temperatures extends the lag phase during subsequent growth on nutrient medium (Hershey, 1939). On TM agar, the colonies produced by heat-treated Aerobacter aerogenes usually varied greatly in size among themselves and all were smaller than colonies from untreated bacteria incubated for the same period. Colony counts did not change significantly after incubation of the plates for more than 48 hr. When viability was determined by slide culture, the growth lag period of bacteria was decreased by using a supplemented agar medium (Postgate et al. 1961), and the incubation period was extended as long as possible without allowing overgrowth of dead organisms by colonies from organisms.
with shorter lag periods. Under these conditions the % viability results were usually close to those obtained by viable counts on TM agar plates (Fig. 2). In experiments reported below, % viability results obtained by only one of the methods are given but all experiments were repeated and results were always confirmed using the other method.

Factors affecting the survival of bacteria at 47°

Preparation of bacterial suspensions. Since certain constituents in culture medium, including Mg²⁺ and sugars (see below), markedly affected the death-rate of bacteria during heating at 47°, bacteria were separated by centrifugation from cultures, then washed and resuspended in diluent before examination of their heat sensitivity. However, the composition of the liquid used to wash bacteria affected their subsequent survival in a given diluent at 47° (Fig. 1). Thus, bacteria washed in distilled water were more resistant than similar bacteria washed in phosphate saline. Further experiments showed that washing bacteria with solutions of NaCl or KCl (0.05–0.15M) decreased their thermal resistance.

Addition of magnesium ions (0.5 mM) to diluents in which bacteria were heated markedly decreased the death-rate and under these conditions survival was little affected by washing in either distilled water or salt solutions. The possibility that salt solutions displaced magnesium from *Aerobacter aerogenes* was examined as follows. A suspension of bacteria (2 × 10¹⁰/ml.) was washed twice with water by

Fig. 1. Effect of washing procedure on the thermal resistance of exponential phase *Aerobacter aerogenes*. Bacteria washed with water (●) or phosphate saline (○) were resuspended in water (about 2.5 × 10⁹/ml.). Samples of washed suspensions (0.2 ml.) were added to 4.8 ml. each of water (a) and phosphate saline (b) aerated at 47°. Viabilities of the four suspensions were determined at intervals by plate counts.

Fig. 2. Effect of growth phase on the thermal resistance of *Aerobacter aerogenes*. Bacterial growth in a defined medium was measured turbidimetrically (∆) and by viable counts (○). At intervals bacteria centrifuged from the culture were washed and resuspended at about 10⁹/ml. in water. Washed suspension (0.1 ml.) was added to phosphate saline (4.9 ml.) at 47° and viability was determined after 0.5 hr. by plate counts (▲) and slide culture (○).
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centrifugation. The resulting pellet was resuspended to the initial concentration in 
MgSO₄ solution (100 µg. Mg²⁺/ml.) and allowed to stand for 15 min. at 20°. The 
bacteria were separated by centrifugation and freed from unadsorbed magnesium 
by washing until the supernatant fluid gave a negative test with the Titan Yellow 
reagent (Gardner, 1946). Five samples (8 ml.) of the magnesium-treated suspension 
(about 10¹⁰ bacteria/ml.) were centrifuged and the pellets resuspended in 5 ml. of 
water, phosphate saline or 1/4, 1/2 and 3/4 dilutions of phosphate saline and water. 
After centrifuging, analyses of the supernatant fluids showed the Mg²⁺ concentra-
tions to be 0·2, 7·4, 6·4, 7·2 and 7·5 µg./ml., respectively. Thus, Mg²⁺ was desorbed 
from A. aerogenes by neat or diluted phosphate saline.

Table 1. Effect of sodium and potassium on magnesium adsorption 
by Aerobacter aerogenes

Organisms harvested from defined medium were washed twice with water and re-
suspended in water (8 x 10¹⁰/ml.). Bacterial pellets centrifuged from 8 samples (1 ml.) 
were resuspended in water (2·5 ml.) and in 82, 8·2 and 0·82 M-KCl or NaCl solutions 
(2·5 ml.). After 15 min. at 20°, 0·82 M-MgSO₄ (2·5 ml.) was added to each suspension. 
After a further 15 min., supernatant fluid was separated from each suspension and 
assayed for Mg²⁺.

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<th>Concentration of Na⁺ or K⁺ (mm)</th>
<th>Concentration of Mg²⁺ (mm)</th>
<th>Ratio Na⁺ or K⁺:Mg²⁺</th>
<th>Mg²⁺ adsorbed (%)</th>
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</table>

Competitive effects of Na⁺ or K⁺ on Mg²⁺ adsorption by Aerobacter aerogenes 
were demonstrated by exposing samples of a washed suspension to a constant 
contentration of Mg²⁺ in the presence of increasing concentrations of Na⁺ or K⁺. 
The results (Table 1) show that the amount of Mg²⁺ adsorbed remained fairly 
constant as the Na⁺ or K⁺ to Mg²⁺ ratio was increased to 10, but at ratios of 10 to 100 there was almost a complete suppression of Mg²⁺ adsorption.

Bacterial growth phase. Several reports (Sherman & Albus, 1928, 1924; Elliker & 
Frazier, 1938; White, 1951, 1953; Lemcke & White, 1959) indicate that the heat 
resistance of bacteria varies with growth phase ('age') of the culture and that 
bacteria in the early exponential phase are the least resistant. It was therefore 
surprising to find that, when grown in a carbon-limiting defined medium and 
washed with distilled water, stationary phase Aerobacter aerogenes were considerably 
less heat-resistant than exponential phase organisms (Fig. 2); but when grown in 
TM broth and tested under similar conditions, stationary phase organisms were the 
more resistant (Fig. 3). Bacteria separated from defined medium cultures by 
centrifugation and examined unwashed or washed with phosphate saline, showed 
least resistance to heating in phosphate saline when in the exponential growth 
phase. Also, bacterial density during heating was important and, irrespective 
of preliminary washing with distilled water or salt solutions, stationary phase
populations of above $4 \times 10^9$ bacteria/ml. survived as well as or better than similar populations of exponential phase organisms.

**Bacterial concentration.** As found with *Escherichia coli* by Sherman & Albus (1928) and Watkins & Winslow (1932), the death-rate of a heated suspension of *Aerobacter aerogenes* was lower the higher the initial bacterial concentration (Fig. 4). This phenomenon occurred under aerobic or anaerobic conditions and was probably mainly due to the protective effect of material leaking from heated bacteria. Cell-free filtrate separated from a heated (30 min./47°C) suspension containing about $10^{10}$ bacteria/ml. phosphate saline protected a sparser population of fresh bacteria.

![Fig. 3](image1.png)

**Fig. 3.** Comparison of the thermal resistance of exponential and stationary phase *Aerobacter aerogenes* grown in defined and complex media. Bacteria were prepared and heated as in Fig. 2. Viabilities of heated suspensions were determined at intervals by slide culture. Exponential phase bacteria from defined medium (△) and TM broth (○); stationary phase bacteria from defined medium (△) and TM broth (○).

![Fig. 4](image2.png)

**Fig. 4.** Effect of bacterial concentration on the thermal resistance of *Aerobacter aerogenes*. Exponential phase bacteria from a defined medium were washed and resuspended at $7 \times 10^{10}$/ml. in phosphate saline. One series of three serial 1/10 dilutions of washed suspension in phosphate saline at 47°C was aerated (a), the other had N_2 passed through (b). Viabilities by plate counts of suspensions initially containing $7 \times 10^9$ (○), $7 \times 10^8$ (△) and $7 \times 10^7$ (□) viable bacteria/ml.

(about $10^8$/ml.) heated in it: viabilities after 10, 20 and 30 min. at 47°C were 98, 93 and 86%, respectively; in filtrate, compared with 87, 15 and 12%, respectively, for a similar bacterial suspension heated in phosphate saline alone. Nelson (1943) found that bacteria subjected to heat at partially lethal values were more demanding in their growth requirements than unheated control organisms. It was possible that leakage products which appeared to exert their influence on bacteria during heating may have in fact acted after heating, by supplying substrates to moribund bacteria so that they were able to grow on nutrient agar. This was shown not to be so by adding leakage products to heated bacteria just before slide culture; the viability of the heated suspension was unaffected by added leakage products.
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Leakage products from heated bacteria consisted of a complex mixture of substances (see below) and the factor(s) responsible for the protective effect has not been identified. Protective material diffused through cellophan on dialysis against water or phosphate saline (Fig. 5) and its activity was unaffected by heating at 100° for 15 min. Protection by filtrates of heated bacterial suspension was markedly decreased but not entirely eliminated by ethylenediamine tetraacetic acid (2 mM-EDTA).

Diluents in which bacteria were heated. Exponential phase bacteria grown in the defined medium were used for these experiments.

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Fig. 5. Effect of dialysable and non-dialysable leakage products from heated bacteria on the thermal resistance of Aerobacter aerogenes. Lyophilized leakage products (60 mg.) from filtrate of an aerated, heated (47°/1 hr.) suspension (equiv. 1·3 g. dry wt. bacteria/125 ml. H₂O) were dialysed against phosphate saline (6 × 5 ml.) at 2°. Diffusates were combined and the sac contents diluted with phosphate saline to 30 ml. Viabilities by plate counts of suspensions (about 10⁶ exponential phase bacteria/ml.) at 47° in phosphate saline (○), diffusate + phosphate saline (1 + 1, by vol.) (□), sac contents + phosphate saline (1 + 1, by vol.) (▲), diffusate + sac contents (1 + 1, by vol.) (△) and undialysed leakage products (1 mg./ml. phosphate saline) (●).

Fig. 6. Effect of various concentrations of KCl, NaCl and equimolar NaCl + KCl on the thermal resistance of Aerobacter aerogenes. Exponential phase bacteria grown in a defined medium were washed and resuspended at 2·5 × 10⁸/ml. in H₂O. Washed suspension (0·2 ml.) was added to diluent (4·8 ml.) at 47°. Viability after 1 hr in KCl solutions (plate counts; ○), equimolar NaCl + KCl solutions (plate counts; □) and NaCl solutions (slide culture; △).

(a) pH value of diluent. As found with Escherichia coli by Jordan & Jacobs (1948) and with Streptococcus faecalis by White (1968), the death-rate of Aerobacter aerogenes subjected to heat was lowest in slightly acid solutions. After 1 hr at 47° in aerated sodium phosphate buffer solutions (0·0875 M), viabilities of suspensions
(5 × 10^7 bacteria/ml.) at pH values of 4.5 (NaH₂PO₄ alone), 5.6, 5.8, 6.25, 6.6, 6.8, 7.0, 7.3, 7.7 and 9.0 (Na₂HPO₄ alone) were 84, 85, 86, 90, 95, 85, 81, 65, < 1 and < 1 %, respectively.

(b) Oxygen tension of diluent. The effect of air or nitrogen on the death-rate of bacteria during heating depended on the diluent and on bacterial concentration. In phosphate saline (pH 6.5) the death-rate was accelerated by aeration when suspensions contained up to 10⁹ bacteria/ml., but above this bacterial concentration air or nitrogen had less effect (Fig. 4). In distilled water aeration decreased the death-rate (Fig. 11).

(c) Concentration of salt in diluent. The effects of various concentrations of NaCl, KCl and an equimolar mixture of NaCl and KCl on the thermal resistance of Aerobacter aerogenes in aerated suspension at 47°C are shown in Fig. 6. At a concentration of 0.1–0.2 M, K⁺ or K⁺+Na⁺ was much more lethal than Na⁺ alone. The lethality of Na⁺+K⁺ (0.1–0.15 M) increased with the relative concentration of K⁺ under aerobic conditions: viabilities after 1 hr. at 47°C of suspensions (10⁹ bacteria/ml.) in aerated 0.15 M- NaCl, 0.1 M- NaCl + 0.05 M- KCl, 0.05 M- NaCl + 0.01 M- KCl and 0.15 M- KCl were 73, 18, 2 and 15 %, respectively. Mg⁰⁺ and/or anaerobic conditions markedly decreased the death-rate of bacteria during heating in otherwise lethal concentrations of K⁺, the concentration of Mg⁰⁺ required for maximum protection increasing with the K⁺ concentration. As a diluent, aerated phosphate saline (pH 6.5; Na:K = 4:1) at 47°C was more lethal to bacteria than distilled water or 0.15 M-NaCl, but replacement of the potassium buffer salts with equivalent sodium salts decreased the death-rate to that which occurred in 0.15 M-NaCl.

(d) Trace metal ions in diluent. The concentration of Mg⁰⁺ required to provide maximum protection to bacteria during heating depended not only on the other salts present but also on the bacterial density (Fig. 7). Mg⁰⁺ was taken up by bacteria suspended in water or phosphate saline at 47°C (Fig. 8) and, in phosphate saline, absorption rather than adsorption probably accounted for this since desorption of the metal ion occurred from bacteria suspended in phosphate saline at 20°C (Fig. 8). The rate of Mg⁰⁺-uptake in phosphate saline at 47°C was much slower than that which occurred in water at 20°C or 47°C. Determination of the small amount of Mg⁰⁺ taken up by bacteria in phosphate saline at 47°C (3–4 μg./10¹⁰ bacteria/ml.) necessitated the use of relatively high bacterial concentrations. Under these conditions, the leakage products available to the bacteria may have affected Mg⁰⁺-uptake by supplying energy sources and phosphate. Rothstein, Hayes, Jennings & Hooper (1958) showed that Mg⁰⁺ can be absorbed by yeast cells provided that glucose is available and that phosphate is absorbed. Mn⁰⁺ (0.25–0.5 mM), Ca⁰⁺ and Co⁰⁺ (5 mM) also protected bacteria during heating in aerated phosphate saline but to a much smaller extent than Mg⁰⁺. Above concentrations of 0.5 mM, Mn⁰⁺ was toxic at 47°C. A mixture of Mg⁰⁺, Co⁰⁺, Ca⁰⁺ (each mM) and Mn⁰⁺ (0.25 mM) provided more protection than Mg⁰⁺ alone.

(e) Other substances in diluent. Both TM broth and the defined medium substantially protected bacteria during heating at 47°C. Viabilities of bacteria washed with phosphate saline and heated for 30 min. at 47°C in phosphate saline, the defined medium, TM broth and distilled water (10⁹ bacteria/ml.) were < 1, 81, 71 and 25 %, respectively. The protective effect of TM broth was decreased by EDTA (2 mM); for example, when 10⁹ bacteria/ml. were heated at 47°C for 10, 20, 30, and 45 min.
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viabilities were 84, 56, 38 and 30%, respectively, in TM broth + EDTA compared with 96, 94, 98 and 88%, respectively, in TM broth alone. Addition of EDTA (0.25–2 mM) markedly increased the death-rate of bacteria in distilled water at 47°: after 10 min. viability was < 1% in the presence and 54% in the absence of EDTA. In phosphate saline at 47° EDTA increased the death-rate slightly. 8-Hydroxyquinoline (0.5 mM) somewhat improved the survival of bacteria in phosphate saline but had little effect in distilled water.

Glucose, mannitol, lactose or ribose (10 mM) markedly increased the death-rate of bacteria in phosphate saline at 47°: viabilities after 10, 15 and 30 min. at 47° of

![Fig. 7](image1)

**Fig. 7.** Effect of Mg²⁺ on the thermal resistance of *Aerobacter aerogenes* in dilute and dense bacterial suspensions. Exponential phase bacteria grown in a defined medium were washed and resuspended at about 10⁹/ml. in phosphate saline. Dilutions (1/10 and 1/1000) of washed suspension were made in phosphate saline + MgSO₄ (0–2 mM), at 47°. Viability (slide culture) after 80 min. of suspension contained about 10⁹ (Δ) and 10³ bacteria/ml. (○).

![Fig. 8](image2)

**Fig. 8.** Mg²⁺-uptake by *Aerobacter aerogenes* at 47°. Exponential phase bacteria grown in a defined medium and washed with phosphate saline were: (1) washed and resuspended in H₂O; (2) washed and resuspended in phosphate saline (1 and 2 equiv. 50 mg. bacterial dry wt./ml.). Suspension 1 (5 ml.) added to aerated H₂O + 0.96 mM-Mg²⁺ (20 ml.) at 20° (○) and 47° (Δ); suspension 2 (5 ml.) added to aerated phosphate saline + 0.96 mM-Mg²⁺ (20 ml.) at 20° (●) and 47° (▲). Supernatant fluids from samples removed at intervals from the four suspensions were assayed for Mg²⁺.

bacteria washed with phosphate saline were 68, 22 and 1%, respectively, in phosphate saline + ribose (10 mM); 81, 60 and 24%, respectively, in phosphate saline alone. Mg²⁺ (mm) reversed the lethal effect of sugars including glucose (Fig. 9) but Mn²⁺ was ineffective in this respect.

*Degradation of RNA and protein in bacterial suspensions at 47°*

Catabolism of RNA and protein which occurs in aerated suspensions of *Aerobacter aerogenes* starved at or below the growth temperature (Strange *et al.* 1961; Postgate & Hunter, 1962; Strange, Wade & Ness, 1963) also occurred at 47° but at a faster
rate. For example, the losses of RNA and protein from bacteria washed with and heated in aerated phosphate saline (pH 6.5) for 1 hr. at 47° were 24 and 5%, respectively, of the initial quantities. No significant change in the concentration of DNA was detected during this period (Table 2). Products released into the suspending fluid included u.v.-absorbing substances, ammonia and other ninhydrin-reacting substances, and traces of magnesium and calcium. The absorption maximum of filtrates from heated suspensions was near 255 mμ, indicating the presence of a high proportion of the deaminated base hypoxanthine; the concentration of pentose-reacting substances was low in proportion to the total u.v.-absorption. At least 80% of the total u.v.-absorbing components of the exudate diffused through Table 2. Effect of Mg²⁺ on viability and on degradation of RNA and protein in an aerated suspension of Aerobacter aerogenes at 47°

Exponential phase bacteria harvested from a defined medium and twice washed with phosphate saline were resuspended (equiv. 2.3 mg. bacterial dry wt./ml.) in phosphate saline and phosphate saline + MgSO₄ (mm). Suspensions were aerated at 47° and samples removed at intervals for analyses and viability determinations. Results refer to a suspension initially containing equiv. 1 mg. bacterial dry wt./ml.

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<th>20</th>
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<tr>
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Bacteria in phosphate saline + Mg²⁺ (mm)

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<td>E₂₆₅ of filtrate</td>
<td>0</td>
<td>0.15</td>
<td>0.25</td>
<td>0.36</td>
<td>0.62</td>
</tr>
<tr>
<td>E₁₄₅ of cold acid bacterial extract</td>
<td>0.32</td>
<td>0.40</td>
<td>0.44</td>
<td>0.43</td>
<td>0.43</td>
</tr>
<tr>
<td>Protein (μg./ml.)</td>
<td>605</td>
<td>607</td>
<td>618</td>
<td>630</td>
<td>630</td>
</tr>
<tr>
<td>Ninhydrin-reacting substances as alanine in filtrate (μg./ml.)</td>
<td>2.4</td>
<td>5.2</td>
<td>6.5</td>
<td>8.1</td>
<td>10.1</td>
</tr>
<tr>
<td>NH₃-N in filtrate (μg./ml.)</td>
<td>0</td>
<td>0.4</td>
<td>0.7</td>
<td>0.9</td>
<td>1.5</td>
</tr>
<tr>
<td>DNA (μg./ml.)</td>
<td>24.6</td>
<td>24.4</td>
<td>25.0</td>
<td>24.8</td>
<td>24.8</td>
</tr>
</tbody>
</table>

cellophan during dialysis against phosphate saline or distilled water. Potassium and inorganic phosphate also leaked from bacteria during heating; this was demonstrated by heating bacteria in aerated water when, after 1 hr at 47°, the released material (equiv. about 4% initial bacterial dry weight) contained potassium (10%) and inorganic phosphate (15%). Although u.v.-absorbing fragments from degraded RNA were released rapidly, cold acid-soluble u.v.-absorbing substances present within the bacteria initially increased and then decreased only after a substantial decrease of viability had occurred (Table 2; Fig. 10). ATP was among the
Thermal resistance of \textit{A. aerogenes}

in intracellular u.v.-absorbing substances which initially increased during heating; values for the ATP-content of neutralized acid-extracts of samples of water-washed bacteria after 0, 10, 20, 30 and 45 min. at 47° in aerated phosphate saline (8.8 mg. dry wt. bacteria/ml.) were 1.5, 2.7, 2.1, 2.0 and 1.5 μg./mg. bacterial dry wt., respectively; viability of the suspension did not change significantly (99–97%).

During heating of bacterial suspensions there was an initial period of zero or small viability loss during which RNA degradation proceeded at the maximum observed rate. Under many conditions, the rate of RNA degradation was related to the survival characteristics of a population as is shown in the following examples. The suspensions initially contained $1 \times 10^9$ viable exponential phase bacteria/ml and

![Fig. 9](image)

**Fig. 9.** Effect of glucose on viability and RNA of \textit{Aerobacter aerogenes} at 47°. Exponential phase bacteria grown in a defined medium were washed and resuspended at $1 \times 10^9$ in H$_2$O. Washed suspension (3 ml.) was added to aerated diluent (27 ml.) at 47°. Viability determinations, open symbols; $E_{255}$, value of filtrate (equiv. 1 mg. dry wt. bacteria/ml.), closed symbols. Diluents: phosphate saline (○); phosphate saline + 10 mM glucose (△, △); phosphate saline + 10 mM glucose + 1 mM-MgSO$_4$ (□, □).

![Fig. 10](image)

**Fig. 10.** Effect of thermal stress on viability and RNA of \textit{Aerobacter aerogenes} before and after starvation. Exponential phase bacteria grown in a defined medium were washed and resuspended in phosphate saline (equiv. 6.9 mg. bacterial dry wt./ml.). (a) Unstarved bacteria: washed suspension diluted 1/10 in aerated phosphate saline at 47°; (b) starved bacteria: washed suspension diluted 1/10 in aerated diluent at 37°; after 19 hr. bacteria (95% viable) were recovered by centrifugation and diluted in aerated diluent at 47° to give the same no. bacteria/ml. as in (a). Viability of slide culture (○); $E_{260}$, value of filtrate (●); $E_{260}$, value of cold acid-extract of bacteria (△). Absorption values equiv. 1 mg. bacterial dry wt./ml.

quantitative results refer to a suspension initially equiv. 1 mg. dry wt. bacteria/ml.

(1) When bacteria washed in water and phosphate saline were heated in aerated phosphate saline for 45 min. at 47°, viabilities were 90 and 14% and $E_{260}$ values for filtrates were 0.79 and 1.35, respectively. (2) In aerated phosphate saline, Mg$^{2+}$ decreased the death-rate, the loss of RNA and also the loss of protein at 47° (Table 2). (3) In aerated phosphate saline, glucose increased and glucose + Mg$^{2+}$ decreased the death-rate and loss of RNA at 47° (Fig. 9). (4) When water-washed bacteria were heated in aerated 0.1 M-, 0.15 M-, 0.2 M- and 0.3 M-KCl for 1 hr,
viabilities were 93, 77, 35 and 24%, respectively, and $E_{450}^{1cm}$ values for filtrates
were 1·1, 1·4, 1·9 and 1·9, respectively. (5) When water-washed bacteria were
heated in aerated phosphate saline and phosphate saline + EDTA (mm) for 80 min.,
viabilities were 90 and 77·5%, respectively, and $E_{450}^{1cm}$ values for filtrates were
0·59 and 0·74, respectively. (6) During heating of phosphate saline-washed bacteria
in phosphate saline viabilities after 10, 20 and 80 min. with vigorous aeration were
58, 4 and 2%, respectively, and $E_{450}^{1cm}$ values for filtrates were 0·8, 1·5 and 2·2,
respectively; under anaerobic conditions (nitrogen) viabilities were 92, 52 and 26%,
respectively, and $E_{450}^{1cm}$ values for filtrates were 0·5, 0·9 and 1·4, respectively.

![Graph](image)

**Fig. 11.** Effect of air and nitrogen on the thermal resistance of *Aerobacter aerogenes* in
H$_2$O at 47°. Exponential phase bacteria grown in defined medium were washed and
resuspended in H$_2$O (equiv. 5·6 mg. bacterial dry wt./ml.). Samples of washed suspension
diluted 1/10 in H$_2$O at 47° gassed with air (O, O) and nitrogen (Δ, Δ). Viability by
plate counts, open symbols; $E_{450}^{1cm}$ values of filtrate (equiv. 1 mg. dry wt. bacteria/ml.),
closed symbols.

The close relationship thus shown to exist between the loss of viability and the
loss of RNA suggested that the lethal effect of heating at 47° on bacteria might be
due to loss of, or irreversible damage to, RNA. If this were true, then decrease of
the RNA-content of bacteria before heating should decrease or have no effect on
their thermal resistance. This was investigated as follows. The death-rate and
RNA degradation was measured in a sample of a washed suspension of bacteria
(about 10$^9$/ml.) in phosphate saline at 47° with aeration; a second sample of sus-
pension was held at 37° with aeration for 19 hr., the bacteria separated by centrifuga-
tion and then treated like those in the first sample. Despite the fact that the starved
bacteria had lost 38% of their RNA, they were still 95% viable and their thermal
resistance was much greater than that of unstarved bacteria (Fig. 10). During
heating, both increase in endogenous u.v.-absorbing substances and leakage was
much less in starved than in unstarved bacteria (Fig. 10).

An exception to the rule that an increased death-rate was associated with in-
creased degradation of RNA was provided by heating bacteria in distilled water
under aerobic and anaerobic conditions. Anaerobiosis increased the death-rate but
reduced RNA degradation (Fig. 11).

The effects of thermal stress on *Serratia marcescens* were similar to those found
Thermal resistance of A. aerogenes

with Aerobacter aerogenes. The death-rate of exponential phase S. marcescens at 47°C was lower in distilled water than in phosphate saline, and Mg²⁺ decreased the death-rate in both diluents, but particularly in the latter. Filtrate from a dense suspension of S. marcescens (about 10¹⁰ bacteria/ml) protected fresh bacteria (about 10⁹/ml) heated in it. When A. aerogenes and S. marcescens harvested in the exponential phase from TM broth were washed and heated under similar conditions at 47°C, losses of viability and leakage occurred as indicated in Table 3. The loss of viability and the leakage were both greater in the case of S. marcescens.

Table 3. Comparison of the effects of heat stress on exponential phase Aerobacter aerogenes and Serratia marcescens

Organisms grown in digest broth, washed with phosphate saline by centrifugation and resuspended in phosphate saline (equiv. 0.5 mg. dry wt. bacteria/ml.) were aerated at 47°C. Samples were removed at intervals for analyses and viability determinations. Results refer to a suspension equiv. 1 mg. dry wt. bacteria/ml.

<table>
<thead>
<tr>
<th>Analytical results</th>
<th>A. aerogenes</th>
<th>S. marcescens</th>
</tr>
</thead>
<tbody>
<tr>
<td>Sampling time (min.)</td>
<td>0</td>
<td>10</td>
</tr>
<tr>
<td>Viability (%)</td>
<td>100</td>
<td>—</td>
</tr>
<tr>
<td>E₅₅₀ of filtrate</td>
<td>0</td>
<td>0.8</td>
</tr>
<tr>
<td>Ninhydrin-reacting substances as alanine in filtrate (µg./ml.)</td>
<td>4</td>
<td>11</td>
</tr>
</tbody>
</table>

DISCUSSION

Bacterial resistance to heat may alter as a result of environmental changes imposed immediately before thermal stress is applied. One effect of washing Aerobacter aerogenes in salt solutions was to desorb magnesium and this may explain the faster death-rate of bacteria at 47°C after such treatment compared with bacteria washed in distilled water. Addition of magnesium to the diluent in which bacteria were heated largely eliminated the differences in thermal resistance resulting from the pre-washing treatment. Although stationary phase populations are generally regarded as being more resistant than exponential phase populations to stresses including heat (see review of Winslow & Walker, 1939), this was not true with A. aerogenes under all conditions. The influence of the growth phase on thermal resistance depended on several factors but particularly on the pre-washing solution and on bacterial concentration during heating. Thus, when grown in a chemically defined medium, washed in distilled water and heated at a concentration of about 10⁸ bacteria/ml. phosphate saline, exponential phase bacteria were much more resistant than stationary phase bacteria; under similar conditions, but after washing in phosphate saline, stationary phase organisms were the more resistant. Assay of bacteria harvested at different times during the growth cycle and washed in distilled water, showed that the magnesium content (on a dry-weight basis) was significantly higher during exponential growth. Thus, the amount of magnesium available after washing with water was probably greater in the case of exponential phase organisms.

The fact that the lethal effect of heating Aerobacter aerogenes increased with the exogenous concentration of potassium above 0·1 M may have been due to a progressive
suppression of the diffusion of this ion out of bacteria. In experiments not reported here, leakage of potassium from bacteria at 47° was shown to be progressively decreased with increasing exogenous potassium (but not sodium) concentration up to 0-05m at least; above this concentration, changes caused by leakage of potassium were too small to measure accurately by chemical analysis. Assays of bacterial potassium were not made because of the possibility that some would be removed during washing to remove suspending fluid. At 47°, maintenance of, and possibly addition to, the normal intracellular ionic concentration may lead to ribosomal instability by a competitive displacement of ribosomally bound magnesium. Addition of magnesium to solutions of potassium which at 47° were otherwise highly lethal to bacteria caused a marked decrease in the rates of death and RNA degradation, probably because the amount of magnesium absorbed by the bacteria was sufficient to counteract the competitive effects. It is of interest that the concentration of exogenous potassium (0-05m) in which A. aerogenes was most stable at 47° was also the minimum concentration in which a measurable ‘optical effect’ (change in turbidity of a suspension as compared with a similar suspension in water; Mager, Kuczynski, Schatzberg & Avi-Dor, 1956) occurred.

The protective effect of high initial bacterial concentration on bacteria subjected to heat stress may be due to many factors among which the heat-stable, dialysable fraction of the leakage products is of major importance. A similar population density phenomenon was observed on chilling exponential phase A. aerogenes (Strange & Dark, 1962) and it is of interest that filtrate from a chilled dense suspension (about 10^10 bacteria/ml.) was found to protect a sparser suspension (about 10^8 bacteria/ml.) not only from the lethal effect of chilling but also from heating at 47°.

In agreement with previous observations by Califano (1952) concerning the effect of heat on various bacteria, the data presented here for suspensions of Aerobacter aerogenes heated at 47° under a variety of conditions strongly suggest a relationship between the rates of death and of RNA degradation. Magnesium, which is known to stabilize isolated ribosomes (see review by McQuillen, 1962), decreased both rates, whereas EDTA, which accelerates ribosomal breakdown (Chao, 1957; Wade, 1961), increased both rates. Magnesium, manganese, cobalt and calcium, which afforded good or some protection to A. aerogenes during heating, all strongly inhibited the auto-degradation at pH 6-5 of purified ribosomes isolated from Escherichia coli by Mr H. E. Wade.

In most cases the shape of the death and leakage curves provided evidence that RNA breakdown preceded bacterial death at 47°, there being an initial period of zero or small death-rate during which RNA degradation occurred at the maximum rate. Other results, not presented here, have shown that at temperatures between 30 and 47° the rate of RNA degradation in Aerobacter aerogenes suspensions increased nearly linearly with temperature while, at least up to 44°, the death-rate was negligible for the first hour or more. However, depletion of RNA to the extent that occurs during the initial period of heating at 47° does not account for the lethal effect. Bacteria with their RNA-content substantially diminished by starvation under conditions where there was little loss of viability showed a much greater thermal resistance than did unstarved bacteria. Since the leaked products from heated bacteria are protective, not toxic, one is left with the possibility that the
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lethal effect associated with rapid RNA degradation results from an effect on bacterial metabolism of a sudden large increase of RNA fragments within the bacteria. It was shown that the ribose moiety of degraded RNA was metabolized by bacteria at $47^\circ$ and also that exogenous ribose (or other sugars) accelerated the death-rate during heating. The lethal effect of exogenous sugars was annulled by the presence of magnesium and these findings appear to be related to the phenomenon of ‘substrate-accelerated death’ which occurs at lower temperatures (Postgate & Hunter, 1963). To what extent the mechanisms are similar remains to be investigated.

We are indebted to Mr F. A. Dark for useful discussion, Mr A. G. Ness for some analyses, Mr B. Phillips for technical assistance and Mr S. Lovett for the use of a scintillation counter.

REFERENCES


