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**INVOLVEMENT OF MEMBRANES IN THE HEAT RESISTANCE OF  
*ESCHERICHIA COLI* AND THEIR DAMAGE BY HEAT**

**1982**

**NORIAKI KATSUI**

INVOLVEMENT OF MEMBRANES IN THE HEAT RESISTANCE OF  
*ESCHERICHIA COLI* AND THEIR DAMAGE BY HEAT

(大腸菌の耐熱性における膜の関与とその熱傷害)

1982

NORIAKI KATSUI

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## PREFACE

Sterilization is an important process in food, brewing, fermentation and medical industries. There are several means of sterilization, such as heat, antimicrobial agents and irradiation. Among them, heat sterilization has been used from the ancient period and occupies the most widely used in the practical sterilization process in respect of effectiveness, safety and economy (64).

The thermal resistance of microorganisms is influenced by a number of factors, e.g. growth temperature, growth phase, cell concentration, and the composition of media for growth, heat treatment and recovery. How the thermal resistance is affected by environmental changes is of practical importance in heat sterilization. However, an exact cause of the thermal cell damage is not well understood yet. Studies on the mechanism are essential for the establishment of fundamental principles in the application of heat sterilization and the knowledge concerning this mechanism should promote the effective use of heat sterilization.

Identification and characterization of various fundamental aspects of injured microorganisms subjected to heat stress should give an insight into the mechanism of thermal inactivation. Several possible targets for the thermal inactivation of a micro-

bial cell, e.g. membranes, ribosomes, RNA, DNA and proteins, have been proposed (1, 7, 29, 74, 83).

Damage to the membranes has been detected by a loss in the selective permeability of the cells, such as leakage of ultra-violet-absorbing substances, carbohydrates, ninhydrin-positive substances, magnesium and potassium ions (22, 30, 32, 60, 61, 65, 70), and increased sensitivity to salt (31) and an antibiotic (77). Release of membrane components such as lipids has also been reported (27, 31, 56).

Degradation of ribosomes and RNA during thermal injury has been studied in Escherichia coli (81), Salmonella typhimurium (73), Bacillus subtilis (45) and Staphylococcus aureus (59). However, it is considered that the degradation of ribosomes is caused by a loss of magnesium due to heat. Magnesium ions are required for the integrity of ribosomes and, moreover, serve to inhibit a ribonuclease. Therefore, the possibility that a ribonuclease activated by heat rather than heat itself is responsible for ribosomal damage has been suggested (8).

Bridges et al. reported that E. coli heated at 52°C suffered damage to the DNA (6) and showed single strand breaks in the bacterial DNA as a consequence of heating and enzyme action (63). Single strand breaks were also reported in mildly heated S. typhimurium (20). Woodcock and Grigg (86) observed double-strand breakage of the DNA in heated E. coli. The overall ten-

tative conclusion drawn from these works is that the strand breaks in DNA are not the direct result of heating but rather the consequence of the activation of (a) nuclease(s). This conclusion is similar to that proposed for RNA degradation.

Inactivation of proteins including enzymes may also be a possible cause of heat injury and cell death. Rosenberg et al. (57) indicated a similar correlation between the thermodynamic parameters for reactions of protein denaturation and for the thermal death of various organisms, and suggested that protein denaturation was a likely cause of cell death.

In this study, I took cell membranes, as a possible site for cell death due to heat and investigated their contribution to the heat resistance of cells and thermal damage of the cell envelope using E. coli.

The cell envelope of E. coli, like other gram-negative bacteria, consists of three layers: outer membrane, peptidoglycan (murein) layer internal to the outer membrane, and cytoplasmic (inner) membrane (9). The outer membrane and peptidoglycan layer are linked by lipoprotein. Peptidoglycan plays roles in maintaining the rigidity and shape of the cell and in protecting the cell from osmotic lysis. Both the outer and cytoplasmic membranes consist of lipids and proteins; the former also includes a specific component, lipopolysaccharide (LPS).

Membrane lipids create a barrier to the free entry and exit

of molecules into and out of the cell and provide a matrix in which (or on which) biochemical reactions take place (67). The cytoplasmic membrane proteins are involved in active transport, oxidative phosphorylation and biosynthesis of envelope macromolecules (9). The outer membrane proteins are involved in diffusion of small molecules, conjugation, and adsorption of phages and colicins (33). The periplasmic space between the outer and cytoplasmic membranes, houses binding proteins involved in the active transport of various metabolites and degradative periplasmic enzymes (9).

The cell envelope has many functions for cell growth and survival, as described above. The membrane damage on heating therefore may significantly affect the survival of cells. In fact, several researchers have suggested that the membranes are closely involved in the heat resistance of bacterial cells (2, 3, 88). Although heat damage to bacterial membranes has been confirmed by many workers, as described before, these workers have merely reported individual phenomena, such as leakage of intracellular materials, release of membrane components or sensitization to some chemical reagents, and have never characterized the process of membrane damage consisting of these events. Direct evidence of the damage to the membrane structure has been provided by only a few investigations.

In this study, using E. coli, I will indicate the contribu-

tion of the membranes to the heat resistance of cells and also characterize thermal damage to the cell envelope from the viewpoint of morphological and structural changes.

This thesis consists of the following three chapters.

In Chapter 1, I describe that the heat resistance of E. coli depended on temperatures of incubation prior and subsequent to the heat treatment. These phenomena seem to be significant in the heat sterilization process of food and in the testing of heat resistance of microorganisms. In addition, the cause of this dependence is examined in relation to the change in fluidity of membrane lipids. These effects of incubation are also investigated using other gram-negative and positive bacteria, and yeasts.

Chapters 2 and 3 deal with the thermal damage to the cell envelope of E. coli. In Chapter 2, as a morphological change of the cell surface due to heat, surface blebs that formed on the cell envelope are described and their morphology and formation patterns are investigated. The increase of hydrophobicity of the cell surface with heating is also examined. In Chapter 3, the released fractions, especially membrane lipids, are characterized chemically. The results obtained indicate that the released lipids are entirely composed of the outer membrane and a part of them shows vesicle-like structures which are probably derived from the surface blebs. And the mechanisms of bleb and vesicle formation due to heat are discussed.

# CHAPTER 1 EFFECTS OF PREINCUBATION AND POSTINCUBATION TEMPERATURES ON THE HEAT RESISTANCE OF MICROORGANISMS

## INTRODUCTION

The effect of growth temperature is well documented as one of the factors which can affect the heat resistance of bacteria (2, 3, 14, 24). This has also been found with yeasts (80). In general, a lower growth temperature produces cells more sensitive to heat. The lowering of the growth temperature also results in an increase in the proportion of unsaturated fatty acid of bacteria (3, 11, 44); the corresponding alteration of heat resistance has therefore been associated with fluidity of the membrane phospholipids (2).

The temperature at which microorganisms are held prior and subsequent to heat treatment may also be a factor influencing heat resistance, but little attention has been paid to these points in the sterilization of food and the testing of heat resistance of microbial cells. Tsuchido et al. (76) have indicated that the viability of E. coli heated at 50°C increased with increasing temperature of preincubation without nutrients.

In this chapter, first, I describe the effect of preincubation temperature on the heat resistance (preincubation effect) of micro-

organisms. Secondly, I examine the preincubation effect on E. coli in which the composition of fatty acids in membrane lipids was modified by changing the growth temperature and by using an unsaturated fatty acid auxotroph, and the results are discussed in relation to the change in fluidity of membrane lipids. Thirdly, I describe the effect of postincubation temperature on the heat resistance (postincubation effect) of microorganisms and discuss the cause of the effect.

#### MATERIALS AND METHODS

Organisms. The following strains were used: Escherichia coli W3110 and K1060, the latter strain, which is a mutant deficient in synthesizing or degrading unsaturated fatty acids and requires them for growth (52), was obtained from the E. coli Genetic Stock Center, Yale University, New Haven, Connecticut, U. S. A., Salmonella typhimurium LT2 and Pseudomonas aeruginosa ATCC 10145 as gram-negative bacteria, Staphylococcus aureus 209P and Bacillus subtilis 168 trp as gram-positive bacteria, and Saccharomyces cerevisiae OUT7020 and Candida utilis OUT6020 as yeasts.

Culture conditions. The growth media used in this study were as follows: EM9 medium, which is M9 minimal medium (consisting of 8.8 g  $\text{Na}_2\text{HPO}_4$ , 1.2 g  $\text{KH}_2\text{PO}_4$ , 5.0 g NaCl, 1.0 g  $\text{NH}_4\text{Cl}$  and 0.25 g  $\text{MgSO}_4 \cdot 7\text{H}_2\text{O}$  per liter) supplemented with 0.2 % (w/v) glucose

and 0.1 % (w/v) vitamin-free casamino acid (Difco), for gram-negative bacteria except E. coli K1060; EM9 medium supplemented with 0.01 % (w/v) unsaturated fatty acid and 0.04 % (w/v) Brij 58 for E. coli K1060, and in one experiment, nutrient broth for E. coli W3110; EM9 medium supplemented with 0.1 % (w/v) yeast extract (Daigo-eiyo-kagaku Co., Ltd.) for S. aureus; Spizizen medium (69) for B. subtilis; and Czapek-Dox medium supplemented with 0.25 % (w/v) Polypepton (Daigo-eiyo-kagaku Co., Ltd.) and 0.25 % (w/v) yeast extract for yeasts. The pH was adjusted to 7.0 for bacteria and to 5.5 for yeasts. Bacteria were grown at 37°C for 16 to 18 h, and yeasts at 30°C for about 30 h in a 100 ml flask containing 20 ml of growth medium. A portion (1.5 ml) of the culture was then inoculated into a 500 ml flask containing 100 ml of fresh medium and incubated at 37°C for bacteria (15°C in an experiment using E. coli W3110) and 30°C for yeasts. All cultures were shaken reciprocally at 100 strokes/min. Cells were harvested by centrifugation at 3,000 g for 5 min during the exponential growth phase. In one experiment, E. coli W3110 at the stationary growth phase was used. Cells were washed twice with 50 mM tris-(hydroxymethyl)aminomethane-HCl buffer, pH 8.0, containing 10 mM MgSO<sub>4</sub> (Tris-Mg buffer) for E. coli W3110, with EM9 medium without fatty acid and Brij 58 for E. coli K1060 or the following suspending media, being growth media from which the nitrogen source was omitted: M9 medium from which NH<sub>4</sub>Cl was omitted for gram-negative

bacteria; EM9 medium for S. aureus; Spizizen medium not supplemented with tryptophan for B. subtilis; and Czapek-Dox medium from which  $\text{NaNO}_3$  was omitted for yeasts. The pHs of these media were adjusted to those of the original growth media. After washing, cells were resuspended in fresh suspending medium. These operations were done at room temperature.

Preincubation, heat and postincubation treatments. When the preincubation effect was examined, the cell suspension ( $2 \times 10^8$  to  $4 \times 10^8$  cells/ml) was incubated in a shaking incubator at various temperatures between 0 and 37°C ( $\pm 0.2^\circ\text{C}$  accuracy) for 30 min. Since the rate of cooling was slow, the injurious effect of cold shock on cells could be avoided. After preincubation, a 2 ml sample was withdrawn and immediately added to a 100 ml flask containing 18 ml of fresh suspending medium which had been preheated to a required heating temperature, and then heat treatment was continued with shaking at 100 strokes/min. Cells were heated at 50°C for gram-negative bacteria and yeasts (in one experiment using E. coli W3110, temperatures of 48, 52 and 54°C were also used), 53°C for B. subtilis, and 60°C for S. aureus.

For investigating the postincubation effect, the cell suspension ( $10^6$  to  $2 \times 10^6$  cells/ml) was incubated at 37°C for 30 min, and heated as described above. At the time of reduction in viability of about 90 %, a 1 ml sample was withdrawn and immediately added to 9 ml of fresh suspending medium kept at various tempera-

tures between 0 and 37°C.

Viable counts. For the experiment on the preincubation effect, 1 ml samples were taken at intervals during the heating period, diluted serially in fresh suspending medium kept at 37°C, and then 0.1 ml diluted samples were plated on the growth medium containing 1 % (w/v) agar and, for E. coli K1060, 0.01 % (w/v) oleic acid and 0.04 % (w/v) Brij 58.

When the postincubation effect was examined, 0.1 ml samples were taken at intervals and directly plated.

The plates were incubated at the same temperature as that of growth for 2 d and the numbers of colonies were counted. The rate of viability loss was expressed as the time required for 90 % reduction of the initial viable count designated as  $T_{90}$ .

Fatty acid analysis. E. coli cells were harvested from 1 l cultures by centrifugation at 4°C and washed twice with deionized water. Phospholipids were extracted from the wet cells by the method of Bligh and Dyer (4). Lipid extracts were dissolved in 10 ml of 3 % (w/v) HCl in anhydrous methanol and the solution was esterified at about 100°C under reflux for 3 h. Methyl esters of fatty acids were extracted twice with 10 ml hexane and the total combined fractions were evaporated. The fatty acid esters were dissolved in acetone and injected into a Hitachi gas chromatograph (model 063) equipped with a thermal conductivity detector. The stainless steel column (2 m x 0.2 cm) was packed with 15 % dieth-

ylene glycol succinate (DEGS) coated on to 60/80 mesh Neopak 1A (Nishio Industry Co.) and operated at 200°C with helium as carrier gas. The temperatures of the injector and detector were 210 and 220°C, respectively. Fatty acid methyl esters were identified by comparing their retention times with those of standard compounds. Areas under the curve were determined by triangulation.

## RESULTS

Effect of preincubation temperature on the heat resistance of microorganisms. E. coli W3110 grown at 37°C was preincubated for 30 min at 0 and 37°C in Tris-Mg buffer (pH 8.0) and then the suspension was transferred, with 10-fold dilution, to fresh buffer preheated to 50°C. Fig. 1-1 shows the resultant survival curves of these cells during the heating period at 50°C. The viability of cells preincubated at 37°C was higher than that of cells preincubated at 0°C in spite of heating at an identical temperature.  $T_{90}$  values were 29 and 15 min, respectively. The level of heat resistance of each cell population was maintained for at least 1 h of prolonged preincubation (data not shown).

When cells were preincubated at 0°C for 30 min, rewarmed to and kept at 37°C for 30 min and then heated at 50°C, the resultant survival curve was similar to that for cells preincubated at 37°C without prior chilling. These observations indicate that the

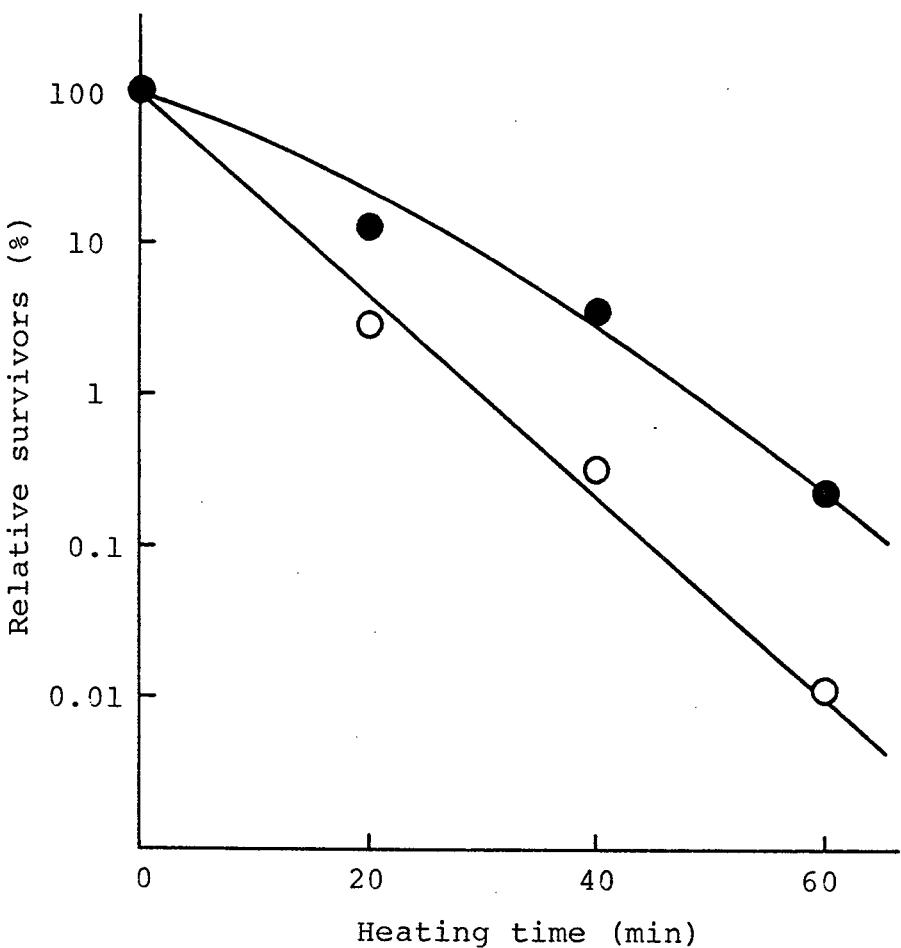


Fig. 1-1. Survival curves of *E. coli* W3110 heated in Tris-Mg buffer. Cells were grown at 37°C, preincubated at 0°C (○) and 37°C (●) for 30 min, and then heated at 50°C. Relative survivors are expressed as (viability after heat treatment / viability before heat treatment) x 100.

alteration of the heat resistance is reversible.

This effect was further examined at different heating temperatures, 48, 50, 52 and 54°C. The preincubation effect on the viability of E. coli W3110 was observed at all of the heating temperatures tested. The Arrhenius plot of the death rate constant obtained from each survivor curve is shown in Fig. 1-2. The activation energy of the death reaction calculated from the slope of the line of the plot was 131 and 134 kcal/mole for cells preincubated at 0 and 37°C, respectively.

The preincubation effect on the stationary phase cells heated at 50°C was more marked than that on the exponential phase cells, although the heat resistance of the former cells was higher than that of the latter (data not shown). The ratios of  $T_{90}$  values for the cells preincubated at 0 and 37°C were 6.3 and 1.6, respectively.

A similar preincubation effect was observed with E. coli W3110 cells grown in either nutrient broth or EM9 medium: the ratio of  $T_{90}$  values for the cells preincubated at 0 and 37°C was 1.6 in both cases, although the heat resistance of the cells grown in nutrient broth was higher than that of the cells grown in EM9 (data not shown).

In addition, I examined the preincubation effect on other bacteria and yeasts. Exponential phase cells of each microorganism were preincubated at 0 and 37°C for 30 min and then heated.

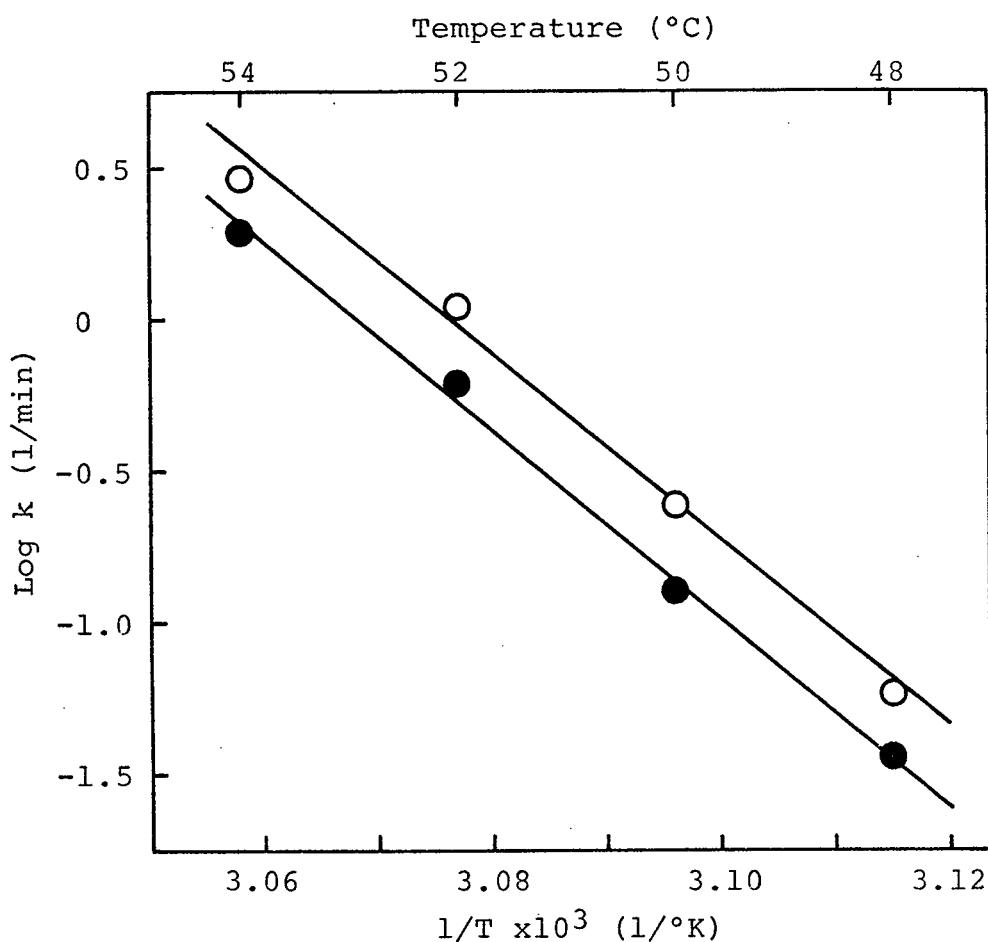


Fig. 1-2. Arrhenius plot of the death rate constant of *E. coli* W3110 heated in Tris-Mg buffer. Cells were grown at 37°C, preincubated at 0°C (O) and 37°C (●) for 30 min, and then heated at 48, 50, 52 and 54°C.

For washing, heating and dilution, the suspending medium, which was described in Materials and Methods, was used to inhibit growth and death during preincubation. In all microorganisms tested, the viability after heat treatment of cells preincubated at 37°C was higher than that of cells preincubated at 0°C, as shown in Table 1-1. The results also indicate that, on the basis of the ratio of  $T_{90}$  values, the preincubation effect on gram-positive bacteria and yeasts was seemingly more marked than on gram-negative bacteria.

In all of these experiments, no growth or death of cells occurred during the preincubation period.

Effect of preincubation temperature on the heat resistance of *E. coli* having different fatty acid compositions. Since the growth temperature affects the fatty acid composition as well as the heat resistance of bacteria, I compared the heat resistance of *E. coli* W3110 preincubated at various temperatures using cells grown at 15°C (15°C-grown cells) and cells grown at 37°C (37°C-grown cells), in relation to the difference in their fatty acid compositions. As shown in Table 1-2, growth of *E. coli* W3110 at 15°C instead of 37°C resulted in an increase in the relative proportions of palmitoleic (16:1) and cis-vaccenic (18:1) acids and a decrease in that of palmitic acid (16:0). Thus, the ratio of the content of total saturated fatty acids to that of the sum of

Table 1-1. Effect of preincubation temperature on the heat resistance of various microorganisms

Microorganism	Preincubation temperature	Heating temperature	T <sub>90</sub> value <sup>a</sup> (min)	Ratio (37°C/0°C)
Gram-negative bacteria				
<u>Escherichia coli</u>	37°C 0°C	50°C	45 30	1.5
<u>Salmonella typhimurium</u>	37°C 0°C	50°C	29 19	1.5
<u>Pseudomonas aeruginosa</u>	37°C 0°C	50°C	9.7 6.6	1.5
Gram-positive bacteria				
<u>Bacillus subtilis</u>	37°C 0°C	53°C	65 22	3.0
<u>Staphylococcus aureus</u>	37°C 0°C	60°C	8.3 2.0	4.2
Yeasts				
<u>Saccharomyces cerevisiae</u>	37°C 0°C	50°C	21 4.5	4.7
<u>Candida utilis</u>	37°C 0°C	50°C	19 7.5	2.5

<sup>a</sup> T<sub>90</sub> value is the time required for a 90 % reduction of the initial viability.

Table 1-2. The fatty acid compositions of E. coli W3110 grown at 15 and 37°C

Growth temperature (°C)	Fatty acid <sup>a</sup> (% w/w, of total)								Ratio	
	14:0	16:0	16:1	17:0cyc	18:0	18:1	19:0cyc	SFA	UFA	(SFA/UFA)
15	1.9	42.4	28.9	1.4	0.0	22.2	2.4	44.3	54.9	0.81
37	2.8	52.5	25.2	3.0	0.7	11.5	2.6	56.0	42.3	1.32

<sup>a</sup> In expression of fatty acids, the first number represents the number of carbon atoms and the second the number of double bonds. cyc means cyclopropane fatty acid. SFA and UFA indicate total saturated and total unsaturated plus cyclopropane fatty acids, respectively. The 18:1 acid is cis-vaccenic acid. The fatty acid composition was determined for two separate samples.

total unsaturated fatty acids and cyclopropane fatty acids was 1.32 for 37°C-grown cells but was 0.81 for 15°C-grown cells. Since fatty acids are mainly located in membrane phospholipids and unsaturated fatty acids largely contribute to the membrane fluidity (10), the above results suggest that the membrane fluidity was different for these cell populations.

There was a marked difference in the preincubation effect on 15°C- and 37°C-grown cells (Fig. 1-3). For 37°C-grown cells, the viability after heating at 50°C for 15 min decreased gradually with decreasing temperature of preincubation below about 20°C, while it was approximately constant above this temperature. On the other hand, the viability of 15°C-grown cells after exposure to heat remained constant in the range of preincubation temperatures tested.

To establish the importance of the fatty acid composition of the cells for the preincubation effect, I investigated this effect further using an unsaturated fatty acid auxotroph, E. coli K1060. In agreement with the results of other investigators (23, 88), this strain incorporated added oleate (18:1) or linolenate (18:3) into the membrane during growth, as shown in Table 1-3. The amounts incorporated were 50.5 and 26.3 %, respectively, of the total fatty acids in the cell and accounted for almost all of the total unsaturated fatty acids.

Also for the heat resistance, strain K1060 showed different patterns when grown in EM9 medium supplemented with oleate or

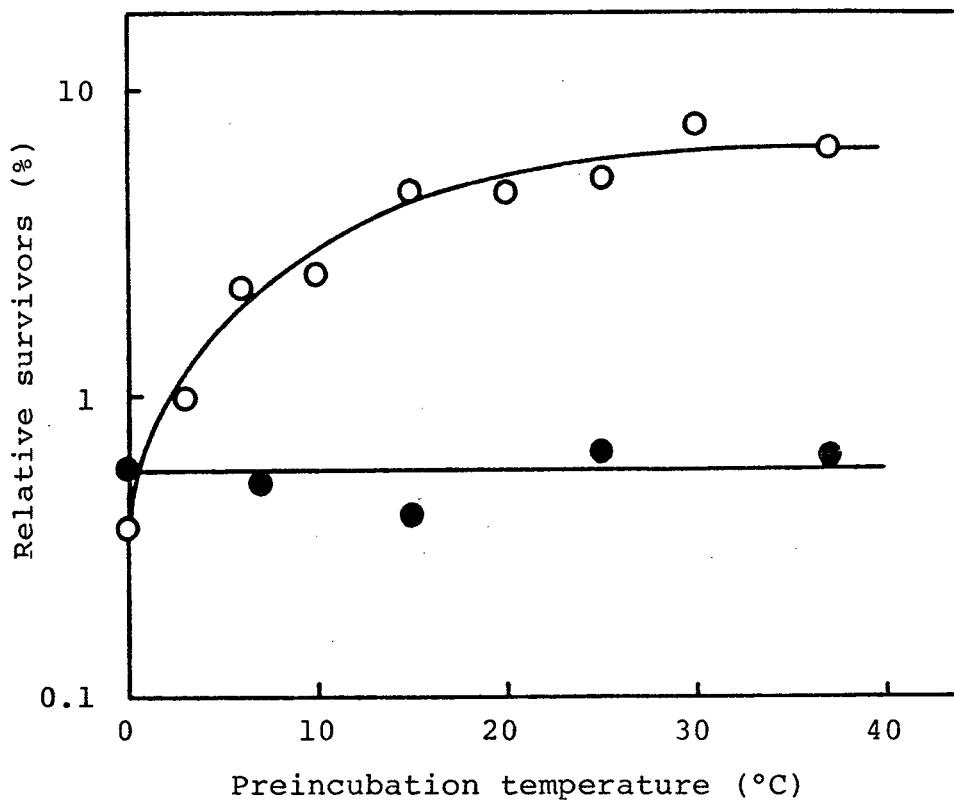


Fig. 1-3. Effect of preincubation temperature on the heat resistance of *E. coli* W3110 cells grown at 15°C (●) and 37°C (○). Cells were preincubated at the indicated temperatures for 30 min and then heated at 50°C for 15 min in Tris-Mg buffer (pH 8.0). Values are means of results from two independent experiments.

Table 1-3. The fatty acid compositions of E. coli K1060 grown with  
oleate and linolenate

Supplemented fatty acid	Fatty acid <sup>a</sup> (% w/w, of total)				
	14:0	16:0	16:1	18:1	18:3
Oleate (18:1)	7.1	38.2	1.1	50.5	0.0
Linolenate (18:3)	7.1	61.1	1.4	0.0	26.3

<sup>a</sup> See Table 1-2 for expression of fatty acids. The fatty acid composition was determined for two separate samples.

linolenate. Fig. 1-4 shows the viability of strain K1060 heated at 50°C for 45 min after preincubation at various temperatures. Cells grown in the presence of oleate (oleate-grown cells) showed higher sensitivity to heat when preincubated at lower temperatures, below about 15°C, but showed similar resistance at temperatures ranging from about 15 to 37°C with a pattern similar to that of 37°C-grown cells of strain W3110. In the case of cells grown in linolenate-supplemented medium (linolenate-grown cells), on the other hand, the heat resistance remained constant, similar to the case of 15°C-grown cells of strain W3110, although a slight decrease was observed at a preincubation temperature of 0°C.

In all of these experiments, no growth or death of cells occurred during the preincubation period.

#### Effect of postincubation temperature on the heat resistance

of microorganisms. The exponential phase cells of E. coli W3110 grown at 37°C were preincubated at 37°C for 30 min in Tris-Mg buffer, heated at 50°C for 10 min, and then held at 0 and 37°C in fresh Tris-Mg buffer. As a result, almost no decrease in the viability occurred during 1 h, when heated cell suspension was incubated at 37°C. When cells were held at 0°C for 1 h after heat treatment, on the other hand, the viability decreased to 25% (Fig. 1-5).

As in the study on the preincubation effect, I compared the effect between cells grown at 37 and 15°C. The viabilities of

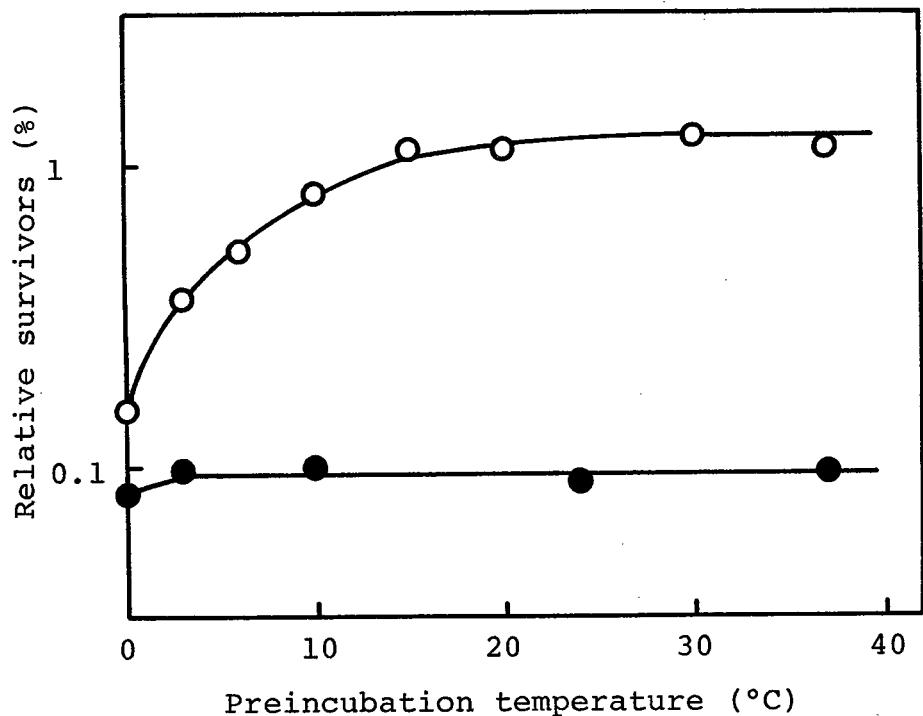


Fig. 1-4. Effect of preincubation temperature on the heat resistance of *E. coli* K1060 cells grown with oleate (O) and linolenate (●). Cells were preincubated at the indicated temperatures for 30 min and then heated at 50°C for 45 min in EM9 medium. Values are means of results from two independent experiments.

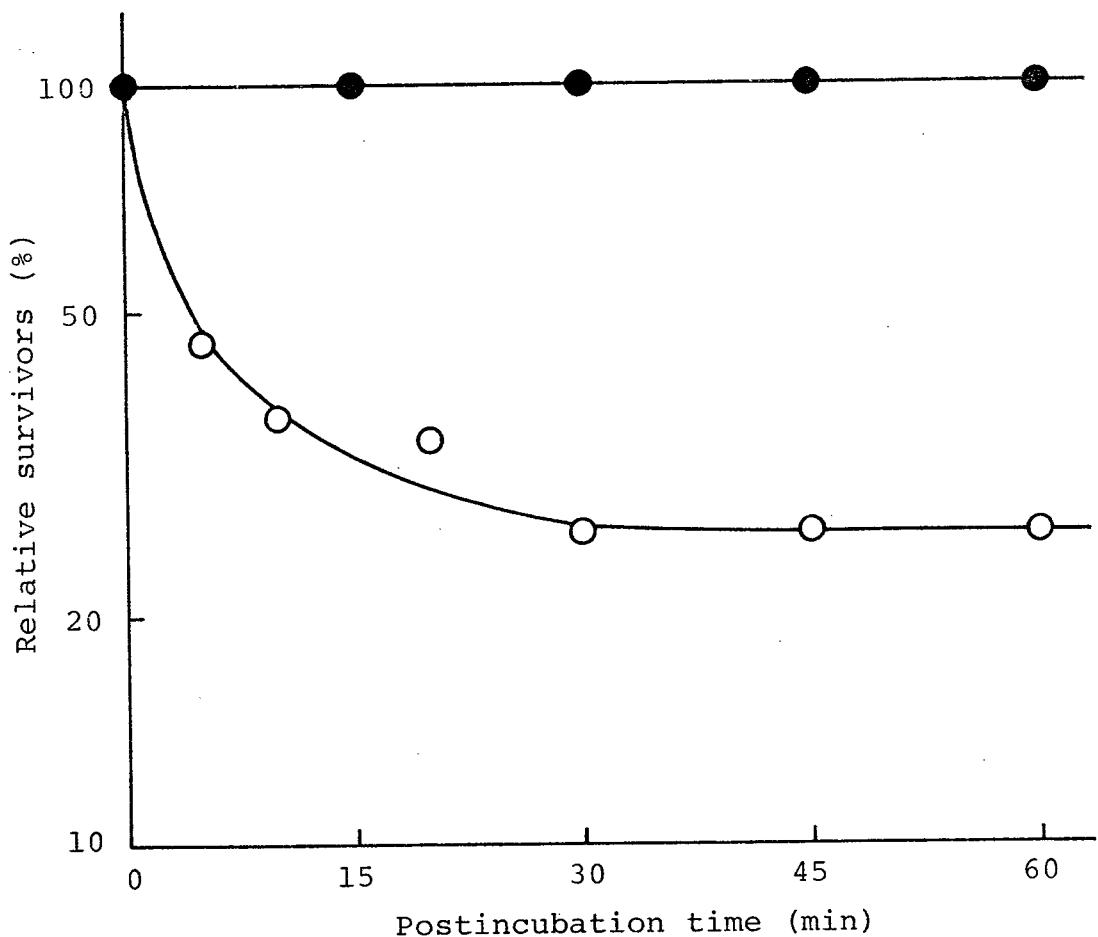


Fig. 1-5. Survival curves of *E. coli* W3110 postincubated in Tris-Mg buffer. Cells were grown at 37°C, preincubated at 37°C for 30 min, heated at 50°C for 10 min, and then postincubated at 0°C (○) and 37°C (●). Relative survivors are expressed as (viability after postincubation / viability immediately after heat treatment) x 100.

E. coli W3110 cells held for 1 h at various temperatures after heat treatment are shown in Fig. 1-6. For 37°C-grown cells, the viability decreased gradually with decreasing postincubation temperature below about 20°C, whereas almost no loss in viability occurred above this temperature. On the other hand, the decrease in the viability of 15°C-grown cells remained almost constant (about 60 %) in the range of postincubation temperatures tested.

The postincubation effect on the viability of heated cells was examined using other bacteria and yeasts. Similar to the experiments on preincubation temperature, for washing and heating the growth medium without the nitrogen source was used. In all micro-organisms tested, the viability of cells incubated at 37°C for 2 h after heat treatment was higher than that of cells incubated at 0°C (Table 1-4).

#### DISCUSSION

In agreement with the report of Tsuchido et al. (76), the heat resistance of E. coli depended on the preincubation temperature. The results obtained indicate that the difference in the heat sensitivity as affected by the preincubation temperature does not arise simply from the difference between the preincubation and heating temperatures, but from the difference in the physicochemical state of cells at various holding temperatures before heating.

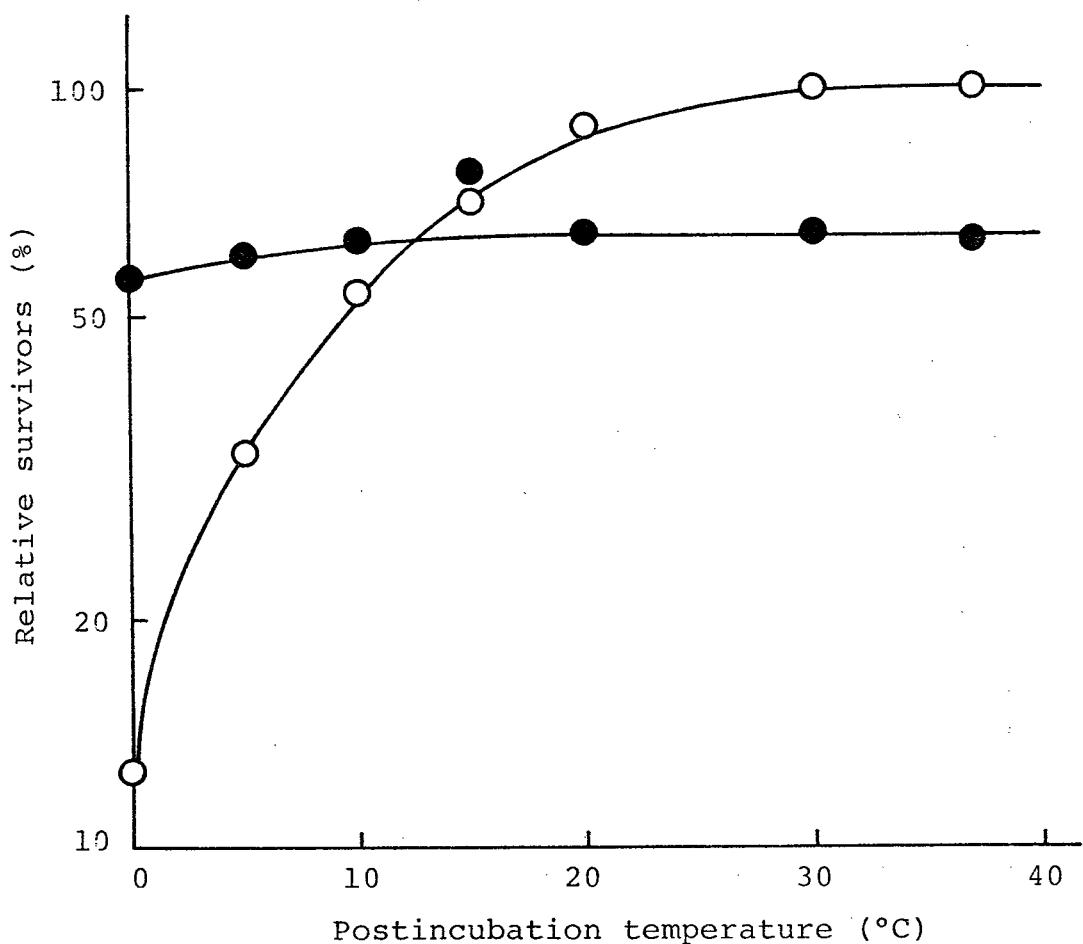


Fig. 1-6. Effect of postincubation temperature on the viability of heat-stressed *E. coli* W3110. Cells were grown at 37°C (○) and 15°C (●), preincubated at 37°C for 30 min, heated at 50°C for 10 min, and then postincubated at the indicated temperatures for 1 h. See the legend to Fig. 1-5 for the definition of relative survivors.

Table 1-4. Effect of postincubation temperature on the viability of various heat-stressed microorganisms<sup>a</sup>

Microorganism	Postincubation temperature	Relative survivors (%) <sup>b</sup> after 2 h postincubation	Ratio (37°C/0°C)
Gram-negative bacteria			
<u>Escherichia coli</u>	37°C 0°C	87 55	1.6
<u>Salmonella typhimurium</u>	37°C 0°C	92 43	2.1
<u>Pseudomonas aeruginosa</u>	37°C 0°C	88 33	2.7
Gram-positive bacteria			
<u>Bacillus subtilis</u>	37°C 0°C	71 32	2.2
<u>Staphylococcus aureus</u>	37°C 0°C	85 38	2.2
Yeasts			
<u>Saccharomyces cerevisiae</u>	37°C 0°C	90 59	1.5
<u>Candida utilis</u>	37°C 0°C	57 25	2.3

<sup>a</sup> See Table 1-1 for heating temperature and heating time corresponding to  $T_{90}$  at a pre-incubation temperature of 37°C for each microorganism.

<sup>b</sup> See the legend to Fig. 1-5.

A decrease in the growth temperature causes an increase in the proportion of unsaturated fatty acids in membrane phospholipids of E. coli (44), as I also observed (Table 1-2). It has been suggested that this change is due to the ability of cells to maintain constant membrane fluidity irrespective of the temperature of growth (67). The unsaturated fatty acid auxotroph of E. coli, K1060, also seemed to maintain constant membrane fluidity by changing the proportion of unsaturated fatty acid incorporated into membrane phospholipids according to the degree of unsaturation, as suggested by Esfahani et al. (15).

In general, lowering the growth temperature is known to result in cells more sensitive to heat (24) and a correlation has therefore been proposed between the growth temperature, the ratio of saturated to unsaturated fatty acid, and the heat resistance (2). Yatvin (88) has also reported that E. coli K1060 grown in linoleate-supplemented medium has a lower resistance to heat than when grown in oleate-supplemented medium. He suggested that the heat resistance of this mutant was correlated with the degree of unsaturation of fatty acid incorporated into membrane phospholipids. In fact, the higher resistance of cells grown at a higher temperature and of oleate-grown cells agrees with our results, except for cells incubated at a low temperature before heating. I observed that at a preincubation temperature of 0°C the order of the heat resistance of strain W3110 was inverted between 15°C- and 37°C-

grown cells (Fig. 1-3) and the difference between oleate- and linolenate-grown cells of strain K1060 diminished markedly.

In E. coli, membrane functions such as substrate transport, the activities of membrane-associated enzymes and the maintenance of cell integrity depend on the membrane fluidity (10). It seems likely that membrane fluidity influences the thermal stability of a certain structure(s) or function(s) essential for survival under heat stress. When the incubation temperature of cells is above a critical level, a gel-liquid crystalline phase transition of their membrane phospholipids should occur. Variation in the growth conditions of cells would influence the temperature of this phase transition as a result of the change in the fatty acid composition. Sinensky (67), using the electron spin resonance spin-label technique, showed that the temperature of the phase transition of membrane lipids in E. coli was usually 14 to 16°C lower than the growth temperature of the cells, i.e. 21 to 23°C for 37°C-grown cells and -1°C for 15°C-grown cells. Moreover, Overath et al. (53) reported from their observations on the efflux of thiomethylgalactoside and force/area isotherms of lipid films that the temperatures of the phase transition for the membrane lipids of the unsaturated fatty acid auxotroph of E. coli were 15°C and 4 to 6°C for oleate- and linolenate-grown cells, respectively. These temperatures are close to those at which the preincubation started to reduce the heat resistance of cells, as observed in this

study. The results suggest that the heat resistance of cells may depend on the physicochemical state of the membranes before heating, or on the phase transition of membrane phospholipids.

In this investigation, I showed that the preincubation effects (the ratio of the death rate constant) were almost identical, even if the heating temperature was changed, since the values of activation energy were almost equal for cells preincubated at 0 and 37°C (Fig. 1-2), and also even if a different growth medium was used.

The preincubation effect observed with the stationary phase cells was more marked than that with the exponential phase cells. Kito et al. (38) reported that the proportion of saturated fatty acids in membrane phospholipids of E. coli is higher in stationary phase cells than in exponential phase cells, suggesting that the phase transition temperature of the former is higher than that of the latter. Therefore, a marked effect of preincubation temperature on the stationary phase cells may result from a larger change in the membrane fluidity.

Heat-injured cells were found to be also affected by the postincubation temperature. When the postincubation effect was compared between 15°C- and 37°C-grown cells, the results shown in Fig. 1-6 resembled those obtained for the preincubation effect. In Fig. 1-6, above the temperatures of postincubation corresponding to the phase transition temperatures, the viability seemingly

remained constant, whereas below those temperatures the viability seems to decrease with decreasing temperature. This result suggests that the viability of heat-injured cells after postincubation depends on the membrane fluidity, similar to the preincubation effect. In the postincubation effect, the stability of the repair function in cells may be influenced by the fluidity of membrane lipids during the incubation after heat treatment.

Although several possible targets of heat action, such as membranes, RNA, DNA and proteins, have been proposed, results obtained suggest that the damage to a certain membrane-associated structure(s) or function(s) which depend(s) on the membrane fluidity may be linked with the heat resistance of E. coli. The involvement of membrane fluidity has also been indicated for bacterial susceptibility to cold shock (17, 54) and  $\gamma$ -irradiation (87).

The effects of incubation temperature prior and subsequent to heat treatment on the heat resistance were observed with various kinds of microorganisms (Tables 1-1 and 1-4), showing that these phenomena seem to be general, at least for mesophiles grown at optimal temperatures.

Based on the results obtained in this study, one should pay attention to the temperatures of preincubation and postincubation in the pasteurization process of food and also in the testing of heat resistance of microorganisms.

SUMMARY

The viability of heat-stressed cells of E. coli was influenced by the temperature of incubation prior and subsequent to heat treatment. I examined the preincubation effect of E. coli in which the composition of fatty acids in membrane lipids was modified by changing the growth temperature and by using an unsaturated fatty acid auxotroph. In each case, above the temperatures of preincubation corresponding to the phase transition temperatures, the viability seemingly remained constant, whereas below those temperatures the viability seems to decrease with decreasing temperature. In the postincubation effect, the results resembled those obtained for the preincubation effect. These results suggest the involvement of the membrane as a cause of the preincubation and postincubation effects. A certain membrane-associated structure(s) or function(s) which depend(s) on the membrane fluidity may be linked with the heat resistance and the repair function of E. coli. These phenomena were observed with other gram-negative and positive bacteria, and yeasts.

## CHAPTER 2 HEAT-INDUCED CHANGES IN THE CELL SURFACE OF ESCHERICHIA COLI

### INTRODUCTION

Heat damage to bacterial membranes has been confirmed by many workers. Destruction of the permeability barrier in the cell envelope causes the leakage of intracellular materials, such as ultraviolet-absorbing substances, carbohydrates, ninhydrin-positive substances, and magnesium and potassium ions (22, 30, 32, 60, 61, 65, 70), and sensitization to an antibiotic, tylosin (77). Membrane components, such as lipids (31) and LPS (27, 56), are also known to be released into the menstruum from cells exposed to heat. Moreover, some workers have presented evidence of damage to substrate transport systems due to heat (21, 55).

However, how the membranes themselves are changed by heat treatment of cells is not well known. From the viewpoint of morphological changes in bacterial cells, only a few investigations have been done. According to de Petris (13), who studied the ultrastructure of E. coli envelopes, electron micrographs of heated E. coli B cells, but at extremely high temperatures (75 to 100°C), indicated the formation of large blebs of the outer membrane. For more mild heat stress, Scheie and Ehrenspeck (62) observed blebs with E. coli B/r and B<sub>s-1</sub> heated at 49 to 55°C

using phase contrast microscopy and suggested that the bleb formation was closely related to the thermal death of cells. However, they provided no direct evidence of damage to the outer membrane.

Following the work in Chapter 1, which indicated the involvement of the membranes in the heat resistance of E. coli, I became interested in how bacterial membranes were damaged by heat. In this chapter, I describe changes in the cell surface of heated E. coli. The results obtained indicate that surface blebs that occurred in E. coli cells, which were heated under sublethal conditions, were mainly surrounded by the outer membrane and that the hydrophobicity of the cell surface was increased with heating.

These facts therefore suggest massive damage to the outer membrane.

#### MATERIALS AND METHODS

Organism and culture conditions. E. coli W3110 was grown for 16 to 18 h at 37°C in EM9 medium, pH 7.0, supplemented with 0.2 % glucose. A portion of this culture was then inoculated (1.5 %) into fresh medium and incubated at 37°C (15°C in one experiment) on a reciprocal shaker at 100 strokes/min. Cells were harvested by centrifugation at 3,000 x g for 5 min during the exponential phase ( $3 \times 10^8$  to  $4 \times 10^8$  cells/ml) (in some experiments, cells at the stationary phase,  $1.8 \times 10^9$  cells/ml, were also used),

washed twice with 50 mM tris(hydroxymethyl)aminomethane-HCl (Tris) buffer at pH 8.0 and then resuspended in fresh buffer, unless otherwise stated. These operations were done at room temperature.

Heat treatment. The cell suspension was incubated at 0°C (37°C for some experiments) for 30 min. After this preincubation, portions of the cell suspension were withdrawn and immediately added with ten-fold dilution to a flask containing an appropriate amount of Tris buffer, unless otherwise stated, which had been preheated to 61.1°C (57.0°C when preincubated at 37°C). With this temperature a final temperature of 55°C was immediately obtained on rapid heating. After mixing, heat treatment at 55°C was continued in an incubator with shaking at 100 strokes/min. For slow heating, a flask containing cell suspension preincubated at 0°C for 30 min was transferred to an incubator preheated to 55°C. In this case, it took about 2.5 min for the cell suspension to reach the final temperature. In one experiment, deionized water adjusted to pH 8.0 with dilute NaOH solution, Tris buffer at pH 8.0, but at concentrations of 100, 200 and 500 mM, and N-2-hydroxyethylpiperazine-N'-2-ethanesulfonic acid (HEPES), N,N-bis(2-hydroxyethyl)glycine (Bicine) and barbital buffers at a concentration of 50 mM, pH 8.0, were also employed as heating menstrua. The same buffer as used for the heating menstruum was used for washing and suspending cells in each case. When cells were heated in EM9 medium, in addition, similar procedures were

carried out without washing and except that Tris buffer was replaced by EM9 medium.

Phase contrast microscopy. After the heated cell suspension was cooled to room temperature, samples (1 drop) were withdrawn and placed on glass-slides, covered with glass cover-slips, and viewed in positive phase contrast with a Nikon Labophot microscope with a x100 objective and x15 oculars. In quantifying bleb formation, at least 300 cells were scrutinized for the presence of blebs.

Freeze-fracturing and electron microscopy. Freeze-fractured specimens were prepared as follows. Cell suspension was cooled to 37°C after heat treatment and then sedimented, without a change in temperature, by low-speed centrifugation at 500  $\times$  g for 10 min. The pellet was prepared on a specimen holder without a change in temperature and then quickly frozen in liquid Freon 22 by the sandwich method (48). The frozen specimens were freeze-fractured at -100°C, etched for 1 to 3 min, and shadowed with platinum-carbon in a vacuum evaporator, type JEE 4AS (Japan Electron Optics Laboratory Co., Ltd.). The replicas were obtained by standard procedures and studied under a JEM-100C electron microscope (Japan Electron Optics Laboratory Co., Ltd.) at x20,000 or x26,000 magnification.

In preparing ultrathin section specimens, to 1 ml samples including cells cooled to 37°C after heating, 4 ml of 2.5 % (v/v)

glutaraldehyde-25 mM phosphate buffer (pH 7.2) was added, and then the samples were allowed to stand at room temperature for 100 min. Fixed cells were centrifuged at 1,000  $\times$  g for 10 min. The cell pellets were then mixed with about 0.1 ml of 2 % agar. After the agar had solidified, it was divided into portions of about 1 mm<sup>3</sup>, washed twice with 25 mM phosphate buffer (pH 7.2), and postfixed with 1 % (v/v) osmic acid-25 mM phosphate buffer (pH 7.2) for 1.5 h. Specimen cores were dehydrated by passage through increasing concentrations of ethanol, embedded in Epon. Sections were cut with a glass knife on a Porter-Blum MT-1 ultra-microtome (Sorvall), mounted on 300-mesh copper grids, stained with uranyl acetate and lead, and examined under a JEM-100C electron microscope.

Viable counts. At intervals during the heating period, 1 ml samples were taken, diluted serially in Tris buffer containing 10 mM MgSO<sub>4</sub> kept at 37°C, and then 0.1 ml diluted samples were plated on EM9 containing 1 % agar. The plates were incubated at 37°C for 1 d and the numbers of colonies were counted. Viability was expressed as the percentage of the initial viable counts.

Assay of cell-surface hydrophobicity. Following the procedure of Rosenberg et al. (58), the hydrophobicity of the cell-surface was measured. To a test tube (18 mm diameter) containing 1.8 ml of cell suspension which was cooled to room temperature, was added 0.3 ml of n-hexadecane (Wako Pure Chemical Industries, Ltd.). After incubation at 37°C for 10 min, the mixture was

agitated uniformly on a Vortex-Genie mixer (Scientific Industries, Inc.) at maximum speed for 2 min and then stood for 15 min. The resultant aqueous layer was carefully removed with a Pasteur pipette and transferred to a half-path cuvette. The absorbance was determined at 400 nm, using a Hitachi Model 100-10 spectrophotometer. Each sample was compared with a control, which was treated as described above but without addition of n-hexadecane.

## RESULTS

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Bleb formation on the surface of heated cells. When a suspension of exponential phase cells of E. coli W3110, which had been kept at 0°C, was rapidly heated to 55°C in 50 mM Tris buffer, pH 8.0, with ten-fold dilution, in the manner described in Materials and Methods, blebs appeared on the surface of cells. On phase contrast microscopy, visible blebs were seen to be mainly (about 80 %) located at the septa of dividing cells and partly at or near polar regions. This confirms the results of Scheie and Ehrenspeck (62) for E. coli B/r and B<sub>s-1</sub>. Fig. 2-1 a-c are freeze-fracture electron micrographs of blebs that occurred on cells heated at 55°C for 10 min. The appearance of the convex fracture face of the blebs resembled that of the outer membrane rather than the cytoplasmic membrane, since no large intramembranous particles, which could be seen in the convex fracture

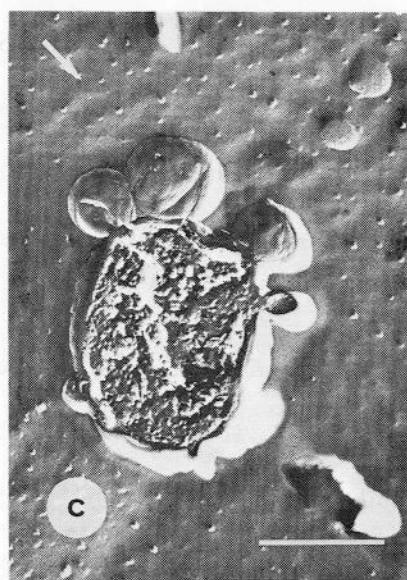
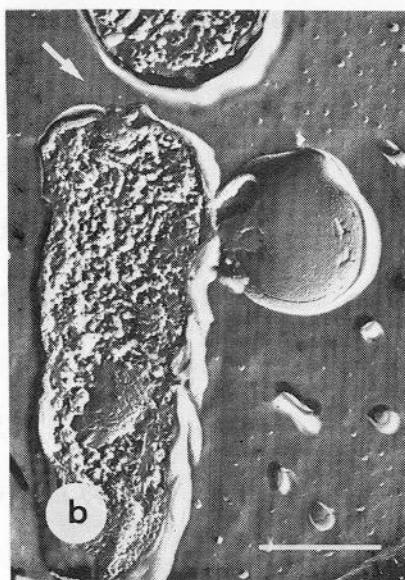
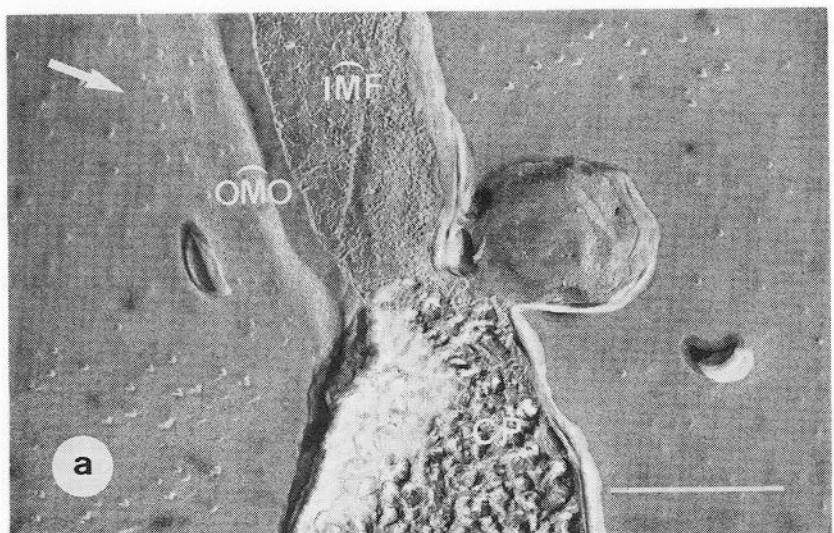


Fig. 2-1. Freeze-fracture faces of *E. coli* cells heated at 55°C for 10 min (a-c). IMF, inner membrane fracture face; OMO, outer membrane, outer surface; CP, cytoplasm;  $\curvearrowright$ , convex aspect. The white arrow at the corner of each micrograph shows the direction of shadowing. The bar in each micrograph represents 0.5  $\mu\text{m}$ .

face of the cytoplasmic membrane (Fig. 2-1 a), were detected.

In part of the fractured face of some blebs, concave regions (Fig. 2-1 b) were also often observed, suggesting a multilayered structure (see Fig. 2-2 a and b). The number of blebs formed was not always one for one cell. As shown in Fig. 2-1 c, two or more blebs occasionally were seen on a cell. In Fig. 2-2 a-c, ultra-thin section electron micrographs of cells heated at 55°C for 10 min are presented. Some of the observed blebs were composed of a single layer of the outer membrane without electron microscopically visible internal materials (data not shown), which was identical to the observation of de Petris (13). It should be noted, however that blebs having a multilayered structure were also observed, as shown in Fig. 2-2 a and b. In such severely damaged cells (Fig. 2-2 a and b), the cytoplasm underwent shrinkage and therefore, the gap between the peptidoglycan layer and the cytoplasmic membrane was widened. In others, moreover, cytoplasmic materials were found to flow out into outer membrane blebs probably due to partial rupture of the cytoplasmic membrane (Fig. 2-2 c). The bleb shown in Fig. 2-2 c also contained some membrane fragments. A boundary line which can be seen between a cell and a bleb is the peptidoglycan layer.

Effect of some factors on bleb formation. Patterns of the bleb formation were examined under various conditions. I counted

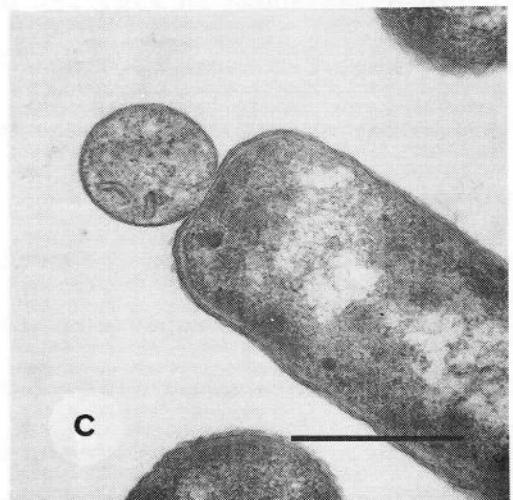
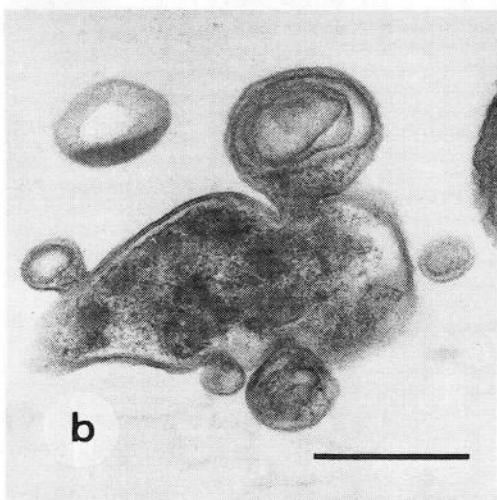
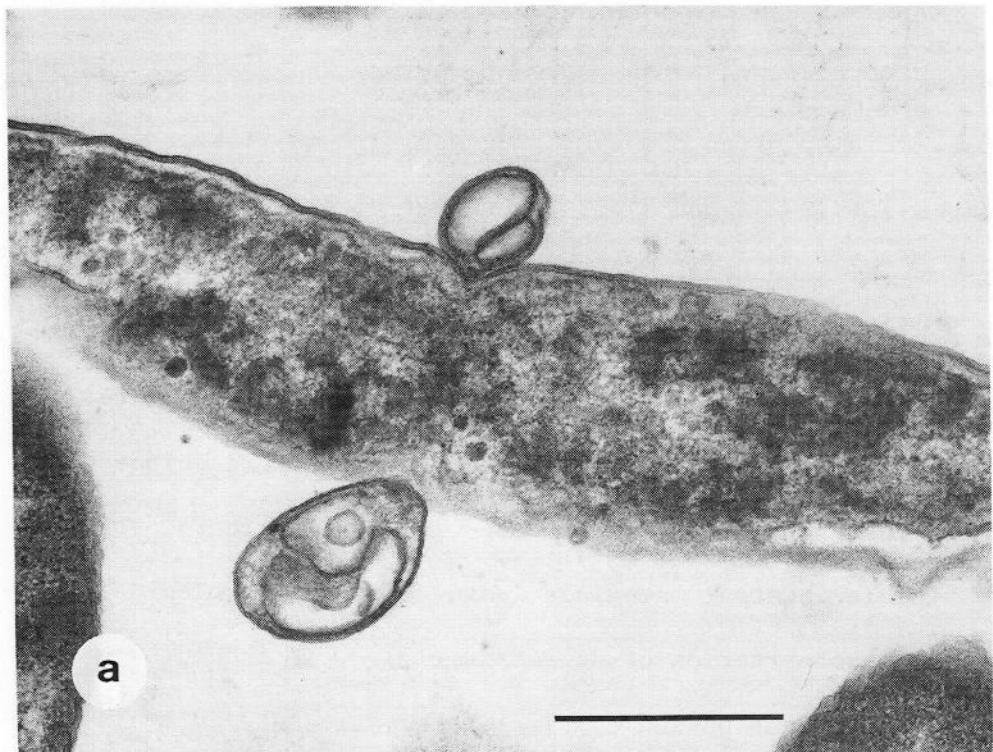


Fig. 2-2. Ultrathin sections of *E. coli* cells heated at 55°C for 10 min (a-c). In a and b, blebs are located at the septa of dividing cells. The bar in each micrograph represents 0.5  $\mu\text{m}$ .

cells having visible blebs under a phase contrast microscope. It should be noted here, however, that the resultant number of these cells is not absolute but only apparent, since very small blebs and blebs on the opposite sides of cells under observation are impossible to see. A significant percentage (11 %) of the total cells were found to have visible blebs on their surfaces immediately after heating (15 s), when exponential phase cells kept at 0°C were rapidly heated to 55°C. After that, however, the proportion of bleb-bearing cells decreased with heating time and reached less than 1 % after 30 min, as shown in Fig. 2-3.

Whether or not rapid heating (heat shock) is required for the bleb formation was investigated. When cells were heated slowly from 0 to 55°C in Tris buffer, taking about 2.5 min, as described in Materials and Methods, the proportion of bleb formation increased with heating time, in contrast to cells heated rapidly, and was about 5 % at 10 min, as shown in Fig. 2-3. And after that it gradually decreased. This, therefore, indicates that rapidity of heating itself is not necessary for the bleb formation, although it affects the amount and the rate of the formation.

In Chapter 1, I indicated that the heat resistance of E. coli cells was influenced by the holding temperature before heating. Also in this experiment, I assumed a similar effect on the bleb formation and examined the effect of preincubation temperature. When cell suspension was kept at 37°C for 30 min and then heated

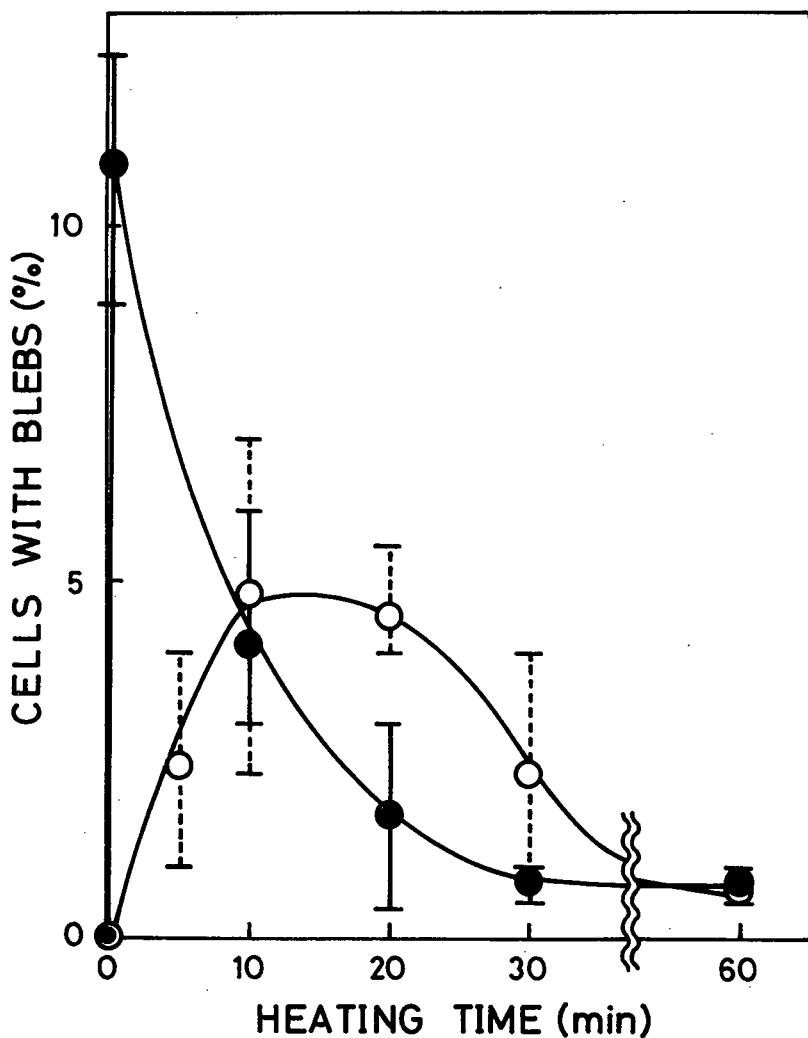


Fig. 2-3. Effect of heating rate on the bleb formation by heating. Cells were kept at 0°C for 30 min and then heated to 55°C rapidly with ten-fold dilution (●) or slowly (○), as described in Materials and Methods. The formation of blebs was determined by phase microscopy. The length of vertical bars represents the spread of values obtained in three independent determinations and the points the average value.

to 55°C, only 2.5 % of the total cells had blebs after 15 s heating, in contrast to 11 % in the cells kept at 0°C as described before, and the proportion increased slightly with heating time until about 20 min (3.6 % at 20 min), but thereafter decreased. In this experiment, no substantial loss of viability was detected after 15 s heating at 55°C for both cells preincubated at 0 and 37°C, although the viability decreased to 0.8 and 4.8 %, respectively, after 5 min. In stationary phase cells, the degree of heat-induced bleb formation was much less than that in exponential phase cells. When stationary phase cells were heated from 0 to 55°C, only 2.3 % of the total cells had visible blebs after 15 s heating whereas the value was 11 % for exponential phase cells, as described above, but the subsequent heat treatment reduced the proportion similarly.

Heat-induced bleb formation was examined using various heating menstrua. No substantial differences in the proportion of bleb-bearing cells were detected between 50, 100 and 200 mM Tris buffer (pH 8.0), the proportions at 15 s heating being 11, 13 and 12 %, respectively. These values were similar to that (13 %) in deionized water adjusted to pH 8.0, although the proportion increased to 22 % in 500 mM Tris buffer. Use of other kinds of buffer, HEPES, Bicine and barbital buffer, instead of Tris buffer but at the same pH (8.0) and at the same concentration (50 mM), also induced no substantial changes in the proportion of bleb

formation (10-12 % at 15 s heating). On using a growth medium, EM9, as heating menstruum, the bleb formation occurred in only a few cells (about 1 %) on 15 s heating, but after that, increased with heating time (32 % at 30 min heating), in contrast to heating in Tris buffer. Furthermore, I found that Tris buffer containing 10 mM  $MgSO_4$  markedly inhibited bleb formation, which was less than 1 % during heating up to 30 min, although  $Mg^{++}$  at 1 mM allowed a gradual increase in bleb formation.

No blebs were detected during the incubation period at 0 and 37°C, when observed by phase contrast and electron microscopy.

Cell-surface hydrophobicity of heated cells. When the suspension of exponential phase cells was heated at 55°C in Tris buffer, the affinity of E. coli toward n-hexadecane changed with heating time. A decrease in the absorbance of the aqueous layer reflects an increase in the cell-surface hydrophobicity.

For 37°C-grown cells, the heat-induced increase of hydrophobicity was more marked in cells preincubated at 0°C rather than those at 37°C (Fig. 2-4 a). For 15°C-grown cells, on the other hand, the increase in the surface hydrophobicity of cells preincubated at 0 and 37°C was almost the same (Fig. 2-4 b). Similar results were obtained by cell counting using a haemacytometer instead of the measurement of absorbance (data not shown).

The increase in the surface hydrophobicity was influenced

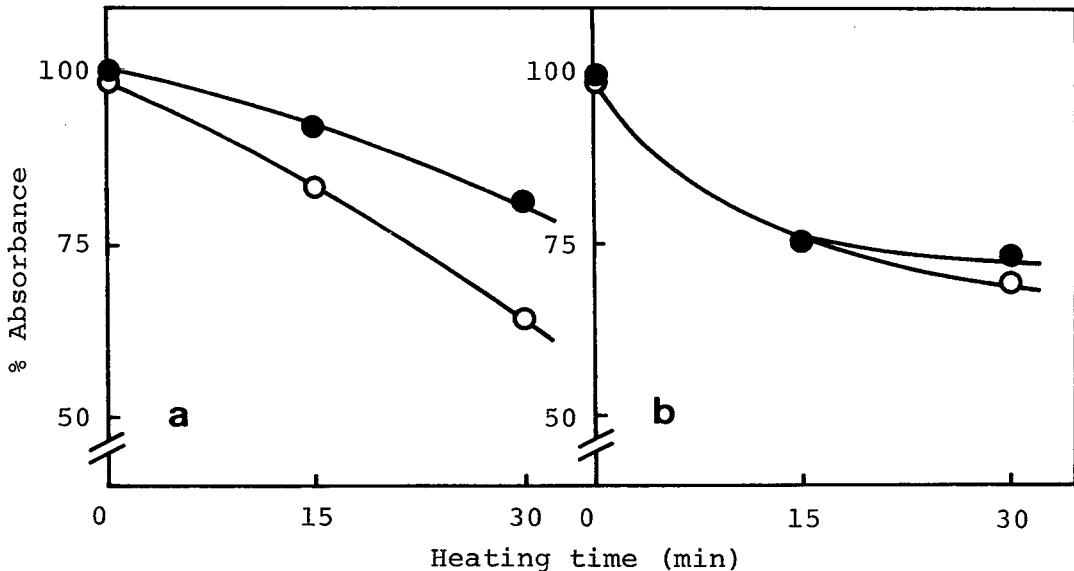


Fig. 2-4. Effect of preincubation temperature on the adherence of heated E. coli W3110 to n-hexadecane. Cells were grown at 37°C (a) and 15°C (b), preincubated at 0°C (○) and 37°C (●) for 30 min, and then heated at 55°C. After mixing the heated cell suspension with n-hexadecane, the turbidity of the lower aqueous layer was measured. The ordinate is the percentage of absorbance of the cell suspension. The results are the average values of three independent determinations.

by the heating menstrua and growth phase. Using EM9 medium and Tris-Mg buffer as heating menstrua, absorbances of the aqueous layer were 81 and 90 % of the control, respectively, after 30 min heating. When stationary phase cells were heated in Tris buffer, absorbance of the aqueous layer was 84 % of the control after 30 min heating whereas it was 64 % for exponential phase cells.

#### DISCUSSION

Surface blebs were observed on heated cells of E. coli W3110 by phase contrast and electron microscopy. A similar phenomenon has been observed by de Petris (13) with E. coli B cells, but cells were heated severely (75-100°C, 5-10 min), and by Scheie and Ehrenspeck (62) with E. coli B/r and B<sub>s-1</sub> cells heated at 49 to 55°C but only with phase contrast microscopy. My electron microscopic studies indicated the occurrence of three types of blebs with different structures: most were multilayered blebs and single-layered blebs without substantial contents, but blebs with cytoplasmic materials were occasionally observed. Most blebs, as illustrated in Figs. 2-1 a-c and 2-2 a-c, seemed to be derived from the outer membrane as judged by microscopic observations. In ultrathin section electron micrographs of gram-negative bacteria, the outer membrane generally shows high electron density and thus is rimmed clearly, whereas the cytoplasmic membrane is seen

less clearly due to its low electron density (47). The outermost layer of a bleb showed a densely stained structure (Fig. 2-2 a-c), implying the outer membrane. The bleb shown in Fig. 2-2 c contained cytoplasmic materials, reflecting additional damage to the cytoplasmic membrane. In addition, the appearance of the freeze-fractured faces of the blebs resembled the outer membrane in the absence of large intramembranous particles (Fig. 2-1 a-c), although the cytoplasmic membrane is in general dominantly fractured rather than the outer membrane when intact cells of gram-negative bacteria were treated (50, 78). This is understandable, if blebs are surrounded only by the outer membrane.

The bleb formation was affected by the heating procedure, heating menstruum and the growth phase of cells. Of cells heated rapidly from 0 to 55°C, 11 % produced blebs visible under a phase contrast microscope immediately after heating (15 s), and the proportion decreased with time during the subsequent heating period at 55°C (Fig. 2-3). This decrease reveals the release of blebs from cells. Therefore, it is likely that, at less than 15 s of heating, more than 11 % of the cells may produce blebs. When the cell suspension was heated from 37 to 55°C, the percentage of bleb formation was lower at 15 s and showed a pattern different from that for cells heated from 0°C. This difference may be due to the change in fluidity of the cell membrane dependent upon the preincubation temperature, similar to the results in Chapter 1

for the heat resistance of cells, and/or due to the degree of heat shock. The stationary phase cells formed much less visible blebs than the exponential phase cells. This may result from the reduction in the number of dividing cells with septa, at which blebbing is apt to occur, as described before, and/or due to the increased rigidity of the cell envelope in the stationary phase.

Tris buffer has recently been reported to alter the outer membrane permeability, resulting in the release of a limited amount of cell envelope components (35). In this investigation, however, the rapid formation of and the subsequent release of blebs in 50 mM Tris buffer at pH 8.0 were not due to a combination effect of heat and the harmful action of the Tris buffer, but due to the heat itself, since the proportion of bleb formation and its change during the heating period were found to be affected neither by higher concentrations of Tris buffer up to 200 mM nor by replacement of Tris buffer with deionized water adjusted to pH 8.0, HEPES, Bicine or barbital buffer at 50 mM and pH 8.0. Furthermore, heating E. coli cells in EM9 medium caused a pattern of bleb formation different from the case of heating in Tris buffer, but similar to the results of Scheie and Ehrenspeck, who used nutrient broth (62): there were only a few bleb-bearing cells (less than 1 %) after 15 s heating and thereafter they gradually increased. Some material(s) in EM9 medium stabilizing bacterial membranes may be responsible for the inhibition of the bleb formation in

earlier stages of heating. Besides, in such an environment blebs might not be easily detached from cells, even if once they are produced. This might possibly explain the bleb formation on many cells after prolonged heat treatment. Magnesium ions are a possible candidate for such a stabilizing substance, since they were found to inhibit blebbing.

No substantial death was detected after 15 s heating, in spite of the formation of visible blebs on 11 % of the total cells. This seems to show that the bleb formation itself is not the direct cause of thermal death, in contrast to the proposal of Scheie and Ehrenspeck (62).

In a study of cell-surface hydrophobicity, intact cells of E. coli showed no significant affinity toward n-hexadecane, suggesting that the cell surface of E. coli is hydrophilic. This result coincides with that of Rosenberg et al. (58). The outer leaflet of the outer membrane in intact cells of gram-negative bacteria is in general occupied by LPS or integral proteins, the outer surfaces of which are hydrophilic and hardly contain any phospholipids (50). The thermal damage to the cell envelope caused the increase in the hydrophobicity of the cell surface. It is likely that the increase in the surface hydrophobicity is due to the release of the outer membrane fragments containing surface blebs and the appearance of the phospholipid molecules on the cell surface. The effects of magnesium ions and the growth

phase of cells on bleb formation and lipid loss (see Chapter 3) caused by heating correspond to the situation for the increase in the surface hydrophobicity. In addition, the results of the cell surface hydrophobicity suggest the influence of the membrane fluidity, in accord with those of the effects of preincubation and postincubation temperatures on the heat resistance of E. coli.

#### SUMMARY

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Surface blebs were formed on the cell envelope of E. coli W3110, when cells were heated at 55°C in Tris buffer. At least 11 % of the cells, as estimated by phase contrast microscopy, possessed such blebs after heat shock treatment of 15 s. From electron microscopic studies, these blebs were found to be mainly located at the septa of dividing cells and to have different types of structures such as single-layered and multilayered blebs, and blebs which included cytoplasmic materials probably resulting from the rupture of the cytoplasmic membrane. Bleb formation patterns were drastically changed with heating rate, heating menstruum and growth phase of cells. In response to bleb formation, the cell-surface hydrophobicity increased with heating. This change seemed to be affected by the membrane fluidity.

# CHAPTER 3 LIPID LOSS AND RELEASE OF OUTER MEMBRANE VESICLES FROM HEAT-STRESSED CELLS OF ESCHERICHIA COLI

## INTRODUCTION

During the heating period parts of membrane components are known to be released from bacterial cells. Hurst et al. (31) found that Staphylococcus aureus cells exposed to sublethal heat lost 30 % of the total cellular lipids in spite of no substantial decrease in colony-forming ability of the cells. Tomlins et al. (72), on the other hand, reported that heating Salmonella typhimurium cells mildly caused no release of lipids, although cells became sensitive to eosine. However, released lipids of S. aureus have not yet been characterized, especially the chemical compositions. For gram-negative bacteria including E. coli, the following questions may be asked: is a substantial amount of membrane lipids in fact released by heat? and, if so, from which layer is it released, the outer or cytoplasmic or both membranes? For other components, Rogers (56) and Hitchener and Egan (27) presented data on the release of LPS-protein complex and LPS, respectively, from heated cells of E. coli, indicating the injury of the outer membrane structure.

In Chapter 2, I described changes of the cell surface of

heated E. coli. I show here the chemical properties of lipids and vesicles released from heated cells of E. coli, and discuss the mechanism of blebbing and vesiculation.

#### MATERIALS AND METHODS

Organism and culture conditions. E. coli W3110 was used throughout this study. Cells were grown at 37°C in EM9 medium, unless otherwise stated, harvested, washed twice and resuspended in 50 mM Tris buffer at pH 8.0. Exponential phase cells ( $3 \times 10^8$  to  $4 \times 10^8$  cells/ml), unless otherwise stated, were employed.

Heat treatment. Aliquots (1 vol) of the cell suspension which was kept at 0°C for 30 min were rapidly added to 9 vol of fresh Tris buffer preheated to 61.1°C for instantaneous heating as previously described, unless otherwise stated. With this operation a final temperature of 55°C can be obtained and thereafter isothermal heating at this temperature was continued in an incubator with shaking at 100 strokes/min.

Lipid extraction and analysis. For the measurement of lipids released by heat treatment, cells were labeled with 1 mM sodium [ $2-^{14}\text{C}$ ]acetate (0.1  $\mu\text{Ci}/\text{ml}$ ) added to the growth medium. After heat treatment the cells were cooled to 0°C and sedimented by centrifugation (3,000  $\times g$ , 5 min, twice) in the cold. The resultant supernatant was re-centrifuged at 12,000  $\times g$  for 10 min.

Lipids were extracted from the resultant supernatant by the method of Bligh and Dyer (4), using commercial phosphatidylcholine (Sigma Chemical Co.) at a concentration of 1 mg/ml of chloroform as a carrier lipid. The lipids extracted were dissolved in chloroform and the solution was absorbed on a glass filter (1.5 x 5 cm, Millipore, AP25). After drying the filter, radioactivity was measured in a Beckman LS-250 liquid scintillation counter. For the determination of total lipids, they were extracted from the cell suspension and the radioactivity was measured as described above.

For the determination of phospholipid composition, I employed two methods, one colorimetric and the other radioautographic. Lipids extracted were separated by thin-layer chromatography on silica gel plates (Merck, no. 5721) with chloroform-methanol-acetic acid (65:25:10, vol/vol) as the solvent. The spots were visualized with either Dittmer reagent or ninhydrin reagent for unlabeled cells. For cells labeled with sodium [ $2-^{14}\text{C}$ ]acetate, the plate was put in contact with a Fuji X-ray film and a radioautogram was thus obtained. Identification of phospholipids was done using authentic samples (Sigma Chemical Co.):  $R_f$  values of spots detected were 0.81, 0.65, 0.48 and 0.15, indicating cardiolipin (CL), phosphatidylglycerol (PG), phosphatidylethanolamine (PE) and lysophosphatidylethanolamine (LPE), respectively. For both methods, the proportion of each phospholipid was estimated using a Shimadzu scanning densitometer CS-910.

Fatty acid composition was determined by gas chromatography as described in Chapter 1.

Freeze-fracturing and electron microscopy. The fraction released by heat treatment was sedimented by high-speed centrifugation (200,000  $\times$  g, 1 h) at 0°C. The pellet was prepared for freeze-fracturing as described in Chapter 2, and the specimen was observed with a JEM-100C electron microscope (Japan Electron Optics Laboratory Co., Ltd.).

Enzyme assay. During the heating period samples were withdrawn, cooled in an ice-bath and then centrifuged at 3,000  $\times$  g for 10 min at 0°C. Each resultant supernatant fraction was divided into two portions. One portion was directly used for the determination of extracellular enzyme activities. The other was sonically treated 3 times at 1.5 A for 5 min each in an ice-bath and then assayed for enzyme activities. Both gave the same results. The following enzymes were assayed: alkaline phosphatase (43) and cyclic phosphodiesterase (49) as periplasmic enzymes, glucose-6-phosphate dehydrogenase (43) and aldolase (12) as cytoplasmic enzymes, and D-lactate dehydrogenase (19) and NADH oxidase (79) as cytoplasmic membrane-associated enzymes. For the determination of total enzyme activities, the cell suspension was sonically treated as described above, centrifuged (6,000  $\times$  g, 10 min) at 0°C, and then the supernatant fraction was assayed. For the assay of membrane D-lactate dehydrogenase in isolated membranes,

cells were grown in nutrient broth. Specific activities of the enzyme in the outer and cytoplasmic membranes isolated were 0.026 and 0.16 units/mg protein, respectively.

Isolation of the outer and cytoplasmic membranes. Following the procedure of Osborn et al. (51), the outer and cytoplasmic membranes were separated. EDTA-lysozyme spheroplasts were lysed by sonication at 1.2 A for 3 min in an ice-bath. After centrifugation, the membrane pellet was resuspended in 30 % sucrose (wt/wt) in 3.3 mM Tris buffer (pH 7.5) containing 5 mM EDTA and then layered on top of a continuous sucrose gradient from 35 to 55 % (wt/wt) containing 5 mM Tris and 5 mM EDTA (pH 7.5). The gradient was centrifuged at 190,000  $\times$  g in a Hitachi RPS-40T rotor at 0°C for 16 h and fractionated by collecting drops from the top of the gradient using an auto Densi-Flow II C (Buchler Instruments), measuring absorption at 280 nm with an ultraviolet monitor. For chemical assays of the outer and cytoplasmic membranes, fractions corresponding to each band were collected, diluted in 20 ml of 50 mM Tris buffer (pH 8.0) and then washed by centrifugation (200,000  $\times$  g, 2 h, 0°C). The resultant pellet was resuspended in 2 ml of distilled water.

Chemical analyses. Total protein was determined by the method of Lowry et al. (41) using bovine serum albumin (Sigma Chemical Co.) as a standard.

Phosphorus was determined by the procedure of Shibuya et al.

(66). Phospholipid content was calculated as 25 times the amount of phosphorus (39).

LPS content was estimated by assaying 2-keto-3-deoxyoctonate using the thiobarbituric acid test (82).

Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis. The electrophoresis of membrane proteins was performed entirely according to Fairbanks et al. (16). Protein samples were incubated at 100°C for 3 min in a mixture of 1 % (w/v) sodium dodecyl sulfate, 10 % (w/v) sucrose, 10 mM Tris buffer (pH 8.0), 1 mM EDTA and 1 % (v/v)  $\beta$ -mercaptoethanol. Solutions (50  $\mu$ l) containing 50-100  $\mu$ g protein were placed on a slab gel (14 cm length, 1 mm thickness) consisting of 7.5 % (w/v) acrylamide, 0.285 % (w/v) bis-acrylamide, 0.1 % (w/v) sodium dodecyl sulfate, 0.025 % (v/v) N,N,N',N'-tetramethylethylenediamine and 0.15 % (w/v) ammonium persulfate and electrophoresed at a constant current of 60 mA. After electrophoresis, the gel was stained with Coomassie brilliant blue and then destained with 10 % (v/v) acetic acid at 37°C for 2 d. Protein standards (BIO-RAD) including (molecular weights in parentheses) phosphorylase B (92,500), bovine serum albumin (66,200), ovalbumin (45,000), carbonic anhydrase (31,000), and lysozyme (14,400) were used for the estimation of molecular weight.

## RESULTS

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Heat-induced release of membrane lipids. Whether membrane lipids are released from E. coli cells on heating was examined. When exponential phase cells labeled with sodium [2-<sup>14</sup>C]acetate were heated rapidly from 0 to 55°C in 50 mM Tris buffer at pH 8.0 as described in Materials and Methods, a significant amount of lipids, estimated by radioactivity, leaked out from cells, as shown in Fig. 3-1. The pattern of the release showed two stages: a rapid initial release caused by heat shock treatment of 15 s and a secondary release at a slower rate during the subsequent heating period. The released amounts were 3.3 and 9.0 % after 15 s and 30 min, respectively. No substantial release of lipids was detected during the incubation at 0 and 37°C even after 30 min (less than 1 %). Magnesium ions added to the heating menstruum at a concentration of 10 mM were shown to inhibit the second stage of the lipid loss but not the initial release (Fig. 3-1). In stationary phase cells, a smaller amount of lipids (2.0 %) was released by the heat shock treatment of 15 s.

### Lipid and fatty acid compositions of the released fraction.

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Table 3-1 shows the phospholipid composition, assayed colorimetrically by phosphorus determination, of the fraction released from E. coli cells heated at 55°C in Tris buffer. As compared with intact cells and heated cells from which the released fraction

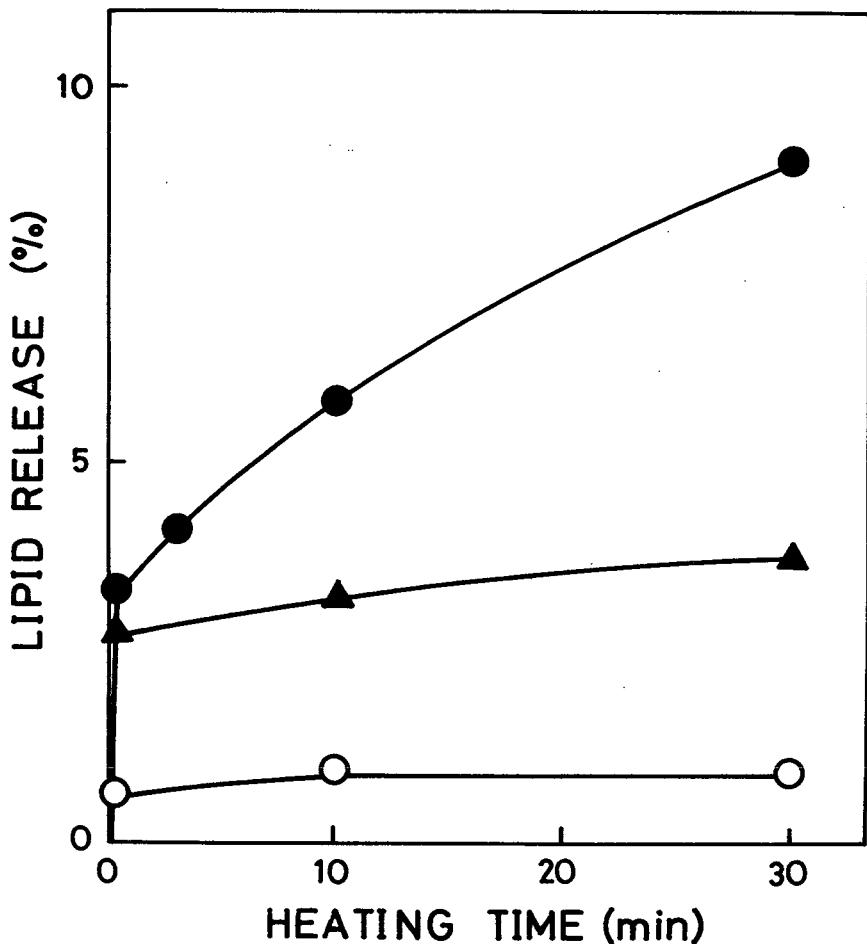


Fig. 3-1. Lipid release from heated cells of *E. coli*. Cells labeled by growing in sodium [ $2-^{14}\text{C}$ ]acetate were kept at  $0^\circ\text{C}$  for 30 min and then heated at  $55^\circ\text{C}$  (●),  $37^\circ\text{C}$  (○) in 50 mM Tris buffer at pH 8.0, and  $55^\circ\text{C}$  in Tris buffer containing 10 mM  $\text{MgSO}_4$  (▲). Heated cell suspension was centrifuged after heating and lipids were extracted from the resultant supernatant, as described in Materials and Methods. Then, the radioactivities of the chloroform layer were counted. The results are the average values of two independent determinations.

Table 3-1. Phospholipid composition of the fraction released from E. coli cells heated at 55°C

| Sample                                      | Phospholipid <sup>a</sup> (% w/w, of total) |     |      |                 | Ratio<br>(PE+LPE)/(PG+CL) |
|---------------------------------------------|---------------------------------------------|-----|------|-----------------|---------------------------|
|                                             | PE                                          | PG  | CL   | LPE             |                           |
| Intact cells <sup>b</sup>                   | 67.6                                        | 6.4 | 26.1 | ND <sup>e</sup> | 2.08                      |
| Heated cells,<br>15 s <sup>c</sup>          | 66.7                                        | 7.1 | 26.1 | ND              | 2.01                      |
| Heated cells,<br>30 min <sup>c</sup>        | 63.8                                        | 6.4 | 26.6 | 3.2             | 2.03                      |
| Released<br>fraction, 15 s <sup>d</sup>     | 70.4                                        | 5.7 | 24.0 | ND              | 2.37                      |
| Released frac-<br>tion, 30 min <sup>d</sup> | 70.1                                        | 4.1 | 22.5 | 3.4             | 2.76                      |

<sup>a</sup> Lipids were extracted from each sample as described in Materials and Methods. The results are the average values of three independent determinations. Abbreviations: PE, phosphatidyl-ethanolamine; PG, phosphatidylglycerol; CL, cardiolipin; LPE, lysophosphatidylethanolamine.

<sup>b</sup> Cells grown in 200 ml of EM9 medium were used for the lipid analysis after washing with 50 mM Tris buffer at pH 8.0.

<sup>c</sup> Cells were sedimented by centrifugation after heating for either 15 s or 30 min and resuspended in fresh Tris buffer.

<sup>d</sup> The fraction was prepared as described in the legend to Fig. 3-1.

<sup>e</sup> ND means not detectable.

was removed by centrifugation, the released fraction was enriched in PE and thus contained less PG and CL. The ratios of PE plus LPE to PG plus CL in the released fraction were 2.37 and 2.76 after 15 s and 30 min of heat treatment, respectively, whereas those in intact and residual heated cells were 2.08 and 2.01-2.03, respectively (Table 3-1). A similar situation of the phospholipid composition was shown by radioautography, using cells labeled with sodium  $[2-^{14}\text{C}]$ acetate. The average contents of PE, PG, CL, LPE and free fatty acids obtained from two independent determinations were 61.3, 12.1, 24.0, <0.1 and 2.5 %, respectively, for intact cells, 54.1, 8.2, 22.9, 3.3 and 11.4 %, respectively, for the released fraction after 15 s heating, and 52.3, 7.9, 18.2, 7.1 and 14.5 %, respectively, for the released fraction after 30 min heating. Therefore, the ratios of PE, LPE plus free fatty acids to PG plus CL were 1.77 for intact cells, and 2.21 and 2.83 for the released fractions after 15 s and 30 min heating, respectively.

During the heating period at 55°C, the content of LPE increased, probably as a result of the deacylation of PE due to the heat-induced activation of (a) phospholipase(s) (Table 3-1). In parallel with this, the proportion of total free fatty acids increased both in residual heated cells and the released fraction, as determined radioautographically by carbon determination.

Table 3-2 shows the fatty acid compositions of intact cells, heated cells and the released lipids. Much saturated fatty acids,

Table 3-2. Fatty acid composition of the fraction released from E. coli cells heated at 55°C

| Sample <sup>a</sup>            | Fatty acid <sup>b</sup> (% w/w, of total) |      |      |      |         |                 |      | Ratio<br>(SFA/UFA) <sup>c</sup> |
|--------------------------------|-------------------------------------------|------|------|------|---------|-----------------|------|---------------------------------|
|                                | 12:0                                      | 14:0 | 16:0 | 16:1 | 17:0cyc | 18:0            | 18:1 |                                 |
| Intact cells                   | 0.2                                       | 3.9  | 47.3 | 34.4 | 1.1     | 0.7             | 12.4 | 1.09                            |
| Heated cells,<br>15 s          | 0.2                                       | 3.0  | 48.1 | 34.8 | 1.6     | ND <sup>d</sup> | 12.2 | 1.06                            |
| Heated cells,<br>30 min        | 0.2                                       | 2.3  | 49.5 | 34.9 | ND      | 0.6             | 14.2 | 1.07                            |
| Released<br>fraction, 15 s     | 5.9                                       | 5.2  | 49.5 | 24.9 | 0.2     | 4.1             | 10.2 | 1.83                            |
| Released frac-<br>tion, 30 min | 3.0                                       | 4.4  | 48.3 | 29.8 | 0.3     | 2.2             | 11.1 | 1.41                            |

<sup>a</sup> See Table 3-1.<sup>b</sup> Fatty acids were analyzed by the method described in Materials and Methods. The results are the average values of three independent determinations. See Table 1-2 for expression of fatty acids.<sup>c</sup> See Table 1-2.<sup>d</sup> ND means not detectable.

lauric (12:0), myristic (14:0) and stearic (18:0) acids, were contained in the released fraction, whereas the contents of unsaturated fatty acids, palmitoleic (16:1) and cis-vaccenic (18:1) acids, and a cyclopropane fatty acid, cis-9,10-methylenehexadecanoic acid (17:0 cyc), were less than in intact and residual heated cells. Therefore, the ratios of total saturated fatty acids to total unsaturated fatty acids (including cyclopropane fatty acid) were 1.83 and 1.41 for the fractions released by heating for 15 s and 30 min, respectively, although the ratios for intact and heated cells were 1.09 and 1.06-1.07, respectively.

Leakage of enzymes. The bleb formation and the release of membrane lipids indicated the damage to the cell envelope of E. coli. One can suppose, therefore, the loss of permeability control in the cell envelope. Then, the leakage of enzymes, which are known to be located at different sites in cells, into the heating menstruum was investigated. Table 3-3 shows the leakage of a periplasmic enzyme, alkaline phosphatase, and a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, during the heating period at 55°C. Both enzymes showed no loss of total activity during heating at 55°C for at least 30 min. The activity of alkaline phosphatase in the fraction released from cells increased with heating time, amounting to 52 % after 30 min. Another periplasmic enzyme investigated, cyclic phosphodiesterase, was also

Table 3-3. Release of enzymes from E. coli cells during heating at 55°C<sup>a</sup>

| Heating time<br>(min) | Activity |       |
|-----------------------|----------|-------|
|                       | APase    | G6PDH |
| 0                     | 0.0      | 0.0   |
| 0.25                  | 2.3      | 0.0   |
| 15                    | 44       | 0.0   |
| 30                    | 52       | 0.0   |

<sup>a</sup> Cells were heated at 55°C for the indicated times in 50 mM Tris buffer (pH 8.0) and then centrifuged. The resultant supernatants were assayed for alkaline phosphatase (APase) and glucose-6-phosphate dehydrogenase (G6PDH). During the heating period tested, no loss of total activity of either enzyme was detected. The results are the average values of two independent determinations.

released, 31 % after 15 min, although this enzyme was inactivated by heat and the total activity decreased to 70 %. On the contrary, no glucose-6-phosphate dehydrogenase activity was detected in the released fraction even after 30 min, as shown in Table 3-3. Also for other enzymes tested, a cytoplasmic enzyme, aldolase, and cytoplasmic membrane-associated enzymes, D-lactate dehydrogenase and NADH oxidase, no activity was detected in the heating menstruum, although these enzymes were inactivated to a large extent during the heating period. These results indicate the massive destruction of only the outer membrane, in response to the profile of damage shown by microscopic observations.

Release of vesicles. The fraction released from heated cells was centrifuged at  $200,000 \times g$  for 1 h and then the amounts of lipids extracted from the resultant supernatant and the precipitate were determined. As shown in Table 3-4, about one-third of the released lipids was sedimented and the release gradually increased with heating time together with the total amount. Furthermore, a freeze-fractured specimen of the fraction sedimented on high-speed centrifugation of a sample after 30 min heating was prepared for electron microscopic observation. Fig. 3-2 is an electron micrograph which evidently indicates the existence of membrane-structured vesicles of various sizes (less than about 0.5  $\mu\text{m}$  in diameter).

Table 3-4. Lipid release from E. coli W3110 cells heated  
at 55°C<sup>a</sup>

| Fraction                 | % Release             |        |        |
|--------------------------|-----------------------|--------|--------|
|                          | 0.25 min <sup>b</sup> | 10 min | 30 min |
| Total <sup>c</sup>       | 3.4                   | 5.6    | 9.1    |
| Supernatant <sup>d</sup> | 2.3                   | 3.9    | 5.1    |
| Precipitate <sup>d</sup> | 0.6                   | 1.1    | 2.6    |

<sup>a</sup> Cells labeled with sodium [2-<sup>14</sup>C]acetate were kept at 0°C for 30 min and then heated at 55°C.

<sup>b</sup> Heating time.

<sup>c</sup> The radioactivity of the released fraction obtained by centrifugation of heated cell suspension was taken as the total.

<sup>d</sup> After high speed centrifugation (200,000 x g, 1 h) of the released fraction, the radioactivities of the supernatant and the precipitate were measured.

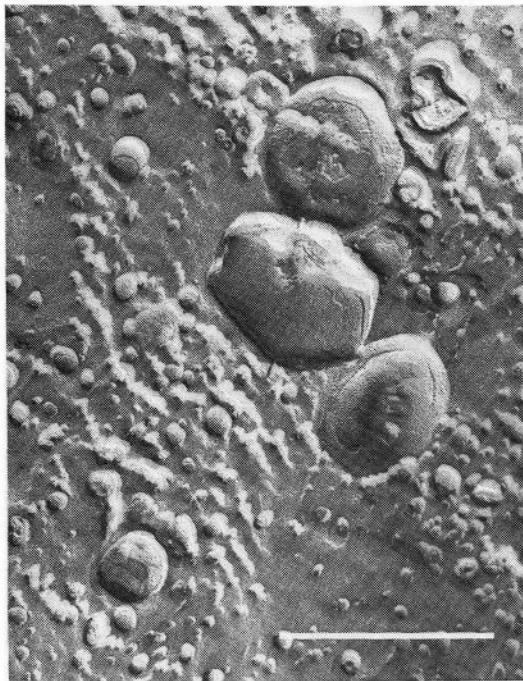


Fig. 3-2. Freeze-fracture electron micrograph of vesicles released from E. coli cells heated at 55°C. The released fraction was centrifuged at 200,000  $\times$  g for 1 h and then a freeze-fracture specimen was prepared from the pellet. The white arrow at the corner of the micrograph shows the direction of shadowing. The bar represents 0.5  $\mu$ m.

Chemical composition of the released vesicle fraction.

Since part of the membrane lipids released by heat treatment into the menstruum was found to form vesicles, as described above, I attempted to analyze their biochemical properties by comparison with those of the outer and cytoplasmic membranes of intact cells. First, I isolated the outer and cytoplasmic membranes from E. coli cells by centrifugation ( $190,000 \times g$ , 16 h) in a sucrose gradient (30 to 55 %) using the method of Osborn et al. (51), as described in Materials and Methods. Fig. 3-3 depicts the separation pattern, showing two bands. The heavier band contained much LPS (Table 3-5), whereas the lighter band contained much membrane D-lactate dehydrogenase, as described in Materials and Methods, thus indicating the outer and cytoplasmic membranes, respectively.

The released vesicle fraction obtained by high-speed centrifugation ( $200,000 \times g$ , 1 h) of a sample heated at  $55^{\circ}\text{C}$  for 30 min was assayed similarly. As shown in Fig. 3-3, this fraction was found to consist of a single band after centrifugation in a sucrose gradient and to have an intermediate buoyant density of  $1.21 \text{ g/cm}^3$  (Table 3-5). The vesicle fraction contained much LPS. Table 3-5 indicates that the LPS/phospholipids ratio of the released vesicle fraction is quite similar to that of the intact outer membrane, whereas the ratios of LPS/protein and phospholipids/protein were approximately twice as large as those of the outer membrane. These results suggest that the vesicles obtained by heating E.

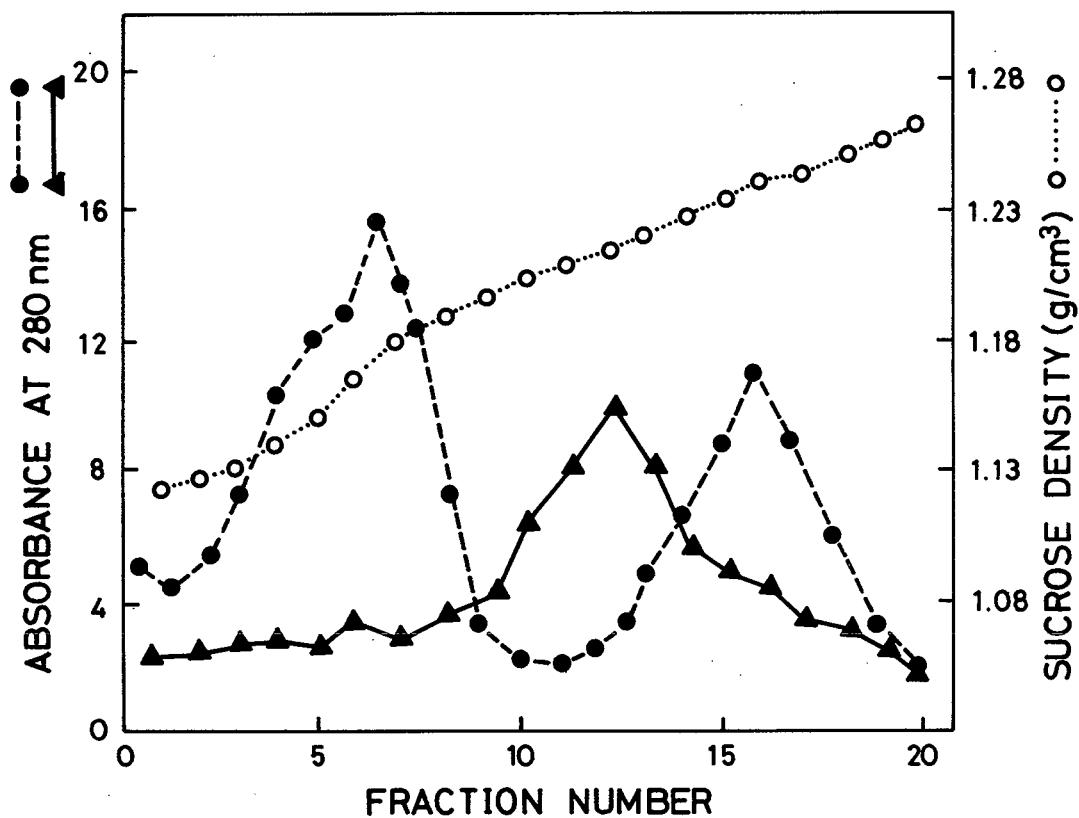


Table 3-5. Chemical compositions of membrane fractions and the released vesicle fraction<sup>a</sup>

| Components                                        | Cytoplasmic membrane | Outer membrane | Vesicle fraction |
|---------------------------------------------------|----------------------|----------------|------------------|
| mg LPS <sup>b</sup> /mg protein                   | 0.07                 | 0.36           | 0.64             |
| mg LPS/mg phospholipid                            | 0.16                 | 1.06           | 0.97             |
| mg Phospholipid/mg protein                        | 0.47                 | 0.32           | 0.66             |
| Buoyant density (g/cm <sup>3</sup> ) <sup>c</sup> | 1.17                 | 1.23           | 1.21             |

<sup>a</sup> Membranes and the vesicle fraction were prepared as described in Materials and Methods. The latter was a sample from cells heated at 55°C for 30 min. The results are the average values of two independent determinations.

<sup>b</sup> LPS, lipopolysaccharide, determined as 2-keto-3-deoxyoctonate.

<sup>c</sup> Buoyant density indicates the value at the peak of each fraction.

coli cells at 55°C are substantially composed of the outer membrane, but that they have a markedly reduced content of protein as compared with the outer membrane.

Sodium dodecyl sulfate-polyacrylamide gel electrophoresis of the vesicle proteins. In Fig. 3-4, sodium dodecyl sulfate-polyacrylamide gel electrophoresis patterns of proteins contained in the vesicle fraction released from E. coli cells after heating at 55°C for 15 s and 30 min are shown together with those of the proteins of the outer and cytoplasmic membranes. The indicated pattern of the vesicle proteins evidently resembles that of the outer membrane rather than that of the cytoplasmic membrane. Bands corresponding to two major proteins of the outer membrane, having apparent molecular weights of 39,000 and 28,500, were found in both vesicle fractions (samples after 15 s and 30 min heating). It should be noted, however, that other bands of outer membrane proteins with apparent molecular weights of 18,000 and 16,500 were much less and one band with an apparent molecular weight of 11,000 was lacking in samples of the vesicle fraction.

#### DISCUSSION

Membrane lipids in E. coli were found to be released into the menstruum on heat treatment under nonlethal conditions (55°C, 15

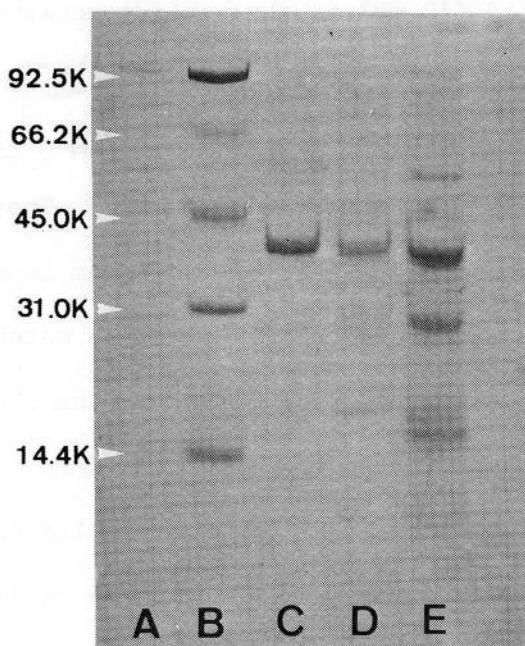


Fig. 3-4. Sodium dodecyl sulfate-polyacrylamide slab gel electrophoresis of the cell envelope proteins of E. coli cells and proteins of the vesicle fraction released from cells heated at 55°C. Lane A, cytoplasmic membrane; lane B, molecular weight marker proteins; lanes C, D, vesicle fractions from samples after heating for 15 s and 30 min, respectively; lane E, outer membrane. The molecular weight markers used and the procedure for electrophoresis are described in Materials and Methods.

s). The initial loss of lipids may result from rapid disturbance of the cell envelope by heat shock and the secondary loss from the time-dependent destabilization by the subsequent heat treatment. Magnesium ions added (10 mM) to the heating menstruum only inhibited the secondary release of lipids, little affecting the initial loss, contrary to the data of Hitchener and Egan (27) on the release of 260 nm-absorbing materials and LPS. These investigators showed that the inhibitory effect of magnesium ions at concentrations of 0.5 and 5 mM on the release of those materials from heated cells of E. coli K-12 was most marked during the first 30 s. These authors, however, did not examine the effect on the lipid loss. The decrease in the loss of membrane lipids on replacing exponential phase cells with stationary phase cells suggests the stabilization of the membranes due to aging of cells.

The heat-induced loss of membrane lipids from E. coli cells may be due to at least part of the outer membrane blebs produced on the cell surface. The kinetics of and the effects of magnesium ions and the growth phase of cells on bleb formation caused by heating correspond to the situation for the lipid loss in this study; especially the secondary release of lipids seems closely related to the reduction in bleb-bearing cells. On electron microscopy, in fact, vesicles of various sizes were found in the pellet obtained on high-speed centrifugation of the fraction released by heat treatment, amounting to about one-third of the

total lipids (Table 3-4 and Fig. 3-2). The remaining two-thirds of the released lipids which were not sedimented are probably extremely small vesicles and/or micelles of lipids.

The lipids in the outer membrane of gram-negative bacteria have in general been reported to contain more PE (37, 42, 51) and saturated fatty acids (37, 42, 85) than the cytoplasmic membrane. Based on this, the fact that the lipid fraction released from heated cells was enriched in PE and also in saturated fatty acids as compared with the lipids in intact cells, which was revealed by thin layer chromatographic analysis (Tables 3-1 and 3-2), may support that the released lipids are substantially derived from the outer membrane, as indicated by microscopic observations.

The hypothesis that almost all blebs produced and the lipids released consisted of the outer membrane was strongly supported by the results of the experiment on enzyme release. E. coli alkaline phosphatase is an enzyme located in the periplasm, the space between the outer membrane and the cytoplasmic membrane, while glucose-6-phosphate dehydrogenase is in the cytoplasm (43). When E. coli was heated at 55°C, the former enzyme was found to leak out from cells, to a large extent, whereas the latter enzyme did not at all (Table 3-3), thus indicating the selective destruction of the outer membrane structure in the sense of inclusion of internal macromolecules. Heat inactivation of enzyme molecules released could be excluded, since the total activities of both

enzymes were completely recovered after sonication even with 30 min of heat treatment. In addition, the lack of leakage of the cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, reveals there was no cell lysis for at least 30 min of heating at 55°C.

The similarity of the relative contents of phospholipids and LPS in the released vesicle fraction to those of the intact outer membrane also supports the hypothesis that the vesicles may be derived from the surface blebs consisting of the outer membrane. However, the vesicle fraction was found to have a relative content of protein markedly lower than that of the outer membrane isolated from intact cells. This may be responsible for the lower density ( $1.21\text{ g/cm}^3$ ) of the vesicles compared to the outer membrane ( $1.23\text{ g/cm}^3$ ) (Table 3-5).

The E. coli outer membrane has been reported to contain several major proteins (25, 50). Such proteins were also observed with the outer membrane isolated in this study. Among them, the protein band with an apparent molecular weight of 39,000, which may be a set of major proteins (50), also clearly appeared in the released vesicle fraction. However, proteins showing lower molecular weights were less and the protein with an apparent molecular weight of 11,000 was lost from vesicle fractions (Fig. 3-4). This suggests that vesicles including a larger relative amount of the protein with an apparent molecular weight of 39,000 were released selectively from cells, leaving other outer membrane pro-

teins on the cell surface, in accord with the result of the reduction in the protein content of the vesicle fraction.

The mechanisms of bleb and vesicle formation due to heat remain to be investigated. The emergence of blebs from cells immediately after starting heat treatment at 55°C (15 s), together with the absence of nutrients, evidently indicates that blebbing does not result from unbalanced synthesis of the outer membrane during heating, as Scheie and Ehrenspeck (62) also indicated.

In order to form blebs and vesicles, part of the outer membrane should be released from the underlying peptidoglycan by breakage of the interaction between these layers. Several proteins have recently been shown to be involved in this interaction including murein lipoprotein (5, 34) and peptidoglycan-associated lipoproteins (46, 84). One can suppose, therefore, that the loss of this association induces the abnormality in bacterial envelopes. In fact, Suzuki et al. (71) and Fung et al. (18) found that E. coli lpo mutants with defects in the lipoprotein produced single-layered blebs of the outer membrane and that the bleb formation was prevented by adding  $Mg^{++}$  to the growth medium. These mutants were also reported to release periplasmic enzymes during growth (18, 26, 71). Interestingly, these events markedly coincide with my results. If these lipoproteins are detached from the outer membrane matrix due to heat treatment, they should not be released into the heating menstruum. This might explain the decrease in

or loss of the contents of proteins with lower molecular weights in the released vesicles in this study, although whether these proteins correspond to peptidoglycan-associated lipoproteins remains unclear.

A similar phenomenon of the partial release of the outer membrane from gram-negative bacteria has been recently observed with other stressed cells, freeze-thawed cells of E. coli B (68) and cells of an E. coli LPS-deficient strain treated with citrate-Tris (36). It was also shown that these stressed cells released periplasmic enzyme(s) in agreement with my results. Moreover, even normally growing cells of E. coli have been reported to release outer membrane fragments into the medium, although the amounts released were markedly less (0.3-0.5 % of the total cell protein) (28) than that from the heated cells in this study.

In conclusion, heating E. coli cells rapidly may make their outer membrane unstable with no membrane-stabilizing substance added and produce blebs of the outer membrane, especially multi-layered ones, probably by inducing the rapid detachment of the outer membrane from the anchoring murein lipoprotein, together with loss of certain amounts of lipids. In addition, under these circumstances, the produced blebs may be fragile and apt to be released from cells into the heating menstruum to form vesicles, which lack or have decreased contents of several outer membrane proteins.

SUMMARY  
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Membrane lipids were released from the cells during the heating period. These lipids were enriched in PE rather than PG or CL, and also in saturated fatty acids rather than unsaturated fatty acids, as compared with lipids from intact cells. After heating bacterial cells at 55°C for 30 min, the resultant leakage of a periplasmic enzyme, alkaline phosphatase, from the cells amounted to 52 % of the total activity, whereas no release of a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, was detected. Part of the released lipids showed vesicle-like structures of the membrane. This vesicle fraction had a ratio of LPS to phospholipids similar to that of the outer membrane of intact cells, whereas it had a lower content of protein than the isolated outer membrane. The results obtained suggest that surface blebs formed by heat treatment almost completely consist of the outer membrane and that the blebs may be gradually released from the cell surface into the heating menstruum to partially form vesicles.

## GENERAL DISCUSSION AND CONCLUSION

The process conditions for heat sterilization are determined by the heat resistance of an objective microorganism, together with the heat penetration data for food. The heat resistance of microorganisms is influenced by a number of factors and its change therefore is a problem for practical use. Although numerous investigations have been done, what changes the heat resistance of cells is unknown.

At the beginning of this investigation, I looked at temperatures at which microorganisms are held prior and subsequent to heat treatment as an instance of factors influencing heat resistance. In Chapter 1, I examined the effect of these temperatures on E. coli cells which have different compositions of fatty acids in membrane lipids by growing them under different conditions. The results obtained suggest that the change of the membrane fluidity due to gel-liquid crystalline phase transition of their membrane phospholipids is the cause of the effects. That is, above the temperatures of preincubation and postincubation corresponding to the phase transition temperatures, implying the membrane is fluid, the viability remains almost constant, whereas below those temperatures, implying the membrane lipids are converted to gel state gradually with decreasing temperature, the viability seems

to decrease in response to the change of membrane fluidity. These phenomena were also observed with other gram-negative and positive bacteria, and yeasts.

The results obtained suggest that for the effective use of the heat sterilization process the object such as food should be heated rapidly from a low temperature and cooled immediately to a low temperature after heat treatment. In addition, in the testing of heat resistance of microorganisms, attention should be paid to the temperatures before and after heat treatment. Otherwise, the lethality evaluation of a heat process will be unreliable, since it is based on the heat resistance data of an objective microorganism.

The possibility that the physicochemical state of the membranes was concerned with the heat resistance of microorganisms was put forward, as described in Chapter 1. Therefore, I became interested in how bacterial membranes were damaged by heat and examined this (Chapters 2 and 3).

When E. coli cells were heated at 55°C, blebs were formed on their surface, mainly at the septa of dividing cells. Most were multilayered blebs and single-layered blebs without substantial contents, although blebs with cytoplasmic materials were occasionally observed. Membrane lipids were released from the cells into the menstruum during the heating period. About one-third of the released lipids was sedimented by centrifugation at 200,000  $\times g$

for 1 h and showed membrane-structured vesicles. These vesicles probably are derived from the surface blebs. It was indicated that the apparent structural changes in the cell envelope observed were found to occur substantially in the outer membrane rather than in the cytoplasmic membrane, based on the following evidence.

- 1) In electron microscopic studies, the structure of blebs was similar to that of the outer membrane.
- 2) The lipid fraction released from heated cells was enriched in phosphatidylethanolamine and also in saturated fatty acids as compared with the lipids in intact cells. This result corresponds to the characteristics of the outer membrane of E. coli.
- 3) During heating, a periplasmic enzyme, alkaline phosphatase, was released from the cells, whereas no release of a cytoplasmic enzyme, glucose-6-phosphate dehydrogenase, was detected.
- 4) In the gel electrophoresis study, the protein bands observed with the released vesicles were similar to those observed with the outer membrane of intact cells, although proteins showing lower molecular weights were less in the vesicle fraction.
- 5) Released vesicles had a ratio of LPS to phospholipids similar to that of the outer membrane of intact cells, but they had a lower content of protein than the isolated outer membrane, in accord with the result of less protein bands in the vesicle fraction.

If the outer membrane lipoproteins, which are found to asso-

ciate with peptidoglycan and have lower molecular weights, are detached from the outer membrane matrix due to heat treatment, the decrease in the relative amount of protein can be explained. As a possible mechanism of blebbing and vesiculation, it is proposed that the outer membrane detached from underlying peptidoglycan forms a more stable spherical structure, namely a bleb, and further, part of the blebs is released from the cell surface into the heating menstruum to form vesicles resulting from the physico-chemical nature of a membrane. The possibility of osmovesiculation by rapid heating also cannot be ruled out.

Such a structural change in the cell envelope caused the increase in the hydrophobicity of the cell surface which is originally hydrophilic mainly due to the presence of LPS in the intact cell surface (50). This may imply the emergence of phospholipids on the cell surface, since phospholipids hardly exist in the outer leaflet of the outer membrane (50). The experimental results of the cell surface hydrophobicity suggested the influence of the membrane fluidity, in accord with those of the effects of preincubation and postincubation temperatures on the heat resistance.

In addition, the sensitization of E. coli and probably other gram-negative cells to a hydrophobic agent by heat treatment can be predicted from this study. Although many reports have been published on combined treatment of food by heating and the addition of an antimicrobial agent (64, 75), there have been few investiga-

tions on characterization in detail of the mechanism of the combined effect. The sensitization to several hydrophobic agents has been observed with EDTA-treated cells (40). If this is the case, systematic investigations on the heat-induced sensitization of gram-negative bacteria to various hydrophobic agents in relation to the degree of hydrophobicity of agents might provide information for a useful method for sterilizing or preserving food by a combination of heat and a potent chemical agent.

As described in Chapters 2 and 3, I proved the structural changes of the outer membrane of E. coli due to heat treatment. However, I did not examine the damage to the cytoplasmic membrane. The cytoplasmic membrane has many functions essential for growth and survival, e.g. active transport, oxidative phosphorylation and biosynthesis of envelope macromolecules (9). Damage to the functions of the cytoplasmic membrane by heat remains to be investigated.

I believe that investigations of various fundamental aspects of injured cells subjected to heat stress explain a variety of responses to different environmental factors on heat treatment and one can find a clue to the mechanism of the thermal inactivation of a microbial cell.

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LIST OF RELATIVE PAPERS BY THE AUTHOR

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