Suggested short title

LOSS OF VIABILITY OF BACTERIAL CELLS ON FREEZING

Kuo

ABSTRACT

Ph.D. Shou-Chang Kuo Microbiology

STUDIES ON THE MECHANISM OF LOSS OF VIABILITY OF BACTERIA CELLS ON FREEZING

Suspensions of Escherichia coli 451B, but not Aerobacter aerogenes (Mac 112), after freezing and thawing gave higher plate counts when a glucose salts medium was supplemented with 2% Trypticase. Hydrolysis with acids partially inactivated the Trypticase. Treatment of an H_2SO_4 hydrolysate with a cation exchange resin greatly improved its count-increasing activity. Aspartic acid was as effective as Trypticase as a supplement to the minimal medium. Glutamic acid and alanine were also very effective. Various other amino acids repressed the count. Other species and strains of Gram negative bacteria responded uniquely to the supplements after freezing.

The effect of inorganic salt solutions as freezing menstrua for survival of A. aerogenes (Mac 112) was studied. NaCl, KCl and $MgCl_2$ were found to be toxic at concentrations above 0.05 M but sulfates were not. Below 0.01 M, NaCl and particularly $NaNO_3$ and Na_2SO_4 gave protection against freezing and thawing.

Freezing and thawing caused cells of A. aerogenes (Mac 112) to leak intracellular solutes and decreased the activity of malic dehydrogenase, ATPase and $NADH_2$ oxidase.

STUDIES ON THE MECHANISM OF LOSS OF VIABILITY OF BACTERIAL CELLS ON FREEZING

by

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A thesis submitted to the Faculty of Graduate Studies and Research in partial fulfilment of the requirements for the degree of Doctor of Philosophy

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Suspensions of Escherichia coli 451B, but not Aerobacter aerogenes (Mac 112), after freezing and thawing gave higher plate counts when a glucose salts medium was supplemented with 2% Trypticase. Hydrolysis with acids partially inactivated the Trypticase. Treatment of an H_2SO_4 hydrolysate with a cation exchange resin greatly improved its count-increasing activity. Aspartic acid was as effective as Trypticase as a supplement to the minimal medium. Glutamic acid and alanine were also very effective. Various other amino acids repressed the count. Other species and strains of Gram negative bacteria responded uniquely to the supplements after freezing.

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CLAIM OF CONTRIBUTION TO KNOWLEDGE

(1) It was found that aspartic acid could replace Trypticase completely as a supplement to the minimal medium for Escherichia coli 451B after freezing. Glutamic acid and alanine were also very effective. Some amino acids depressed the counts.

(2) The requirement for aspartic acid by a portion of the Escherichia coli 451B population was shown not to be due to the formation of stable auxotrophic mutants.

(3) The response of other species and strains of Gram negative bacteria to the supplements after freezing was found to be unique for each organism.

(4) The comparative effect of glass-distilled water and various inorganic salt solutions as freezing menstrua on the survival of Aerobacter aerogenes (Mac 112) was investigated and it was found that chloride salts at concentrations above 0.05 M caused damage to the cells during freezing and thawing. The sulfate salts tested did not cause damage.

(5) At concentrations below 0.01 M, NaCl and particularly $NaNO_3$ and Na_2SO_4 were found to exert a protective

effect on the survival of Aerobacter aerogenes (Mac 112) during freezing and thawing.

(6) It was found that freezing and thawing caused cells of Aerobacter aerogenes (Mac 112) to leak K^+ and small amounts of malic dehydrogenase, and decreased the activity of malic dehydrogenase, ATPase and $NADH_2$ oxidase. The loss of enzyme activity correlated in a general way with loss of cell viability.

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GENERAL INTRODUCTION

Interest in studies on freezing and freeze-drying of bacteria has increased tremendously during the past decade. If a population of cells is frozen or frozen and lyophilized under appropriate conditions a proportion of the cells in the population can remain viable and apparently unchanged for indefinite periods. Therefore, freezing and freeze-drying as a means for preservation of a large variety of bacteria has become a common practice. However, bacterial cells subjected to subzero temperature do not always remain undamaged.

It has been observed repeatedly with a number of bacterial species that exposure to subzero temperature causes the organisms to become more exacting in their nutritional requirements. Suspensions of the cells give the same counts before freezing on both minimal and enriched agar media. After freezing and storage, a reduction of the total number of viable cells is observed, but plate counts are always higher on the enriched medium. Those cells growing only on the enriched medium are referred to as "metabolically injured" cells. Some attempts have been made

to identify the nature of the component(s) in enriched media which enhance the survival (Straka and Stokes, 1959; Arpai, 1964; Moss and Speck, 1966a; MacLeod et al., 1966, 1967). However, the nature of the effective component(s) involved still remains in controversy. Escherichia coli 451B, which has been used by Straka and Stokes (1959), and Moss and Speck (1966a), and several strains of Gram negative organisms were therefore employed to investigate further the nature of the component involved.

In previous studies of the effect of various freezing menstrua on the viability of Aerobacter aerogenes (Mac 112), Kuo (1966) confirmed previous findings that glass-distilled water provided a relatively innocuous environment for freezing. The most lethal effects were observed when NaCl was used as a suspending fluid. In the present study, the effect of various salts as freezing menstrua on the loss of viability of the cells has been examined. An effort has been made to localize the action of NaCl in bacterial cells by determining internal and external concentrations at the various levels of salt solutions used as freezing menstruum.

When suspended cells are frozen and thawed, a proportion is unable to grow. This is evidenced by their inability to form colonies on enriched agar media. Many

hypotheses have been put forward concerning the cause of death of cells by freezing, but none has been substantiated. One aspect of the present study was to investigate whether the loss of viability of cells by freezing could be correlated with the loss of particular intracellular materials or enzyme activities.

REVIEW OF LITERATURE

Effect of freezing menstrua on viability of bacteria

Keith (1913), while studying factors affecting survival of bacteria at freezing temperatures, found that the suspending fluid in which bacteria were frozen was of importance in relation to the survival of the organisms. He observed that food materials, e.g., egg or milk, provided conditions which permitted greater bacterial survival than plain water. He also suggested that 5 to 42% aqueous glycol solutions allowed bacteria to survive to six months at $-20^{\circ}C$; 20% or more glycerol was antiseptic above freezing but very protective below freezing.

Squires and Hartsell (1955) demonstrated that the survival of defrosted Escherichia coli was affected by the menstrua used for frozen storage. They found that a phosphate buffer-glycerol-water solution provided the best protection during freezing. Harrison (1956) noted that the survival of Escherichia coli stored at $-22^{\circ}C$ was increased by decreasing the amount of solute present in the suspending medium and was best in distilled water. NaCl was found to

be strongly lethal. Glycerol was able to counteract to some extent the lethality of NaCl.

Straka and Stokes (1957) stated that the percentage of survivors after freezing, storage and thawing varied with the nature and pH of the suspending medium. They found that 0.5% aqueous beef extract provided greater protection to Escherichia coli than an aqueous solution of 1% peptone, 1% yeast extract or 10% skim milk.

Postgate et al. (1961) observed that 100% survival was obtained after dropping bacteria, suspended in 10% aqueous glycerol, into liquid nitrogen and thawing. The most lethal medium was dilute NaCl solution; broth, water or a dilute salt mixture (9 parts 0.137 M NaCl + 1 part 1:5 mixture of 0.066 M KH_2PO_4 + 0.066 M Na_2HPO_4; pH 7.4) was moderately lethal.

Arpai (1962), studying the reconstitution of motility of organisms which had been frozen showed that the protective action of a suspending fluid against death of an organism and the rate of reconstitution of motility decreased in the order of the following suspending fluids: 10% skim milk; 1% yeast extract; 0.5% beef extract; 0.5% peptone and distilled water. He stated that the nature of

the suspending fluid and the pH have a marked effect on the survival and on the extent of bacterial injury by freezing. He found a pH of 6.8 to 7.2 to be the most satisfactory for survival and protection against injury, as expressed by the loss of motility.

Sokolski et al. (1964) found that the presence of NaCl in the suspending medium was highly detrimental to rapidly frozen cells of Lactobacillus leichmanii. Only about 1% survived freezing in a medium containing 0.9% NaCl, whereas nearly 100% survived freezing in a NaCl-free vitamin B_{12} basal medium.

Of interest in this respect are the results of studies by Kuo (1966) of the relative effect of glass-distilled water, NaCl (0.05 M), and NaCl + glycerol (10%) as freezing menstrua on the viability of Aerobacter aerogenes (Mac 112) and of the sensitivity of frozen cells to Cu^{++}. Cells suspended in glass-distilled water for freezing resulted in 15% loss of viability, and only a small amount of cells was injured. On the other hand, about 80% of cells suspended in 0.05 M NaCl for freezing were killed after being frozen and stored for three weeks at $-20^{o}C$. Glycerol and several other hydrophilic non-electrolytes are known to protect cells of bacteria from damage by freezing and thawing. Cells

suspended in NaCl plus glycerol for freezing lose only 15% of their viability after being frozen and stored for three weeks at -20°C. The lethal effect of NaCl on frozen cells was entirely eliminated by addition of glycerol to the freezing menstruum. A comparison of the sensitivity of cells to Cu^{++} showed that unfrozen cells, suspended in either 0.05 M NaCl, or NaCl plus glycerol were more resistant to the germicidal action of Cu^{++} than cells suspended in glass-distilled water. However, cells that were frozen and stored, and suspended in 0.05 M NaCl were more sensitive to the germicidal action of Cu^{++} than those cells suspended in either glass-distilled water or NaCl plus glycerol.

Effect of the plating diluents on the viability of frozen bacteria

Even in the case of non-frozen cells, the diluents used for making dilutions for plate counts have been shown to have an effect upon the count obtained. Winslow and Brooke (1927) noted that unwashed cells remained viable in diluents much longer than washed cells, and that the lethal effect of distilled water as a diluent could be negated by the addition of 1% nutrient broth to the diluent. Butterfield (1932) found that the mixed bacterial populations from river water may decrease as much as 60% in 30 minutes when

diluted with distilled water, and found that phosphate buffer (3×10^{-4} M; pH 7.2), or phosphate buffer containing $CaCl_2$, $MgSO_4$ or $FeCl_3$, when used as diluents, gave rise to more consistent bacterial counts. King and Hurst (1963) observed that the best diluent fluid for four bacterial species, i.e., Staphylococcus aureus, Streptococcus pyogenes, Salmonella typhimurium, and Escherichia coli, was 0.1% peptone solution. Tap water, tap water treated with charcol, quarter-strength Ringer's solution, or glass-distilled water were bactericidal to one or more of the test species. Viability of all test species was sharply decreased by 0.85% NaCl.

The nature of the diluent fluid used has also been found to be important when cells which have been frozen and thawed are being counted. Straka and Stokes (1957) demonstrated that distilled water or tap water as the plating diluents rapidly lowered the viability of natural populations of bacteria in frozen meat pies. Phosphate diluent (3×10^{-4} M; pH 7.2) was the least toxic but nevertheless still remarkably so. A diluent consisting of 0.1% peptone in the pH range of 6.0 to 7.3 was satisfactory for the determination of bacterial numbers in frozen meat pies.

Bretz and Hartsell (1959) found that the plate-count

survival of frozen and thawed Escherichia coli was increased by adding 20% sucrose to the plating diluent. However, Postgate and Hunter (1963), studying the frozen and thawed cells of Aerobacter aerogenes, showed that plating diluents such as hypertonic sucrose solutions, hypotonic environments (distilled water or glycerol solution) as well as an isotonic buffer (saline-phosphate buffer), resulted in similar viabilities. Therefore, the osmosensitivity of frozen cells proposed by Bretz and Hartsell (1959) was excluded.

Working with Aerobacter aerogenes (Mac 112), MacLeod, Kuo and Gelinas (1967) found that when the plating diluent consisted only of distilled water, even unfrozen cells of the organism developed symptoms of metabolic injury as evidenced by increased counts when a complex supplement was added to the minimal medium. The effect produced by the distilled water has been traced to its content of toxic trace elements. One of the elements, Cu^{++}, when added to redistilled water at the concentration present in distilled water, i.e., 1×10^{-7} M, reproduced the effect of the distilled water. High cell density, supernatant solutions from suspensions of the organism, buffers and salts overcame the effect of the distilled water and also counteracted the toxicity of Cu^{++}. Mg^{++} and, to a lesser extent, phosphate buffer protected unfrozen cells, but not cells which had been

frozen and stored, against the loss of capacity to grow on minimal medium.

Effect of cell concentrations on the viability of frozen bacteria

Major et al. (1955) demonstrated that there was a direct relation between the density of cells of Escherichia coli, Salmonella gallinarum, Serratia marcescens, Micrococcus pyogenes var. aureus and Bacillus pumilus in the freezing suspension and the percentage survival. On the other hand, the percentage survival of Lactobacillus fermenti, Lactobacillus acidophilus, Microbacterium flavum, Chromobacterium organgium, and Bacillus coagulans was not related to the concentration of cells. Bretz (1961), studying freezing and storage of Escherichia coli suspended in phosphate buffer solution, found that 60% survived 7 days storage at -9°C when the original population was 2×10^9 per ml., but only 4% survived when it was 2×10^5 cells per ml.

The protection against freezing obtained at high cell densities has generally been attributed to the solutes that escape from cells injured by freezing. Bergmann et al. (1957) studied the freeze-drying of Br. abortus and extracted a lecithin from the organism. This substance was able to protect cells of Br. abortus when the organism was freeze-

dried in tryptose suspensions. Bretz and Ambrosini (1966) demonstrated that a protective factor from heavy suspensions of Escherichia coli was carbohydrate in nature.

Packer et al. (1965) provided evidence that the higher sensitivity of a diluted cell suspension of Escherichia coli to repeated freezing was due not to the reduction in the number of cells but to the dilution of substances in the spent growth medium. They found that the viabilities were constant at cell concentrations ranging from 10^4 to 10^9 per ml. if the plating diluents were made with a cell-free spent growth medium.

Nonlethal injury of the bacteria after freezing and thawing - Nutritional requirements

Bacterial populations that have been partially killed by physical or chemical agents (e.g., ultraviolet light, heat, $HgCl_2$ or phenol) show different proportions of survival according to the character of the medium used to assay viability. Enriched media generally yield greater recoveries than minimal media. Bacteria that have been frozen and thawed demonstrate a similar phenomenon.

Straka and Stokes (1959) first described survivors able to grow on an enriched medium but not on a minimal

medium as "metabolically injured" cells, the injury being expressed as a nutritional requirement for materials that were probably peptide in nature. Organisms among which such metabolically injured cells have been observed include Escherichia coli (Straka and Stokes, 1959; Arpai, 1962, 1964; MacLeod et al., 1966; Moss and Speck, 1966a), Aerobacter aerogenes (Postgate and Hunter, 1963; MacLeod et al., 1966, 1967), Streptococcus lactis (Moss and Speck, 1963), Shigella sonnei (Nakamura and Dawson, 1962), and Pseudomonas species (Straka and Stokes, 1959).

Some attempts have been made to identify the specific components in enriched medium that enhance survival. Straka and Stokes (1959) found that the most effective compound was Trypticase and attributed its effect to the activity of the peptide components since enzymatic digest of casein was active, but acid hydrolyzed casein was not. They concluded that peptides might be the active substances in the enzymatically digested casein and might be required by injured cells for the resynthesis of essential protein denatured by the subzero temperature.

Arpai (1964) observed that recovery of injured cells of Escherichia coli on enriched media after freezing and storage was due to active components in yeast extract or in

Trypticase, which have not been positively identified. Acid or alkaline hydrolysis of yeast extract resulted in little if any change in activity. Autoclaving had little effect on activity. Sixteen individual amino acids at a concentration of 0.01 per cent tested were inactive. An amino acid mixture was found to be partially effective.

Recently, Moss and Speck (1966a) claimed to have identified the nutritional components responsible for the recovery of Escherichia coli injured by freezing and storage as peptides in the complex medium, Trypticase soy agar. MacLeod et al. (1966, 1967), on the other hand, found that mixtures of amino acids or cysteine were as effective as more complex supplements to the minimal medium in increasing the bacterial count on suspensions of Aerobacter aerogenes (Mac 112) or Escherichia coli (Mac 22) which had been frozen and stored. They found that the supplements did not increase the count after freezing if steps had been taken to remove toxic metals from the plating diluent. They proposed that freezing and storage damaged the cytoplasmic membrane of a proportion of the cells rendering them more penetrable by toxic metal ions present as trace contaminants. The enriched medium would permit growth of damaged cells by providing compounds capable of chelating the toxic ions.

Permeability damage of frozen and thawed bacteria

Sato (1958) observed that freezing and thawing increased the concentration of water-soluble materials in the fluid surrounding cells of Bacillus megatherium. Kohn and Szybalski (1959) showed that Escherichia coli which was not normally sensitive to lysozyme, was rendered so after freezing. The immediate addition of lysozyme after thawing, or the addition of lysozyme prior to freezing, was necessary to obtain lysis of the cells since the lysozyme sensitivity of the cells was rapidly lost at 37°C. At this temperature complete resistance to lysozyme was regained within 60 minutes in nutrient broth or 120 minutes in synthetic medium. However, at 0°C or in nutrient-free medium the sensitivity to lysozyme was retained. They speculated that the freezing and thawing of Escherichia coli caused tearing of the outer lipoprotein layer which thus permitted the lysozyme to penetrate to the lysozyme-sensitive rigid component of the cell wall.

Lindeberg and Lode (1963) found that ultraviolet absorbing materials appeared in the extracellular fluid of frozen and thawed suspensions of Escherichia coli in amounts proportional to the loss in viability. Mazur (1963) showed that the increase in the concentration of solutes in

external medium of Escherichia coli was roughly proportional to the loss in viability caused by freezing and thawing. Strange and Postgate (1964) observed that frozen and thawed cells of Aerobacter aerogenes were more permeable to exogenous RNAse than unfrozen cells. MacLeod, Kuo and Gelinas (1967) found that Aerobacter aerogenes (Mac 112) after freezing and storage were rendered much more sensitive to toxic trace contaminants such as Cu^{++} in the plating diluents.

Razin and Argaman (1963) observed that normal cells of Micrococcus lysodeikticus and spheroplasts of Escherichia coli remain unlysed after ten successive cycles of freezing and thawing between -24^{o} and $40^{o}C$. On the other hand, protoplasts of Micrococcus lysodeikticus lysed almost completely after several cycles. Pleuropneumonia organisms possess no rigid cell wall, but they were completely resistant to lysis when 0.25 to 1.0 M sucrose was used as a suspending fluid for freezing.

Release of biologically active peptides from Escherichia coli at freezing temperature was demonstrated by Moss and Speck (1966b). They observed that leakage material from frozen cells contained protein in the form of peptides of relatively small molecular weight. These compounds

protected a diluted cell suspension from the lethal effects of freezing and also possessed biological activity for the recovery of cells which had been injured by freezing.

Enzymatic and metabolic damage of frozen and thawed bacteria

The possibility that deep freezing might affect the respiratory system was postulated by Postgate and Hunter (1961). A population of Aerobacter aerogenes (equivalent to 10 mg. dry wt. per ml.) frozen in 10% glycerol was thawed in 10 volume of saline phosphate and the QO_2 was determined manometrically at 40°C. It was found that cells which had been frozen had a respiratory activity 20 to 25% lower than control cells which had been chilled to -4°C in glycerol. Unfortunately, the plate count survivals were not reported in their experiments.

Sato (1958) studied the relationship between viability and the respiratory activity of the cells of Bacillus megatherium before and after freezing. He observed that the endogenous and exogenous respiration of the organism was less reduced by freezing and thawing than were colony counts; after one cycle of freezing and thawing, the former dropped to 75-90% of normal whereas survival dropped to 25-40%.

Kuo (1966) studied the effect of freezing and storage on the respiratory activity of Aerobacter aerogenes (Mac 112) using glucose, yeast extract, or an amino acid mixture approximating the amino acid composition of yeast extract as oxidizable substrate respectively. In the case of glucose, respiratory activity correlated well with viability as determined by bacterial count on minimal medium. With the amino acid mixture, and yeast extract, freezing and storage decreased viability more than respiratory activity. Ahn et al. (1964) observed that freezing and thawing in diluted albumin solution had no effect on viability and lactic oxidation of Staphylococcus aureus, but the oxidation rates of alanine, glutamic acid, or glucose were decreased.

Hansen and Nossal (1955) studied the effect of freezing and thawing on the enzymatic activity of Saccharomyces cerevisiae, and found that the activity of the dehydrogenases of a number of substrates (succinate, lactate, citrate, malate, fumarate, and glutamate) were about the same as with extracts from unfrozen cells. Brodley (1963) observed that the ability of Saccharomyces pastorianas to ferment glucose and sucrose after freezing was closely correlated with the per cent survival.

Genetic stability of bacteria to freezing and thawing

The replicate plate technique has been employed to investigate whether the metabolic injury caused by freezing and storage was permanent and hence inheritable. Postgate and Hunter (1963) have reported that out of 2135 survivors in suspensions of Aerobacter aerogenes which had been frozen and thawed only three auxotrophic mutants unable to grow on minimal medium were obtained. MacLeod, Smith and Gelinas (1966), studying Escherichia coli (Mac 22) and Aerobacter aerogenes (Mac 112), also concluded that stable auxotrophic forms of bacteria were not formed by freezing.

Ashwood-Smith (1965), working with Pseudomonas species and Escherichia coli, studied the effect of repeated cycles of freezing using liquid air at -196^{o}C and thawing on the sensitivity of the cells to a wide range of antibiotics and sulfonamides. He found that there was no change in the response of these organisms to freezing and thawing damage or to drugs over the course of 18 cycles of treatments, and suggested that freezing and thawing was not, in itself, mutagenic to bacteria. However, Arpai and Tomisheva (1966) observed that freezing was able to enhance the induction of streptomycin resistance in Escherichia coli by chemical mutagens, such as caffein, theobromine and theophylline.

There is little information concerning the effect of freezing and thawing on the stability of deoxy-ribonucleic acid. Shikama (1965) observed that the ultraviolet absorbance of DNA was not increased after freezing and thawing. He therefore, suggested that the double-stranded helical structure of DNA would not be broken down by freezing and thawing.

Action of freezing and causes of injury in frozen cells

The action of freezing on bacterial cells has been the subject of much speculation by many authors. Keith (1913) proposed that the death of bacteria under freezing conditions was due to the piercing of the cells by ice crystals. Haines (1938) isolated protein of Pseudomonas aeruginosa frozen rapidly at -70°C, and then stored at -20°C or -2°C. After a few days, a flocculent precipitate settled out of the tubes on thawing. At -2°C, a rapid accumulation of precipitate occurred, amounting in 1 day to nearly 17% of the "total coagulable protein nitrogen" in the solution and in 8 days to about 50%, after which time little further change appeared to take place. However, there was little or no precipitate at -20°C, or -70°C. He also observed that the maximum death rate of Pseudomonas aeruginosa occurred

at $-1^{o}C$ and $-2^{o}C$, and death was comparatively slow at $-20^{o}C$. He therefore, suggested that the death of bacteria in the frozen state was due to some change, or a complex of changes, leading to denaturation and subsequent flocculation of one moiety of the cellular proteins owing to the particular salt concentration and pH of the medium.

Weiser and Osterud (1945) presented evidence that the death of Escherichia coli by freezing involves a rapid-acting or "immediate" death, caused by freezing and thawing per se, and a "storage" death, which is a direct function of time and temperature. Evidence was also presented that immediate death results principally from the mechanical action of extracellular ice. Hollander and Nell (1954) also suggested that the death which occurs during freezing and thawing of bacteria is from mechanical compression, and that the protection afforded by glycerol is explainable by its physical characterization.

Harrison and Cerronie (1956), on the other hand, demonstrated that there was no correlation between the physical strength of bacterial cells and their susceptibility to the lethal effect of freezing and thawing. Harrison (1956) further suggested that the primary cause of death in frozen suspension was not mechanical but appeared to be due

to solute which was concentrated concomitantly with the solidification of the suspension.

A correlation between freezing injury and the liquid-to-solid phase transition of eutectic salt solutions was reported by Zimmerman (1964). When cells of Serratia marcescens were suspended in various concentrations of NaCl up to 20% and were frozen at temperatures at which phase-transition crystallization of NaCl did not occur, little effect on the viability of any suspension was observed. However, freezing these suspensions through the phase-transition of the salt solution produced high mortality among the viable cells. Other inorganic salts that underwent phase transition during freezing were also found to be as toxic as NaCl to Serratia marcescens.

Recently, Mazur (1966) suggested that most low temperature injury to Escherichia coli, both immediately and during storage, is ascribable to the action of concentrated extracellular solutes, especially NaCl.

Lovelock (1953a, 1953b, 1954) explained freezing injury to erythrocytes as the result of exposure to concentrated electrolytes arising when water was converted to ice. Glycerol and other small molecular weight solutes appeared to protect by "buffering" the concentration of

salts in equilibrium with ice at any sub-freezing temperature above the eutectic point. It was suggested that hemolysis of erythrocytes after freezing and thawing occurred because concentrated NaCl removed membrane phospholipid and cholesterol, thereby enhancing the permeability of the cells to cations.

Levitt (1966), studying higher plants, has developed a hypothesis of freezing injury that is based on the removal of water. The proteins are the substances that undergo changes leading the cells to injury or death. This has been explained by the "SH ⟷ SS" hypothesis, according to which intermolecular SS bonds formed between protein molecules are broken during injurious freezing, leading to denaturation of protein. Unfortunately, there is little experimental evidence as yet to demonstrate the specific proteins that when modified would lead to freezing injury.

The mechanism of freezing injury of yeasts has been intensively studied by Mazur (1965, 1966). On the basis of his experimental data he concluded that the following factors were involved: (1) Low temperature per se - the yeast cells are not susceptible to thermal shock. Therefore, the mere action of cooling seems not to be detrimental. (2) Extracellular ice formation - extracellular freezing is a

prerequisite for injury, yet it seems not to be lethal by itself. (3) The concentration of solutes during freezing - the concentrating of extracellular or intracellular solutes does not appear to be a major contributor to the immediate injury of yeast, but it is probably a factor in storage death. (4) Intracellular ice formation - since other factors just described seem not to be important causal agents and this, by elimination, leaves intracellular freezing. He also found that the freezing conditions that produce internal ice kill a high proportion of the cells. Conversely, conditions that prevent internal freezing usually kill a much smaller proportion of the cells. Therefore, he suggested that intracellular ice crystals might be chiefly responsible for the immediate low temperature death of yeast.

PART I

A DETAILED INVESTIGATION OF NUTRITIONAL REQUIREMENTS OF BACTERIAL CELLS AFTER FREEZING AND STORAGE

INTRODUCTION

It has been reported many times that bacteria, after being subjected to freezing and freeze-drying, appear to become more exacting in their nutritional requirements. In 1959, Straka and Stokes reported the results of studies on the extent and nature of non-lethal injury to several strains of Pseudomonas species and a strain of Escherichia coli 451B by freezing and storage. They found that a proportion of the cell population after freezing could no longer grow on a minimal, glucose-salts agar medium, but could develop on a rich, complex medium such as Trypticase Soy Agar. These cells, which were referred to as being metabolically injured, constituted as much as 40 per cent of the bacterial population. Using Pseudomonas fluorescens, they noted that an enzymatic digest of casein was active as a supplement to a minimal medium able to increase the count after freezing but not the acid hydrolyzed casein. From this the authors suggested that the recovery of injured cells on Trypticase Soy Agar was due to the activity of special peptide components of the medium. Straka and Stokes further concluded that the peptides might be required by injured cells for resynthesis of essential

protein denatured by the subzero temperatures. The most recent exponents of this hypothesis (Moss and Speck, 1966a) claim to have identified the nutritional components of the medium, Trypticase Soy Agar, as peptides which are responsible for the recovery of Escherichia coli Y injured by freezing. On the other hand, in studies with Aerobacter aerogenes (Mac 112) MacLeod et al. (1966) found that cysteine added to a minimal medium was equally as effective as more complex supplements in increasing the bacterial counts on suspensions of cells which had been frozen and stored. Further investigation (MacLeod et al., 1967) showed that a suspension of unfrozen cells in a plating diluent consisting of distilled water from a laboratory tin-lined still caused the development of symptoms of metabolic injury as evidenced by increased viable counts on supplemented as compared to minimal plating medium. Cysteine was as effective as yeast extract in supplementing the minimal medium to increase the viable counts. Mg^{++} and, to a lesser extent, phosphate buffer at the concentrations tested protected unfrozen cells, but not cells which had been frozen and stored, against the loss of capacity to grow on minimal medium. When the plating diluent consisted of distilled water redistilled in an all-glass still, the symptoms of metabolic injury did not appear. Spectrographic

analysis revealed the presence of 10^{-7} M Cu^{++} in the distilled water, and Cu^{++} added to redistilled water serving as the plating diluent reproduced the metabolic injury effects induced by distilled water. It was concluded that freezing and storage damaged the cell membrane rendering it more penetrable by toxic elements. The toxic elements were thereby enabled to act at sites where Mg^{++} and other solutes in the plating diluent could not serve as effective antagonists. Increased recovery of cells on supplemented medium could be asoribed to the capacity of the supplements to remove toxic elements which had become bound to the cells during suspension in the plating diluent.

Since the results obtained with Aerobacter aerogenes (Mac 112) have shown that cysteine could replace completely more complex supplements to the minimal medium in the recovery of cells injured by freezing, it was of interest to see if it was also active for one of the cultures, Escherichia coli 451B used by Straka and Stokes (1959), and by Moss and Speck (1966a).

MATERIALS AND METHODS

Test organisms

Escherichia coli 451B (Mac 614) was obtained from Dr. R. P. Straka of the U.S. Department of Agriculture, Western Regional Research Laboratory, Albany, California.

Escherichia coli ATCC 11246 (Mac 236), *Escherichia coli* B ATCC 11303 (Mac 237), *Escherichia coli* ATCC 9723 (Mac 317), *Aerobacter aerogenes* (Mac 112), *Aerobacter aerogenes* (Mac 1), *Aerobacter aerogenes* (Mac 438), *Aerobacter aerogenes* (Mac 613), *Serratia marcescens* (Mac 21), *Serratia marcescens* (Mac 274), *Pseudomonas aeruginosa* (Mac 436) and *Pseudomonas fluorescens* (Mac 9) were chosen from the culture collection of the Department of Microbiology, Macdonald College.

Serratia marcescens (8UK) was obtained from Defence Research Board of Canada.

The *Escherichia coli* 451B and *Aerobacter aerogenes* (Mac 112) organisms were used in the majority of the experiments.

Maintenance of Cultures

A culture of the test organism was maintained by weekly transfer on slopes of Trypticase Soy Agar. The Trypticase Soy Agar used was a product of Baltimore Biological Laboratories (BBL). Prior to the inoculation of the culture medium, a fresh slope was inoculated and incubated for 24 hr. at the optimum temperature for growth as follows: 37°C for *Escherichia coli* and *Pseudomonas aeruginosa*, 30°C for *Aerobacter aerogenes* and *Serratia marcescens*, and 25°C for *Pseudomonas fluorescens*. A loopful of cells from this culture was then used to inoculate the medium.

Media

(a) Liquid medium for culturing the organism

Cells for preparing suspensions were grown in Trypticase Soy Broth the same as that used by Moss and Speck (1966), containing 1.5% Trypticase (BBL), 0.5% Phytone (BBL) and 0.5% NaCl (Fisher Scientific Co.).

(b) Solid media used for plate counts

The following two minimal media designated as A and B were prepared.

Minimal medium A was the same as that used by Straka and Stokes (1959) and Moss and Speck (1966a) and contained (in grams per liter): glucose (Baker Chemical Co.), 2.0; K_2HPO_4 (Fisher Scientific Co.), 7.0; KH_2PO_4 (Fisher), 3.0; $(NH_4)_2SO_4$ (Fisher), 1.0; sodium citrate (Fisher), 0.1; $MgSO_4.7H_2O$ (Fisher), 0.1; and agar (Difco), 15.

Minimal medium B, which was used by MacLeod, Kuo and Gelinas (1967) was the same as minimal medium A except that it contained only one-quarter as much KH_2PO_4 and K_2HPO_4, and twice as much glucose.

For preparing the minimal media, the salt portion containing K_2HPO_4, KH_2PO_4, $(NH_4)_2SO_4$ and $MgSO_4.7H_2O$, and the organic portion containing glucose and agar were prepared separately in double strength, dispersed in 5 ml. portions, autoclaved and then added together just before use.

To test the effect of supplements such as 1 mM L-cysteine (pH 7.0), 1% yeast extract (Difco), 2% Trypticase (BBL) etc., the supplements were added at double strength to the organic portion of the medium.

Preparation of cell suspensions

Ten ml. of the liquid medium in a 50 ml. Erlenmeyer flask was inoculated from a culture growing on an agar slant

and incubated for 8 hr. at the temperature optimum for the organism in a stationary culture. This liquid culture was then transferred to 250 ml. of medium in a 2-liter flask and incubated for 15 hr. in a stationary culture. The cells were then harvested by centrifugation at 10,000 x Gravity for 20 minutes in a refrigerated centrifuge keeping the temperature at 4^{o}C. The supernatant fraction was poured off and the cells were washed three times by resuspension in and centrifugation from 0.85% NaCl.

Viability determinations

The viability of unfrozen, and of frozen and stored cell suspensions was determined by the application of standard pour plate methods.

The diluents used to make dilutions for plating contained 2×10^{-3} M $MgSO_4$ in glass-distilled water, except where otherwise indicated. The original suspensions were serially diluted to produce a suspension containing approximately 30 to 300 cells per ml. One ml. portions of the final dilutions were used as inoculum in each petri dish. All petri dishes were poured in quadruplicate using 10 ml. of plating medium per plate. Colonies were counted after 48 hr. of incubation. Longer plating periods were tested and found not to increase the total count.

In the study of Pseudomonas species, the surface plate method instead of the pour plate method was used. To surface plate, minimal and enriched medium were prepared and poured into the petri dishes 24 to 36 hr. before use to permit free water to evaporate from the surface. The plates were placed on a dish turntable (Fisher Scientific Co.) and were inoculated by spreading quickly and evenly 0.2 ml. of sample on the surface of a spinning plate. The total count on the surface of 4 plates was recorded.

Freezing procedure and storage

Prior to freezing, the washed bacterial cells were suspended in 0.85% NaCl. Five ml. aliquots of this suspension containing about 200×10^5 cells per ml. were dispersed into polypropylene tubes, frozen at $-20^{\circ}C$ and stored at this temperature. The suspending menstruum for freezing and the method of storage were the same as were used by Moss and Speck (1966a), except where otherwise indicated.

Thawing procedure

The frozen suspensions were thawed by placing the polypropylene tubes in a beaker of water at room temperature. The melting process was observed closely and immediately

upon the disappearance of the last trace of ice in the tubes, the appropriate dilutions for plating were prepared.

Distilled water and glass-distilled water

The distilled water was obtained from a Barnstead still described as being lined with block tin to protect against metal ion contamination. The glass-distilled water was prepared by redistilling the distilled water from the Barnstead still in a Corning AG-2 all-glass still.

Preparation of glassware

All glassware used to prepare dilutions of the cells for plating was held a minimum of 3 hr. in a mixture of concentrated sulfuric acid and nitric acid (2:1 v/v). This treatment was followed by 5 rinses with tap water and finally by 10 rinses with glass-distilled water.

Preparation of acid-hydrolyzed Trypticase

The following two methods were used:

(i) HCl-hydrolyzed Trypticase

Fifty gm. of Trypticase (BBL) were suspended in 200 ml. of 6 N HCl in a 1-liter Erlenmeyer flask and digested for 12 hr. at 121^{o}C in an autoclave at 15 lb. steam pressure.

The acid hydrolysate was evaporated to dryness under reduced pressure at 60°C, redissolved in 100 ml. of glass-distilled water and re-evaporated to dryness. This process was repeated 5 times to remove the free hydrochloric acid. The acid hydrolysate was then kept under vacuum again for 24 to 48 hr. in a dessicator with KOH pellets at the bottom.

The hydrolysate was subsequently suspended in 500 ml. of glass-distilled water and divided into 2 parts. One portion of the hydrolysate was further decolorized by treatment with 1% of activated carbon (Atlas Powder Co., Wilmington, Delaware). The other portion was left untreated.

Each hydrolysate solution was adjusted to a pH of 7.0 by using KOH, and the final volume was brought to 250 ml. with glass-distilled water. The resulting solution was equivalent to that of a 10% solution of the original Trypticase.

(ii) H_2SO_4-hydrolyzed Trypticase

Fifty gm. of Trypticase (BBL) were suspended in 250 ml. of 6 N H_2SO_4 in a 1-liter Erlenmeyer flask and digested for 12 hr. at 121°C in an autoclave at 15 lb. steam pressure. The sulfate in the hydrolysate was removed by precipitating out with saturated $Ba(OH)_2$ (Ca. 10%). To remove any excess

Ba^{++} which might be left in the solutions, carbon dioxide was bubbled in. The precipitate of $BaCO_3$ was filtered with the aid of a Buchner funnel and washed with glass-distilled water. The hydrolysate was brought to a final volume of 500 ml. at a pH of 7.0. The resulting solution was equivalent to that of a 10% solution of the original Trypticase.

Treatment of H_2SO_4 hydrolysate with cation exchange resin

To remove any trace of Ba^{++} which might be left in the hydrolysate solution, a cation exchange resin of Dowex 50 x 8 (H^+ form) was added to 100 ml. of 10% hydrolysate until the pH indicated 4.0. The resin was removed by filtration and the solution adjusted to a pH of 7.0 using KOH.

Resin column preparation and elution

Dowex 50 x 8 (200 to 400 mesh, H^+ form) and Dowex 1 x 8 (200 to 400 mesh, Cl. form) were used.

Dowex 50 x 8 (H^+ form) was soaked in glass-distilled water for 30 minutes and then washed on a Whatman No. 1 filter with 6 N HCl until the filtrate was colourless. The

resin was then washed with copious volumes of glass-distilled water until the filtrate was free from Cl^- ions as shown by the $AgNO_3$ test. A slurry of washed resin was prepared in glass-distilled water and poured into a column with an inside diameter of 2.2 cm. and a packing length of 80 cm.

A 50-ml. volume of H_2SO_4 hydrolysate equivalent to 10% of original Trypticase was allowed to run through the Dowex 50 x 8 (H^+ form) resin column which was then washed with 10 bed volumes of glass-distilled water, keeping the flow rate at 2 ml. per minute. The collected eluate was evaporated by vacuum distillation at 55^oC to a volume of 50 ml. adjusted to pH 7.0.

Dowex 1 x 8 (Cl^- form) was prepared in the same way as Dowex 50 x 8 (H^+ form). The eluate from the Dowex 50 x 8 column was applied to the Dowex 1 x 8 column which was then washed with 10 bed volumes of glass-distilled water. The eluate from the second column was treated in the same way as mentioned with Dowex 50 x 8 (H^+ form).

Preparation of an amino acid mixture approximating the composition of casein

The amino acid composition in casein of cow's milk is shown in Table 1. An amino acid mixture approximating the amino acid composition of casein was prepared using L forms (except for DL methionine) of the free amino acids (as opposed to their HCl salts). The only amino acid unobtainable in free form was lysine which was used as the monohydrochloride salt. The mixture was dissolved with the aid of a small amount of H_2SO_4 and diluted to a concentration equivalent to a 10% solution of casein (Table 2). Before use the solution was adjusted to pH 7.0 with KOH.

TABLE 1

Quantitative amino acid composition in the casein of cow's milk*

Amino acid	gm/100 gm casein	Amino acid	gm/100 gm casein
Alamine	2.3	Histidine	2.25
Aspartic acid	5.8	Isoleucine	6.1
Cysteine	-	Leucine	10.8
Cystine	0.34	Lysine	6.8
Glutamic acid	21.7	Methionine	2.88
Glycine	0.4	Phenyl alanine	5.55
Proline	9.8	Threonine	4.35
Serine	5.4	Tryptophane	1.22
Tyrosine	5.96	Valine	6.6
Arginine	3.77		

*Published by National Research Council, National Academy of Sciences, "The composition of milks." Publ. 254, Revised 1953.

TABLE 2

Amino acid mixture* used to approximate the amino acid composition of casein

Amino acid**	gm.	Amino acid**	gm.
L-Alamine	0.460	L-Isoleucine	1.220
L-Aspartic acid	1.160	L-Leucine	2.160
L-Cystine	0.068	L-Lysine HCl	1.700
Glycine	0.080	DL-Methionine	1.152
L-Proline	1.960	L-Tryptophane	0.244
L-Serine	1.080	L-phenyl alanine	1.100
L-Tyrosine	1.192	L-Threonine	0.870
L-Arginine	0.754	L-Valine	1.320
L-Histidine	0.045	Equivalent to 20 gm. of casein	
L-Glutamic acid	4.340		

*200 ml. of this solution per 500 ml. of final medium provided an amino acid mixture equivalent to that supplied by 4% casein.

**Amino acids were obtained from the Nutritional Biochemical Corporation.

RESULTS

(I) Re-examination of metabolic injury in Aerobacter aerogenes (Mac 112) after freezing and storage

In order to duplicate the conditions used by Moss and Speck (1966a) as closely as possible, their freezing procedure was employed. Cells of Aerobacter aerogenes (Mac 112) were washed three times with 0.85% NaCl and resuspended in the same solution for freezing at -20°C in a commercial deep-freeze. The results in Table 3 show that before and after freezing, the cells showed no response to any supplements added to minimal medium A or B. In fact, Trypticase (2%) depressed the count on minimal medium B which contained one-quarter of the concentration of phosphate, and twice the amount of glucose present in minimal medium A.

It was of interest to determine why Trypticase was inhibitory when added to minimal medium B but not A. Since both the concentrations of phosphate and of glucose were different in the two media, another two minimal media were therefore prepared. Minimal medium A' contained a high

TABLE 3

Effect of various compositions of plating medium on the viable count of a suspension of Aerobacter aerogenes (Mac No. 112) before and after freezing

Supplement to Minimal medium A or B	Bacterial count**	
	Before freezing	After freezing*
Minimal medium A	181 ± 5	180 ± 8
+ Cysteine (1mM)	183 ± 5	189 ± 7
+ Yeast extract (1%)	185 ± 4	195 ± 4
+ Trypticase (2%)	170 ± 8	180 ± 5
Minimal medium B	170 ± 4	178 ± 4
+ Cysteine (1 mM)	183 ± 6	189 ± 5
+ Yeast extract (1%)	177 ± 7	184 ± 4
+ Trypticase (2%)	33 ± 6	34 ± 6

*Frozen and stored at $-20^{\circ}C$ for 1 day.

**$MgSO_4$ (2×10^{-3} M) in glass-distilled water was used as plating diluent. Minimal medium B contained only one-quarter as much KH_2PO_4 and K_2HPO_4 and twice as much glucose as in minimal medium A.

Different dilutions were used before and after freezing.

phosphate level as in minimal medium A and a high glucose level as in minimal medium B, whereas minimal medium B' contained a low phosphate level as in minimal medium B and a low glucose level as in minimal medium A. The results in Table 4 show that Trypticase depressed the count in a low phosphate medium, irrespective of the glucose concentration involved.

Another experiment was carried out to re-examine the metabolic injury of Aerobacter aerogenes (Mac 112) by using the freezing procedure of MacLeod et al. (1966). The cells suspensions were suspended in 0.5% beef extract, frozen with liquid air (-196°C) and stored at -20°C. A suspension of Aerobacter aerogenes (Mac 112) before and after freezing was diluted for plating with either glass-distilled water, 3×10^{-4} M phosphate buffer in glass-distilled water, or 2×10^{-3} M $MgSO_4$ in glass-distilled water and the effect of supplements of cysteine and Trypticase to minimal medium A was compared. The results in Table 5 show that the supplements to minimal medium A did not produce any increase in viable count with frozen cells irrespective of the plating diluent.

Thus, so far as Aerobacter aerogenes (Mac 112) was concerned there was no evidence of a requirement for special

TABLE 4

The effect of Trypticase on the viable count of Aerobacter aerogenes (Mac No. 112) in the high or low level of phosphate and glucose contained plating medium

Plating medium	Bacterial count** ***	
	Before freezing	After freezing*
Minimal medium		
A (high phosphate, low glucose)	225 ± 5	234 ± 6
A' (high phosphate, high glucose)	215 ± 2	232 ± 5
B (low phosphate, high glucose)	225 ± 3	220 ± 8
B' (low phosphate, low glucose)	216 ± 6	225 ± 10
Minimal medium A + 2% Trypticase	199 ± 7	238 ± 5
" " A' + " "	191 ± 4	221 ± 5
" " B + " "	48 ± 6	45 ± 5
" " B' + " "	39 ± 3	43 ± 4

*Frozen and stored at -20°C for 1 day

**$MgSO_4$ (2×10^{-3} M) in glass-distilled water was used as plating diluent. Plating medium see Table 3.

***Different dilutions were used before and after freezing.

TABLE 5

Effect of various compositions of plating diluent, and composition of plating medium on the viable count of a suspension of Aerobacter aerogenes (Mac 112) before and after freezing

Supplement to minimal medium A	Bacterial count ***		
	Additions to glass-distilled water as plating diluent		
	0	KH_2PO_4 buffer** (3×10^{-4}M)	$MgSO_4$ (2×10^{-3}M)
Before freezing			
0	117 ± 5	124 ± 6	129 ± 5
Cysteine (1 mM)	117 ± 4	126 ± 4	125 ± 4
Trypticase (2%)	119 ± 6	122 ± 5	127 ± 4
After freezing*			
0	175 ± 8	166 ± 5	178 ± 9
Cysteine	176 ± 5	172 ± 6	186 ± 4
Trypticase (2%)	179 ± 7	175 ± 6	183 ± 6

*In this experiment cells were frozen in liquid air and stored at -20°C for 1 week; approximately 30% of the cells in the suspension before freezing remained viable after freezing.

**KH_2PO_4 buffer (3×10^{-4}M) adjusted to pH 7.2 with NaOH.

***Different dilutions were used before and after freezing.

peptides to repair and to enhance the viable count of the cells after freezing and storage.

(II) Efforts to isolate active component(s) which promote the viable count of Escherichia coli 451B after freezing and storage

An experiment was carried out to investigate the effect of supplements to minimal medium A and B on the viable count on a suspension of Escherichia coli 451B before and after freezing. The results are shown in Table 6. Supplements to minimal medium A or B did not alter the plate count on a suspension of Escherichia coli 451B before freezing when 0.002 M $MgSO_4$ in glass-distilled water was the plating diluent. After freezing and storage, the viable count was higher when the minimal media were supplemented with yeast extract or Trypticase. Cysteine, however, had no supplementary capacity under these conditions.

The possibility was considered that Escherichia coli 451B might be rendered unusually sensitive to some toxic trace element in the plating medium which could not be adequately detoxified by cysteine. For this reason the effect of adding to the minimal medium traces of Cu^{++} and various supplements such as nitrilotriacetic acid, RNA and DNA which would be expected to chelate and hence detoxify

TABLE 6

Effect of various compositions of plating medium on the viable count of a suspension of Escherichia coli 451B before and after freezing

Supplement to minimal medium A or B	Bacterial count**	
	Before freezing	After freezing*
Minimal medium A	170 ± 5	78 ± 5
+ Cysteine (1 mM)	179 ± 4	80 ± 4
+ Yeast extract (1%)	177 ± 3	154 ± 4
+ Trypticase (2%)	179 ± 5	149 ± 3
Minimal medium B	172 ± 4	79 ± 3
+ Cysteine (1 mM)	172 ± 3	74 ± 3
+ Yeast extract (1%)	172 ± 3	162 ± 5
+ Trypticase (2%)	180 ± 4	145 ± 5

*Frozen and stored at $-20^{\circ}C$ for 1 week.

**$MgSO_4$ (2×10^{-3} M) in glass-distilled water was used as plating diluent.

toxic elements was tested. The results in Table 7 show that all the supplements both before and after freezing had some capacity to protect the cells against 1×10^{-5} M of added Cu^{++} while RNA, DNA and Trypticase could protect against 1×10^{-4} M Cu^{++}. There was still an enhanced response to Trypticase which could not be duplicated by the other supplements at the concentrations tested. These results show that although the various supplements can chelate toxic heavy elements such as Cu^{++}, only Trypticase caused enhanced growth of cells after freezing in a minimal medium without added Cu^{++}. This suggests that the action of Trypticase in the absence of added Cu^{++} is not due to its ability to chelate toxic metals in the plating medium. Both RNA and DNA were not inhibitory for the cells before freezing, but after freezing, however, lower counts were obtained on minimal medium supplemented with RNA or DNA than on minimal medium alone.

Other workers had found that acid hydrolyzed casein did not replace Trypticase as a supplement to the minimal medium in increasing the viable count on a suspension of Escherichia coli or Pseudomonas fluorescens after freezing and storage. They concluded, therefore, that the active factor was a peptide. To investigate whether the factors in Trypticase which were active in increasing the viability of

TABLE 7

Effect of various supplements on the toxicity of Cu^{++} added to the plating medium for Escherichia coli 4518 before and after freezing

Supplement to minimal medium A*	Bacterial count		
	Cu^{++} added		
	0	1 x 10^{-5} M	1 x 10^{-4} M
Before freezing			
0	244 ± 4	204 ± 12	86 ± 9
Cysteine	233 ± 5	226 ± 4	32 ± 6
Nitrilotriacetic acid	232 ± 6	229 ± 6	86 ± 5
RNA	221 ± 7	219 ± 2	229 ± 6
DNA	216 ± 7	226 ± 4	229 ± 5
Trypticase	232 ± 3	238 ± 2	228 ± 2
After freezing**			
0	105 ± 4	19 ± 3	1 ± 1
Cysteine	107 ± 4	44 ± 3	2 ± 1
Nitrilotriacetic acid	103 ± 2	41 ± 2	1 ± 1
RNA	80 ± 5	74 ± 4	39 ± 2
DNA	30 ± 4	27 ± 4	11 ± 3
Trypticase	149 ± 3	126 ± 7	105 ± 4

*Nitrilotriacetic acid, RNA and DNA (Sigma Chem. Co.) adjusted to pH 7.0 with KOH.

**Frozen and stored for 1 day at -20°C.

Escherichia coli 451B were sensitive to hydrolysis, a sample of Trypticase was hydrolyzed with hydrochloric acid. The results in Table 8 show that the Trypticase hydrolysate either decolorized with charcoal or not, at 5% had no capacity to enhance the viable count over the minimal medium alone but at 2.5% had some effect, though the response did not approach that obtained with either Trypticase or yeast extract.

Trypticase was subsequently hydrolyzed with hydrochloric acid which was purified by boiling reagent grade hydrochloric acid (Fisher Sci. Co.) and allowing the HCl gas released to dissolve in glass-distilled water. Another sample of Trypticase was hydrolyzed with sulfuric acid and the sulfate was removed by precipitating with $Ba(OH)_2$. Since at the end-point of the $BaSO_4$ precipitation it was anticipated that some of Ba^{++} might be left in the hydrolysate, a part of the hydrolysate after treatment with $Ba(OH)_2$ was treated with Dowex 50 x 8 (H^+ form) at pH 4.0 and neutralized with KOH. When these preparations were used results were obtained which varied depending on the supplement tested (Table 9). It is clear that the H_2SO_4 hydrolysate, particularly after treatment with Dowex 50 x 8 resin had a capacity to increase the viable count on a suspension of Escherichia coli 451B after freezing and

TABLE 8

Effect of HCl hydrolyzed Trypticase as a supplement to minimal medium A on the bacterial count on a suspension of *Escherichia coli* 451B before and after freezing

Supplement to minimal medium A	Bacterial count	
	Before freezing	After freezing**
0	165 ± 9	80 ± 8
Cysteine (1 mM)	164 ± 5	95 ± 4
Trypticase (2%)	162 ± 5	195 ± 5
Yeast extract (1%)	162 ± 6	213 ± 7
HCl hydrolyzed Trypticase*		
5%	170 ± 7	76 ± 7
2.5%	162 ± 9	121 ± 9
HCl hydrolyzed Trypticase* (decolorized)		
5%	165 ± 5	71 ± 6
2.5%	169 ± 9	140 ± 6

*The amount of hydrolysate added is expressed in terms of the percentage of Trypticase in the medium to which it was equivalent.

**Frozen and stored for 1 day at -20°C; approximately 20% of the cells in the suspension before freezing remained viable after freezing, based on the colony count obtained on the best medium tested.

TABLE 9

Effect of HCl and H_2SO_4 hydrolyzed Trypticase as a supplement to minimal medium A on the bacterial count on a suspension of *Escherichia coli* 451B before and after freezing

Supplement to minimal medium A*	Bacterial count	
	Before freezing	After freezing**
0	203 ± 11	62 ± 3
Cysteine	218 ± 5	81 ± 3
Yeast extract	204 ± 11	155 ± 11
Trypticase	214 ± 4	152 ± 7
H_2SO_4 hydrolyzed Trypticase		
1%	212 ± 5	114 ± 3
2%	197 ± 7	81 ± 8
3%	202 ± 7	81 ± 5
H_2SO_4 hydrolyzed Trypticase (resin treated)		
1%	209 ± 6	142 ± 5
2%	212 ± 5	141 ± 8
3%	202 ± 6	151 ± 8
HCl hydrolyzed Trypticase		
2%	198 ± 8	117 ± 3
3%	195 ± 7	85 ± 4
HCl hydrolyzed Trypticase (decolorized)		
2%	201 ± 7	120 ± 4
3%	197 ± 4	92 ± 9

*See Table 8.

**Frozen and stored for 1 day at -20°C.

storage. And again the HCl hydrolysate had some effect when tested at a level equivalent to 2% Trypticase but not 3%.

Further exploratory work was carried out to determine if any forms of the ion exchange resins available had a capacity to remove the factor(s) present in H_2SO_4 hydrolyzed Trypticase which was increasing the viable count on suspensions of Escherichia coli 4518. For this purpose a solution of H_2SO_4 hydrolysate was added to the top of a column of Dowex 50 x 8 (H^+ form). The column was washed with ten bed volumes of glass-distilled water. The eluates were collected and concentrated for use as a supplement for the minimal medium. Another sample of the eluate from the Dowex 50 x 8 (H^+ form) resin was added to the top of a column of Dowex 1 x 8 (Cl^- form) resin. Ten bed volumes of glass-distilled water were passed through this column and the eluates were pooled and concentrated. Samples of the H_2SO_4 hydrolysate, of the eluate from the Dowex 50 x 8 column and of the Dowex 1 x 8 column were ashed and solution of the ash prepared. The effects of these fractions used as supplements to the minimal medium in increasing the viable count on a suspension of Escherichia coli 4518 after freezing and storage were determined. The results are recorded in Table 10. The results indicate that the active component(s) were still present in the eluate from the Dowex 50 x 8 column but

TABLE 10

Effect of column treatment with ion exchange resins, and ashing of eluates upon the viable count of frozen cells of *Escherichia coli* 451B from H_2SO_4 hydrolyzed Trypticase

Supplement to minimal medium A*	Bacterial count**
0	111 ± 6
Cysteine (1 mM)	118 ± 6
Yeast extract (1%)	264 ± 7
Trypticase (2%)	242 ± 5
H_2SO_4 hydrolyzed Trypticase (resin treated)	
1%	235 ± 8
2%	252 ± 6
Dowex 50 x 8 (H^+ form) (eluate)	
1%	240 ± 9
2%	237 ± 7
Dowex 1 x 8 (Cl^- form) (eluate)	
1%	116 ± 7
2%	117 ± 5
Ash from H_2SO_4 hydrolyzed Trypticase (resin treated)	
1%	88 ± 5
2%	84 ± 8
Ash from Dowex 50 x 8 (eluate)	
1%	104 ± 5
2%	93 ± 5
Ash from Dowex 1 x 8 (eluate)	
1%	104 ± 4
2%	104 ± 5

*See Table 8.

**Frozen and stored for 1 day at -20°C.

not after the eluate was passed through the Dowex 1 x 8 column. The ash from these preparations either had the count depressing action or showed no effect at all.

An amino acid mixture approximating the amino acid composition of casein was tested for activity in promoting the growth of cells of Escherichia coli 451B which had been injured by freezing and storage. The amino acid mixture was prepared using L-forms (except for DL-methionine) of the free amino acids (as opposed to their HCl salts). The mixture was tested as a supplement to the minimal medium. It was found (Table 11) that the amino acid mixture was quite effective in replacing Trypticase as long as the concentration at which it was tested was kept below the equivalent of 2% casein. Surprisingly, concentrations as low as 0.1% were the most effective in increasing the counts on the cell suspensions after freezing.

The amino acid mixture was then divided into three groups and the groups were tested alone and in combination. The results (Table 12) show that Group A at a level equivalent to 0.5% casein was very active in replacing the amino acid mixture and almost as effective as Trypticase. Group C was also quite active, whereas Group B had some count depressing action. It should be noted that these effects were observed after freezing, not before.

TABLE 11

Effect of an amino acid mixture as a supplement to minimal medium A on the bacterial count on a suspension of *Escherichia coli* 451B before and after freezing

Supplement to minimal medium A	Bacterial count	
	Before freezing	After freezing**
0	221 ± 7	124 ± 4
Cysteine (1 mM)	221 ± 4	134 ± 9
Yeast extract (1%)	238 ± 7	258 ± 6
Trypticase (2%)	228 ± 6	247 ± 7
Amino acid mixture		
2% casein*	223 ± 6	102 ± 4
1%	219 ± 5	144 ± 9
0.5%	232 ± 11	171 ± 6
0.1%	230 ± 7	198 ± 7
0.05%	223 ± 8	178 ± 6
0.01%	232 ± 5	154 ± 8

*The amounts of amino acid mixture are expressed in terms of the percentage of casein in the medium to which they were equivalent.

**Frozen and stored for 3 days at -20°C.

TABLE 12

Effect of subgroups of the amino acid mixture as supplements to minimal medium A on the bacterial count on a suspension of Escherichia coli 451B before and after freezing

Supplement to minimal medium A*		Bacterial count	
		Before freezing	After freezing**
0		196 ± 8	133 ± 6
Trypticase (2%)		192 ± 9	256 ± 10
Amino acid mixture (0.1%)		189 ± 4	226 ± 5
Group A	0.5%	194 ± 10	234 ± 6
	0.1%	182 ± 5	207 ± 7
	0.05%	195 ± 8	187 ± 7
Group B	0.5%	184 ± 4	90 ± 3
	0.1%	196 ± 6	90 ± 5
	0.05%	183 ± 5	80 ± 3
Group C	0.5%	184 ± 6	202 ± 6
	0.1%	198 ± 9	169 ± 7
	0.05%	187 ± 5	154 ± 5

*See Table 11

**Frozen and stored for 1 day at -20°C.

Group A: L-alanine, L-aspartic acid, L-threonine, L-glutamic acid, L-serine, glycine

Group B: L-lysine, DL-methionine, L-cystine, L-arginine, L-proline, L-histidine

Group C: L-phenylalanine, L-tyrosine, L-tryptophane, L-valine, L-leucine, L-isoleucine

Group A of the amino acids was subdivided into two further subgroups, which were examined in more detail to determine which of their components might be active. The results in Table 13 show that Subgroup A_1 was active but A_2 was not. Aspartic acid alone was as effective as Trypticase as a supplement to the minimal medium for increasing the viable count on the cell suspensions after freezing. Glutamic acid, alanine and threonine were also quite effective while serine was inhibitory. The inactivity of Subgroup A_2 seems to be due to the fact that the effects of threonine and glycine were abolished in the presence of serine.

Further experiments were carried out to investigate the effect of aspartic acid and serine as supplements to the minimal medium. A number of concentrations were tested to determine their effect on the bacterial count on suspensions of Escherichia coli 451B before and after freezing. The results were recorded in Tables 14 and 15. Aspartic acid was equally effective from 1 mM to 10 mM, while serine again had no capacity for increasing the viable count on the cells after freezing and storage. In fact it exerted some count depressing action.

TABLE 13

Effect of the amino acids of subgroup A alone and in combination as supplements to minimal medium A on the bacterial count on a suspension of Escherichia coli 451B before and after freezing

Supplement to minimal medium A*	Bacterial count	
	Before freezing	After freezing**
0	212 ± 3	125 ± 7
Trypticase (2%)	213 ± 7	263 ± 7
Amino acid mixture (0.1%)	214 ± 4	257 ± 6
Group A (0.5%)	204 ± 8	263 ± 5
Subgroup A_1 1%	192 ± 5	255 ± 5
0.5%	201 ± 10	272 ± 3
Subgroup A_2 1%	196 ± 8	136 ± 4
0.5%	206 ± 5	139 ± 5
L-alanine 1%	193 ± 3	227 ± 5
0.5%	211 ± 5	174 ± 6
L-aspartic acid 1%	190 ± 5	260 ± 8
0.5%	188 ± 6	265 ± 5
L-glutamic acid 1%	197 ± 7	242 ± 6
0.5%	215 ± 3	221 ± 8
L-serine 1%	194 ± 6	91 ± 8
0.5%	206 ± 6	93 ± 7
L-threonine 1%	206 ± 4	182 ± 5
0.5%	195 ± 7	198 ± 8
Glycine 1%	204 ± 7	172 ± 5
0.5%	199 ± 11	124 ± 8

*See Table 12.

**Frozen and stored for 1 day at -20°C.

Subgroup A_1: L-alanine, L-aspartic acid, L-glutamic acid.

Subgroup A_2: L-threonine, L-serine, glycine.

TABLE 14

Effect of various concentrations of L-aspartic acid as supplement to minimal medium A on the bacterial count of *Escherichia coli* 4518 before and after freezing

Supplement to minimal medium A	Bacterial count	
	Before freezing	After freezing*
0	183 ± 5	55 ± 7
Trypticase (2%)	190 ± 3	179 ± 6
Aspartic acid (0.1 mM)	190 ± 2	47 ± 6
" " (0.5 mM)	190 ± 9	66 ± 2
" " (1.0 mM)	187 ± 7	121 ± 7
" " (2.5 mM)	182 ± 6	165 ± 5
" " (5.0 mM)	179 ± 3	168 ± 6
" " (10 mM)	183 ± 4	166 ± 7

*Frozen and stored for 1 week at -20°C.

TABLE 15

Effect of various concentrations of L-serine as supplement to minimal medium A on the bacterial count of *Escherichia coli* 451B before and after freezing

Supplement to minimal medium A	Bacterial count	
	Before freezing	After freezing*
0	186 ± 11	107 ± 3
Trypticase (2%)	198 ± 6	255 ± 6
Aspartic acid (2.5 mM)	193 ± 6	273 ± 7
Serine (1.0 mM)	192 ± 9	85 ± 9
" (2.5 mM)	196 ± 7	86 ± 9
" (5.0 mM)	183 ± 8	96 ± 5
" (10 mM)	193 ± 7	93 ± 8

*Frozen and stored for 1 day at -20°C.

(III) Permanency of the metabolic injury occurring in Escherichia coli 451B

It was of importance to know if the metabolic injury caused by freezing and storage was permanent and hence heritable. To investigate this problem the replica plating technique was employed. Equal aliquots of the suspension were spread on the surface of minimal medium and minimal medium supplemented with 2.5 mM aspartic acid. After incubation the numbers of colonies appearing on both media were counted. The colonies on the plates of the minimal medium supplemented with aspartic acid were then transferred by the replica plating technique to plates on minimal medium. The results, Table 16, show that before replication the expected difference in counts on the two media was obtained. All of the colonies on the supplemented medium, however, could be replicated onto the minimal medium. Thus a stable heritable requirement for aspartic acid was not produced in this organism by freezing.

(IV) Effect of composition of plating diluents on the viable count and development of metabolic injury in unfrozen, and frozen and stored cells of Escherichia coli 451B

The present results show that when the plating diluent is a solution of 0.002 M $MgSO_4$ in glass-distilled

TABLE 16

Capacity of cells of Escherichia coli 451B which had been frozen and stored to grow on minimal medium and minimal medium supplemented with aspartic acid, and of colonies to be replicated onto minimal medium after growth on enriched medium

Days for freezing at -20°C	No. of colonies appearing*		
	A	B	C
2	587**	1213	1213
6	367	1024	1024

* A on minimal medium A

B on enriched medium (minimal medium A supplemented with 2.5 mM aspartic acid)

C on minimal medium A after replication from enriched medium (column B)

** Each result represents the sum of colonies appearing on 14 plates.

water a nutritional requirement can be detected in a part of the population of Escherichia coli 451B after freezing that is not satisfied by cysteine but rather by aspartic acid, glutamic acid and some other amino acids. It was of interest to investigate the effect of the composition of the plating diluent on the development of metabolic injury in Escherichia coli 451B. A suspension of Escherichia coli 451B before and after freezing was diluted for plating with either glass-distilled water, 3×10^{-4} M phosphate buffer in glass-distilled water (the plating diluent used by Straka and Stokes (1959) and MacLeod et al. (1967)), or with 2×10^{-3} M $MgSO_4$ in glass-distilled water. The results are recorded in Table 17. The recovery of the frozen cells on the enriched medium A or B was best with $MgSO_4$ (2×10^{-3} M) as the plating diluent, poorer with phosphate (3×10^{-4} M), and poorest with glass-distilled water. In all cases, cysteine added to the minimal plating medium was inactive in increasing the count after freezing, whereas aspartic acid was as effective as Trypticase.

Previous studies with Aerobacter aerogenes (Mac 112) showed that when distilled water from a tin-lined still served as the plating diluent, cells of Aerobacter aerogenes (Mac 112) developed symptoms of metabolic injury as evidenced by increased counts when supplements were added to the

TABLE 17

Effect of various compositions of plating diluent, composition of plating medium on the viable count on a suspension of Escherichia coli 451B before and after freezing (I)

Supplement to minimal medium A or B	Bacterial count: Additions to glass-distilled water as plating diluent		
	0	KH_2PO_4 buffer (3×10^{-4}M)	$MgSO_4$ (2×10^{-3}M)
Before freezing			
Minimal medium A	189 ± 3	194 ± 8	204 ± 9
+ Cysteine (1 mM)	193 ± 8	194 ± 5	211 ± 9
+ Aspartic acid (2.2 mM)	190 ± 7	199 ± 6	192 ± 7
+ Trypticase (2%)	214 ± 13	207 ± 10	208 ± 5
Minimal medium B	195 ± 7	200 ± 7	202 ± 8
+ Cysteine (1 mM)	193 ± 7	186 ± 8	201 ± 8
+ Aspartic acid (2.2 mM)	201 ± 8	204 ± 8	185 ± 6
+ Trypticase (2%)	199 ± 8	197 ± 6	211 ± 10
After freezing*			
Minimal medium A	66 ± 4	75 ± 6	73 ± 7
+ Cysteine (1 mM)	69 ± 8	91 ± 5	81 ± 4
+ Aspartic acid (2.2 mM)	96 ± 8	124 ± 8	164 ± 7
+ Trypticase (2%)	104 ± 6	131 ± 6	167 ± 7
Minimal medium B	78 ± 6	85 ± 5	79 ± 8
+ Cysteine (1 mM)	78 ± 8	95 ± 9	88 ± 5
+ Aspartic acid (2.2 mM)	116 ± 6	125 ± 7	168 ± 7
+ Trypticase (2%)	117 ± 7	140 ± 7	174 ± 6

*Frozen and stored for 1 week at $-20^{\circ}C$.

minimal plating medium (MacLeod et al., 1967). In the present study, an effort was made to examine the effect of distilled water obtained from the same tin-lined still on the development of metabolic injury in a population of Escherichia coli 451B before and after freezing. The results are shown in Table 18. When the plating diluent was distilled water, even unfrozen cells of Escherichia coli 451B developed metabolic injury. This had also been observed with Aerobacter aerogenes (Mac 112) (MacLeod et al., 1967). The viable count on minimal salts-glucose medium was less than 75% of the count obtained on the enriched medium, and none of the cells was viable after a 2-hour incubation period at 25°C. When the plating diluent consisted of either glass-distilled water, phosphate buffer in glass-distilled water, or $MgSO_4$ in glass-distilled water, no variation in plate counts with composition of the plating medium was obtained, and there was no loss of viability after 2 hours incubation at 25°C. With the cells after freezing and storage, only a few viable cells were recovered when distilled water from the tin-lined still was used as the plating diluent. The recovery of frozen cells with glass-distilled water as the plating diluent was not as high as when phosphate buffer or $MgSO_4$ served as the diluent. However, there was no loss in viable count on the cells

TABLE 18

Effect of composition of plating diluent, composition of plating medium and time of suspension in plating diluent on viable count of *Escherichia coli* 451B before and after freezing (II)

Plating diluent	Incubation time at 25°C (hr.)	Bacterial count			
		Supplement to minimal medium A			
		0	+ Cysteine (1 mM)	+ Aspartic acid (2.5 mM)	+ Trypticase (2%)
Before freezing					
Distilled water*	0	134 ± 3	194 ± 9	189 ± 6	186 ± 3
	2	0	1 ± 1	1 ± 1	2 ± 2
Glass-distilled water	0	186 ± 7	184 ± 6	189 ± 6	182 ± 7
	2	181 ± 11	182 ± 6	181 ± 2	180 ± 4
Glass-distilled water + KH_2PO_4 buffer (3×10^{-4}M)	0	182 ± 9	189 ± 6	181 ± 5	179 ± 4
	2	177 ± 8	184 ± 5	184 ± 5	178 ± 2
Glass-distilled water + $MgSO_4$ (2×10^{-3}M)	0	179 ± 5	195 ± 2	187 ± 5	181 ± 6
	2	189 ± 9	191 ± 5	188 ± 7	190 ± 5
After freezing**					
Distilled water*	0	0	3 ± 2	4 ± 2	2 ± 1
	2	0	0	0	0
Glass-distilled water	0	47 ± 6	52 ± 5	96 ± 8	102 ± 5
	2	58 ± 6	50 ± 5	88 ± 4	100 ± 4
Glass-distilled water + KH_2PO_4 buffer (3×10^{-4}M)	0	65 ± 5	67 ± 7	128 ± 7	137 ± 6
	2	57 ± 9	55 ± 7	130 ± 8	134 ± 7
Glass-distilled water + $MgSO_4$ (2×10^{-3}M)	0	70 ± 4	62 ± 3	138 ± 6	146 ± 7
	2	70 ± 6	69 ± 6	142 ± 7	145 ± 7

*From Barnstead still (for detail see MacLeod, Kuo and Gelinas, 1967)

**Frozen and stored for 1 week at -20°C

suspended in glass-distilled water for a 2-hour period.

A subsequent experiment was run to investigate whether increased concentrations of phosphate buffer or $MgSO_4$ in the plating diluent would enhance the recovery of the cells which had been frozen and stored. The results in Table 19 show that the recovery of frozen cells was improved by the addition of 3×10^{-4} M KH_2PO_4 or 2×10^{-3} M $MgSO_4$ but not significantly further improved by increasing the concentration of phosphate buffer or $MgSO_4$ above these levels. In fact, phosphate buffer and $MgSO_4$ at concentrations of 3×10^{-2} M and 2×10^{-1} M respectively, reduced the count on the suspension of cells after freezing.

Since phosphate buffer improved the recovery of cells after freezing it was of interest to know if the effect was due to the specific anion, cation or both supplied by the buffer. The effect of adding to the plating diluent either KCl or KH_2PO_4 adjusted to pH 7.2 with KOH or with NaOH, or NaH_2PO_4 adjusted with NaOH was examined. The results in Table 20 show that there was no specific cation or anion responsible for the recovery of the cells which had been frozen and stored. All of the additives to the plating diluent improved the recovery of cells to much the same extent after freezing. It was also of interest to determine

TABLE 19

Effect of composition of plating diluent, composition of plating medium on the viable count of a suspension of *Escherichia coli* 4518 before and after freezing (III)

Plating diluent	Bacterial count		
	Supplement to minimal medium A		
	0	+ Aspartic acid (2.5 mM)	+ Trypticase (2%)
Before freezing			
Glass-distilled water	191 ± 9	189 ± 5	194 ± 8
KH_2PO_4 buffer (3×10^{-4} M)	177 ± 4	184 ± 7	187 ± 5
" " (3×10^{-3} M)	178 ± 5	177 ± 2	185 ± 5
" " (3×10^{-2} M)	181 ± 4	186 ± 3	185 ± 5
$MgSO_4$ (2×10^{-3} M)	176 ± 6	177 ± 4	181 ± 5
" (2×10^{-2} M)	172 ± 6	171 ± 4	173 ± 5
" (2×10^{-1} M)	172 ± 4	199 ± 7	183 ± 8
After freezing*			
Glass-distilled water	46 ± 4	134 ± 8	155 ± 11
KH_2PO_4 buffer (3×10^{-4} M)	51 ± 2	190 ± 11	195 ± 4
" " (3×10^{-3} M)	63 ± 5	198 ± 6	209 ± 5
" " (3×10^{-2} M)	69 ± 4	152 ± 5	151 ± 5
$MgSO_4$ (2×10^{-3} M)	62 ± 6	233 ± 7	249 ± 13
" (2×10^{-2} M)	61 ± 4	224 ± 7	240 ± 6
" (2×10^{-1} M)	23 ± 8	146 ± 4	151 ± 5

*Frozen and stored for 1 week at $-20^{\circ}C$

TABLE 20

Relative effect of K^+, Na^+ and $PO_4^{\equiv}$ in plating diluent on the viable count of Escherichia coli 451B before and after freezing

Plating diluent	Bacterial count		
	Supplement to minimal medium A		
	0	+ Aspartic acid (2.5 mM)	+ Trypticase (2%)
Before freezing			
Glass-distilled water	198 ± 4	197 ± 5	189 ± 5
KCl ($3x10^{-3}$M)	196 ± 6	196 ± 14	194 ± 7
KH_2PO_4 ($3x10^{-3}$M, KOH neutralized)	205 ± 6	201 ± 7	196 ± 3
KH_2PO_4 (3x10-3M, NaOH neutralized)	191 ± 2	205 ± 7	194 ± 5
NaH_2PO_4 (3x10-3M, NaOH neutralized)	197 ± 7	202 ± 7	201 ± 9
After freezing*			
Glass-distilled water	82 ± 7	165 ± 9	176 ± 7
KCl ($3x10^{-3}$M)	94 ± 7	223 ± 11	212 ± 10
KH_2PO_4 ($3x10^{-3}$M, KOH neutralized)	96 ± 3	232 ± 6	226 ± 7
KH_2PO_4 (3x10-3M, NaOH neutralized)	81 ± 5	218 ± 7	220 ± 10
NaH_2PO_4 (3x10-3M, NaOH neutralized)	91 ± 7	216 ± 9	221 ± 7

*Frozen and stored for 1 day at -20°C

if the recovery of cells would be increased if aspartic acid was included in the plating diluent. The results of an experiment to determine this are recorded in Table 21. The recovery of frozen cells in the medium without added supplement was higher when aspartic acid was added to the plating diluent. This is to be expected since some carry-over of aspartic acid from the plating diluent to the plating medium would occur in plating. If a supplement was present in the medium, however, there was not a significant increase in count on the suspension after freezing if aspartic acid was present in the plating diluent. The results also show that the effects of $MgSO_4$ and phosphate buffer in the plating diluent are not additive.

(V) Metabolic injury in several species and strains of Gram negative organisms after freezing and storage

Since *Aerobacter aerogenes* (Mac 112) and *Escherichia coli* 4518 showed marked differences in their nutritional response after freezing and storage, it was decided to examine several species and strains of Gram negative organisms to see how they responded to supplements after freezing. In this survey, the effect of adding aspartic acid, cysteine or Trypticase at one arbitrarily

TABLE 21

Relative effect of glass-distilled water, $MgSO_4$, phosphate buffer, and Aspartic acid in combining as plating diluent on the viable count of *Escherichia coli* 451B before and after freezing

Plating diluent	Bacterial count		
	Supplement to minimal medium A		
	0	+ Aspartic acid (2.5 mM)	+ Trypticase (2%)
Before freezing			
Glass-distilled water	178 ± 3	173 ± 4	183 ± 5
$MgSO_4$ ($2x10^{-3}$m)	173 ± 4	179 ± 3	172 ± 5
$MgSO_4$ + KH_2PO_4 ($3x10^{-3}$M)	177 ± 4	180 ± 3	175 ± 5
$MgSO_4$ + Aspartic acid (2.5 mM)	175 ± 3	178 ± 5	179 ± 5
$MgSO_4$ + KH_2PO_4 + Aspartic acid	173 ± 4	178 ± 7	177 ± 5
After freezing*			
Glass-distilled water	69 ± 4	139 ± 4	136 ± 5
$MgSO_4$ ($2x10^{-3}$m)	91 ± 4	209 ± 4	203 ± 6
$MgSO_4$ + KH_2PO_4 ($3x10^{-3}$M)	87 ± 4	193 ± 4	189 ± 6
$MgSO_4$ + Aspartic acid (2.5 mM)	142 ± 5	222 ± 5	206 ± 5
$MgSO_4$ + KH_2PO_4 + Aspartic acid	111 ± 5	198 ± 4	194 ± 3

*Frozen and stored for 1 day at $-20^{o}C$

selected level of each supplement was determined. Aspartic acid was added at 2.5 mM, the concentration optimum for increasing the count on Escherichia coli 451B after freezing, cysteine was added at 1 mM, the level used to protect Aerobacter aerogenes (Mac 112) from the effect of toxic trace elements in the plating diluent when water from a tin-lined still was used to prepare the diluent. In this survey, however, water distilled from an all-glass still was used and the plating diluent was a solution of 0.002 M $MgSO_4$. The results are recorded in Tables 22, 23, 24 and 25. All of the organisms tested produced equivalent numbers of colonies before freezing on both minimal and enriched agar media, except one of the strains of Aerobacter aerogenes (Mac 1) which was extremely sensitive to the presence of aspartic acid in the plating medium. The results in Table 22 show that all of the strains of Escherichia coli exhibited symptoms of metabolic injury after freezing and storage. Cysteine was not effective for most of the strains tested except for strains Mac 22. Aspartic acid or aspartic acid plus cysteine was as effective as Trypticase as a supplement to the minimal medium for increasing the viable count on the cell suspensions after freezing and storage. In Table 23, it was confirmed that suspensions of Aerobacter aerogenes (Mac 112) after freezing showed little difference

TABLE 22

Effect of composition of plating medium on recovery of various strains of *Escherichia coli* before and after freezing

Organism	Treatment of cells*	Relative number of colonies**				
		Supplement to minimal medium A				
		0	Cysteine	Aspartic acid	Aspartic acid + Cysteine	Trypticase
Escherichia coli 451B	Unfrozen	100	101	100	101	100
	Frozen for 1 day	100	91	173	182	190
	Frozen for 1 week	100	106	174	184	186
Escherichia coli Mac No. 22	Unfrozen	100	98	100	97	93
	Frozen for 3 days	100	156	148	140	149
	Frozen for 4 days	100	130	134	131	136
Escherichia coli ATCC11303	Unfrozen	100	95	99	95	96
	Frozen for 3 days	100	113	136	119	135
	Frozen for 1 week	100	111	143	134	140
Escherichia coli ATCC9723	Unfrozen	100	98	106	102	101
	Frozen for 1 day	100	102	147	175	167
	Frozen for 3 days	100	93	149	164	164
Escherichia coli ATCC11246	Unfrozen	100	101	104	100	101
	Frozen for 1 day	100	101	119	120	123
	Frozen for 1 week	100	109	132	132	167

*Frozen and stored at $-20^{\circ}C$

**2×10^{-3} M $MgSO_4$ was used as plating diluent. The bacterial count on minimal medium A was taken as 100. Each result represents the average of the count on four plates.

TABLE 23

Effect of composition of plating medium on recovery of various strains of *Aerobacter aerogenes* before and after freezing

Organism	Treatment of cells*	Relative number of colonies**				
		Supplement to minimal medium A				
		0	Cysteine	Aspartic acid	Aspartic acid + Cysteine	Trypticase
A. aerogenes Mac No. 112	Unfrozen	100	101	103	106	102
	Frozen for 1 day	100	100	102	100	99
	Frozen for 1 week	100	117	105	114	113
A. aerogenes Mac No. 613	Unfrozen	100	98	94	100	103
	Frozen for 2 days	100	122	95	125	127
	Frozen for 1 week	100	117	120	112	126
A. aerogenes Mac No. 438	Unfrozen	100	104	104	99	102
	Frozen for 2 days	100	179	144	184	184
	Frozen for 1 week	100	177	145	180	176
A. aerogenes Mac No. 1	Unfrozen	100	93	8	34	91
	Frozen for 1 day	100	141	9	46	123

*See Table 22

**See Table 22

in plate count on adding supplements to the minimal medium. A similar result was obtained with Aerobacter aerogenes (Mac 613). The count on suspensions of Aerobacter aerogenes (Mac 1) both before and after freezing was markedly depressed by the addition of aspartic acid to the plating medium, though cysteine showed a capacity to promote the count after freezing. A strain of Aerobacter aerogenes (Mac 438) developed a high extent of metabolic injury. Cysteine was as effective as Trypticase, whereas aspartic acid was not so effective in enhancing the viable count of the cells after freezing and storage. The results with the strains of Serratia marascens recorded in Table 24 show that supplementing the plating medium with cysteine plus aspartic acid was as effective as with Trypticase for recovery of the cells of Serratia marcescens (8 UK) after freezing and storage, but neither cysteine nor aspartic acid alone was effective. Both Serratia marcescens (Mac 21) and Serratia marcescens (Mac 274) responded best to Trypticase. The results with the two Pseudomonas species tested (Table 25) show that the count on suspensions of Pseudomonas fluorescens after freezing was not increased consistently by adding any of the supplements to the minimal medium while cysteine depressed the count. Pseudomonas aeruginosa, on the other hand, developed a marked capacity to respond to Trypticase after freezing though, again, cysteine depressed the count.

TABLE 24

Effect of composition of plating medium on recovery of various strains of *Serratia marcescens* before and after freezing

Organism	Treatment of cells*	Relative number of colonies**				
		Supplement to minimal medium A				
		0	Cysteine	Aspartic acid	Aspartic acid + Cysteine	Trypticase
S. marcescens 84K	Unfrozen	100	101	102	100	100
	Frozen for 1 day	100	105	117	156	153
	Frozen for 4 days	100	114	126	177	182
S. marcescens Mac No. 21	Unfrozen	100	100	99	102	96
	Frozen for 1 day	100	105	134	129	182
	Frozen for 1 week	100	112	124	170	229
S. marcescens Mac No. 274	Unfrozen	100	101	108	97	97
	Frozen for 1 day	100	113	132	129	142
	Frozen for 4 days	100	97	142	120	168

*See Table 22

**See Table 22

TABLE 25

Effect of composition of plating medium on recovery of *Pseudomonas fluorescens* and *Pseudomonas aeruginosa* before and after freezing

Organism	Treatment of cells*	Relative number of colonies**				
		Supplement to minimal medium A				
		0	Cysteine	Aspartic acid	Aspartic acid + Cysteine	Trypticase
P. fluorescens Mac No. 9	Unfrozen	100	94	101	97	97
	Frozen for 1 day	100	49	99	56	93
	Frozen for 3 days	100	40	90	39	93
	Frozen for 1 week	100	62	105	51	119
P. aeruginosa Mac No. 436	Unfrozen	100	98	94	99	99
	Frozen for 1 day	100	52	116	54	159
	Frozen for 3 days	100	31	113	21	170
	Frozen for 1 week	100	34	138	77	275

*See Table 22

**See Table 22

DISCUSSION

The results of the studies with Aerobacter aerogenes (Mac 112) show that there was no response to either cysteine, yeast extract or Trypticase as supplement to minimal medium when suspensions of the cells after freezing were plated irrespective of the additives to the plating diluent, if the diluent was prepared with water distilled in an all-glass still. This corroborates previous findings with this organism (MacLeod, Kuo and Gelinas, 1967) that if toxic trace elements were removed from the plating diluent no metabolic injury effects could be demonstrated after freezing.

When Escherichia coli 451B was tested, under the condition described by Moss and Speck (1966a), the metabolic injury was manifested even after the toxic trace elements in the plating diluent had been removed. With this organism, after freezing and thawing of the suspension, higher counts were obtained on minimal medium supplemented with yeast extract or Trypticase than on minimal medium alone. However, cysteine had no supplementary capacity under these conditions. Neither chelating agents such as nitrilotriacetic acid nor the nucleic acids DNA and RNA were effective

as supplements to the minimal medium. Thus, the enhanced response to Trypticase on the recovery of Escherichia coli 451B after freezing and storage seems to be due to more than an ability to detoxify toxic elements.

Straka and Stokes (1959), and Moss and Speck (1966a) reported that acid hydrolyzed casein is ineffective in bringing about the recovery of injured cells after freezing and storage. They, therefore, concluded that the active component(s) in the Trypticase was one or more peptides. In their experiments they tested acid hydrolyzed casein at only one level, 2%. The results in this study show that the HCl hydrolyzed Trypticase had some effect in bringing about the recovery of the cells of Escherichia coli 451B after freezing and storage, but the concentration which was effective seemed to be quite critical. When a 3% concentration of HCl hydrolyzed Trypticase was used as a supplement in the minimal medium, it depressed the count on suspension of cells after freezing and storage but not before. Since, with HCl hydrolyzed Trypticase, a considerable amount of alkali is needed to neutralize the HCl left after removal of all that can be removed by evaporation, the amount of salts formed might be toxic. Nakamura and Dawson (1962) have shown that casamino acid is able to cause the recovery of as many cells of Shigella sonnei as blood heart infusion agar or nutrient

agar when the cells are frozen in nutrient broth or milk. Unfortunately, the concentrations used by them were not clearly reported. Arpai (1964) observed that acid and alkali hydrolysis of yeast extract resulted in little change to its activity in the recovery of the cells of Escherichia coli injury by freezing.

H_2SO_4 hydrolyzed Trypticase after treatment with Dowex 50 x 8 (H^+ form) was as effective as Trypticase in promoting the recovery of Escherichia coli 451B after freezing and storage. Dowex 50 x 8 (H^+ form) resin did not remove active component(s) present in hydrolyzed Trypticase. However, further treatment of the eluate from Dowex 50 x 8 with Dowex 1 x 8 (Cl^- form) caused the sample to lose activity. This fact suggested that the active component was not a neutral compound, and most probably was one or more of the amino acids with an excess of carboxyl over amino groups. Subsequent experiments with an L-amino acid mixture which showed aspartic and glutamic acids to be the most active compounds support this initial conclusion.

In studies with the L-amino acid mixture, a number of different concentrations were tested to determine their effect on the bacterial count of Escherichia coli 451B before and after freezing. There was no difference in the bacterial

count on the addition of any concentration of amino acid mixture before freezing. After freezing, however, a concentration of amino acid mixture equivalent to 2% casein was inhibitory, and even the concentration equivalent to 1% was not effective. The amino acid mixture at 0.1% was found, however, to be effective, but when concentrations as low as 0.01% were tested the recovery of the frozen cells of Escherichia coli 451B was no better than with minimal medium alone. Thus, the effect of concentration of amino acid on the recovery of the cells after freezing should not be overlooked, otherwise some inappropriate conclusions could be drawn. Arpai (1964), for instance, tested 16 amino acids at a concentration of 0.01% (w/v) individually supplemented to minimal medium on the recovery of Escherichia coli after freezing and storage, and concluded that all of these amino acids were not effective. And again, unfortunately, no explanation was given of why such a low concentration was used in his studies. Moreover, in his studies, DL-amino acid mixture instead of an L-amino acid mixture was tested. DL-amino acids have been found to be toxic for Pseudomonas denitrificans while the L-forms of the same amino acids were either required or innocuous for the growth (Daniels, 1966). The results could have been quite different if an L-amino acid mixture had been used.

When the individual amino acids were tested, aspartic acid alone was found to be as effective as Trypticase or the amino acid mixture as a supplement to the minimal medium. Glutamic acid and alanine were also quite effective. Those cells which after freezing were able to grow on the minimal medium supplemented with aspartic acid but not on the minimal medium alone appeared not to be stable auxotrophic mutants since all of the colonies on the supplemented medium could be replicated onto the minimal medium. MacLeod et al. (1966) and Postgate and Hunter (1963) have arrived at a similar conclusion with other organisms.

Serine alone, or an amino acid mixture containing lysine, methionine, cystine, arginine, proline and histidine were found to be inhibitory to the bacterial cells after freezing, but not before. Threonine and glycine tested individually as supplements to the minimal medium have some count increasing capacity, but the activity seems to be masked by the presence of serine. These findings suggest that the cells become more permeable to toxic amino acids after freezing.

The effect of the composition of the plating diluent on viability and the development of metabolic injury

in unfrozen and frozen cells of Aerobacter aerogenes (Mac 112) has been intensively studied by MacLeod et al. (1967). In the present study, the results obtained with Escherichia coli 451B also show that when the plating diluent was distilled water from a tin-lined still, even unfrozen cells of Escherichia coli 451B could develop symptoms of metabolic injury as evidenced by an increased count on the minimal medium, glucose-salts agar medium when it was suitably supplemented. Cysteine, aspartic acid, and Trypticase were equally effective as supplements under these conditions. The cells after freezing and storage became much more sensitive when distilled water from a tin-lined still was used as the plating diluent. This water has been shown to contain toxic trace contaminants (MacLeod, Kuo and Gelinas, 1967).

When glass-distilled water alone was used as the plating diluent to determine the viability of the cells of Escherichia coli 451B after freezing and storage, low viable counts were obtained in either enriched medium A or B relative to those obtained with a plating diluent consisting of 2×10^{-3} M $MgSO_4$ in glass-distilled water. Since there was no loss in viability of cells held in the plating diluent of glass-distilled water for a 2-hour period at $25^{\circ}C$, the loss, therefore, appeared not to be due to trace element contaminants. When glass-distilled water containing 3×10^{-4} M

phosphate buffer was used as the plating diluent, the recovery of the frozen cells of Escherichia coli 451B was increased relative to those obtained with glass-distilled water alone. However, the recovery was even better when 2×10^{-3} M $MgSO_4$ was used as the plating diluent. The recovery of frozen cells was not improved by increasing the concentration of phosphate buffer to 3×10^{-3} M. When solutions of KH_2PO_4, NaH_2PO_4 or KCl at a concentration of 3×10^{-3} M were used as diluting fluids to investigate whether any cations or anions are responsible for the effect on the recovery of Escherichia coli 451B after freezing and storage, the results show that no ionic specificity was involved under the conditions studied.

Aspartic acid was found capable of enhancing the viable count when it was used as a supplement to the minimal medium, but there was no evidence of increased recovery of frozen cells of Escherichia coli 451B on enriched agar medium when a plating diluent containing aspartic acid plus $MgSO_4$ was used as compared with a solution of $MgSO_4$ alone or $MgSO_4$ plus phosphate buffer. However, it was found that the count on minimal medium was higher when aspartic acid was added to the plating diluent. The effect seems to be due to some carry-over of aspartic acid from the plating diluent to the plating medium.

A comparison of several strains of Gram negative organisms, Escherichia coli, Aerobacter aerogenes, Serratia marcescens and Pseudomonas species as regards their response to enrichment of the plating medium before and after freezing, indicates that the extent of metabolic injury and the activity of the various enriching supplements in the minimal medium varied from strain to strain. In the case of three strains of Escherichia coli tested, aspartic acid, or aspartic acid plus cysteine when added to the minimal medium had the same count increasing effect as Trypticase. Two strains of Aerobacter aerogenes did not develop metabolic injury after freezing and storage for one week under the conditions studied. However, another two strains showed metabolic injury to a great extent after freezing and storage, and the viable count was particularly promoted by cysteine or Trypticase. These findings suggest that cysteine, in addition to its action as a chelating agent to remove toxic trace elements present in plating diluents or media, in the case of certain strains of Aerobacter aerogenes, also exhibits other beneficial functions which enhance the viable count of other strains of Aerobacter aerogenes after freezing and storage. Studies with three strains of Serratia marcescens and two species of Pseudomonas indicated that most of them did not respond positively to the presence of

aspartic acid in the medium after freezing. Since the effect of adding aspartic acid at the one level of 2.5 mM was tested in these experiments, it is not possible to conclude that aspartic acid would not be effective in increasing the count of these organisms after freezing and storage at other concentrations which could be tested.

Lindeberg and Lode (1963) found ultraviolet absorbing material appearing in the extracellular fluid of frozen and thawed suspensions of Escherichia coli in amounts proportional to the loss in viability. Mazur (1963) also studied Escherichia coli and showed that the loss in viability caused by freezing and thawing was roughly proportional to the increase in the concentration of solutes in the external medium. Moss and Speck (1966b) observed that freezing and storage of Escherichia coli Y at -20°C in phosphate buffer resulted in loss of cell viability and a pronounced leakage of cellular material which had maximal absorption at 260 mμ. They also found that greater loss in cell viability occurred when the cells were frozen in distilled water, but only small amounts of 260 mu absorbing material were detected in the supernatant solution. In their studies, the appearance of peptide material in the supernatant fractions of frozen and thawed cells paralleled losses in cell viability.

Strange and Postgate (1964) have shown that cells of Aerobacter aerogenes after freezing and storage were more permeable to exogenous RNAase than the cells before freezing. MacLeod et al. (1967) observed that freezing and storage rendered the cells of Aerobacter aerogenes (Mac 112) much more sensitive to the toxic metal contaminants in the plating diluent.

Based on the observation that leakage of peptides into the extracellular fluids paralleled losses in viability of Escherichia coli and that these compounds possessed activity for the recovery of the cells which had been injured by freezing, Moss and Speck (1966b) suggested that this fact could explain why the peptides from Trypticase, when added to minimal medium, effected a recovery of Escherichia coli after freezing and storage. The results in the present studies with Escherichia coli 451B found that no special peptide is required to repair the damage to the cells caused by freezing. The efficacy of Trypticase could be due to the fact that toxic amino acids bound in peptides may be less toxic than when free and would have less tendency to mask the growth promoting effects of aspartic acid, glutamic acid or their peptides.

The basic lesion in the cells injured by freezing

and storage seems to be membrane damage, leading to increased cell penetrability, and therefore to increased sensitivity of the cells to environmental changes which do not have any influence on unfrozen cells. Thus certain amino acids, non-toxic before freezing, become toxic after freezing since they can penetrate the cell membrane. There are at least two possible explanations for the count increasing action of aspartic acid or glutamic acid on Escherichia coli 451B. One is that damage to the cell membrane causes the intracellular pool of these amino acids to leak from the cells. If a critical concentration of at least one of these amino acids is required inside the cells for the cells to grow and divide, the appropriate concentration of these amino acids must be supplied in the medium for growth to take place. Alternatively, one or other of these active amino acids may be required specifically to repair the damage caused by freezing before growth and division can take place.

Metabolic injury as evidenced by an increased viable count on enriched as compared to a minimal medium would manifest itself if the enriched medium contained a factor(s) capable of rendering innocuous any toxic penetrating solutes present in the plating diluent or plating medium. These factors may be able to reconstitute the

intracellular pool of solutes required for growth, or permit the resynthesis of the cellular materials such as the membrane which were damaged by exposure to freezing. It is evident from the results with organisms other than Escherichia coli 451B that a supplement favoring the recovery of one organism may not be applicable to the others.

PART II

EFFECT OF GLASS-DISTILLED WATER AND VARIOUS SALTS AS FREEZING MENSTRUA ON THE VIABILITY OF *AEROBACTER AEROGENES* (MAC 112) AFTER FREEZING AND STORAGE

INTRODUCTION

There are a large number of reports on the striking influence of the freezing menstruum on the survival of bacterial cells during freezing. The survival is usually lower if the freezing medium is a solution of NaCl than when it is distilled water (Kuo, 1966; Major et al., 1955; Harrison, 1956; Postgate and Hunter, 1961). However, it is not known whether the effect of NaCl is due to the Na^+, the Cl^- or to the nonspecific action of a salt. A limited amount of information is available on the effects of other inorganic salts on the survival of bacterial cells during freezing. It seemed pertinent, therefore, to compare the effect of distilled water, solutions of NaCl and other inorganic salts as freezing menstrua on the viability of Aerobacter aerogenes (Mac 112). An attempt was made to localize the action of NaCl by determining the internal Na^+ and Cl^- concentration at various levels of NaCl in the solution used to suspend the cells during freezing. It was thought that more information regarding the effect of various salts during freezing on the survival of bacterial cells after freezing may lead to a better understanding of the cause of death of bacterial cells due to freezing.

MATERIAL AND METHODS

Test organisms

Aerobacter aerogenes (Mac 112) was used throughout in these experiments.

Media

(a) Liquid medium for culturing the organisms

Cells for preparing suspensions were grown in Trypticase Soy Broth (BBL).

(b) Solid medium for plate counts

The plating medium used was minimal medium containing 1% yeast extract (Difco). The composition of the minimal medium was the same as that employed by MacLeod, Kuo and Gelinas (1967). It contained K_2HPO_4, 1.75 gm.; KH_2PO_4, 0.75 gm.; $(NH_4)_2SO_4$, 1.0 gm.; $MgSO_4.7H_2O$, 0.1 gm.; Sodium citrate, 0.1 gm.; Glucose, 4.0 gm. and Agar, 15 gm., dissolved in one liter of glass-distilled water.

Preparation of cell suspensions

Ten ml. of the liquid medium in a 50-ml. Erlenmeyer flask were innoculated from a culture growing on an agar slant and incubated for 8 hours at 30°C in a stationary culture. This liquid culture was then transferred to 250 ml. of medium contained in a 2-liter flask and incubated for 16 hours at 30°C on a rotary shaker. The cells were then harvested by centrifugation at 10,000 x G for 20 minutes in a refrigerated centrifuge, keeping the temperature at 4°C. The cell pellet was resuspended in the same volume of glass-distilled water and centrifuged again in the same fashion. The procedure was repeated three times.

Viability determinations

The number of viable cells in suspensions of both unfrozen and of frozen and thawed cells was determined as described in Part I. The plating diluent used to make dilutions for plating was glass-distilled water.

Preparation of a sterile supernatant from unfrozen, and frozen and thawed cell suspensions

A 250-ml. volume of washed cell suspension in glass-distilled water containing 1 x 10^{10} cells per ml. was divided

into two parts. One part was left unfrozen and held at 4°C in a refrigerator. The other part was dispensed in 60-ml. aliquots into 250-ml. screw-capped polypropylene bottles. The bottles were then placed in liquid air (-196°C) until the suspensions were frozen solid (after 5 minutes). The frozen suspensions were thawed by placing the polypropylene bottles in a beaker of water at 25°C. The cells were centrifuged off and the supernatants were combined and sterilized by filtration through a 0.45 micron HA millipore filter.

A supernatant from the suspension of unfrozen cells was prepared by removing the cells by centrifugation and filtering the supernatant through a sterile 0.45 micron HA millipore filter.

Freezing procedure and storage for viability studies

Prior to freezing, the washed bacterial cells were suspended in the suspending medium appropriate for the experiment at a concentration of 2×10^{10} cells per ml. Five ml. aliquots of this suspension were dispersed into 12 x 100 mm. polypropylene tubes. The tubes were then placed in liquid air (-196°C) until the suspensions were frozen solid. The suspensions were thawed immediately, or were

then transferred to a commercial deep freeze unit and stored at a temperature of -20°C until required.

Thawing procedure

The procedures were the same as were described in Part I.

Measurement of intracellular ion concentrations

To measure intracellular ion concentrations the total amount of a particular ion in a packed cell preparation was measured. The volume of extracellular fluid in this packed cell preparation was determined and the amount of the ion associated with this volume was subtracted from the total obtained for the packed cells. The intracellular fluid volume was estimated and the intracellular ion concentration calculated by assuming that all of the ion associated with the cells was present in solution in the intracellular fluid. The procedures used were essentially those described by Matula (1964).

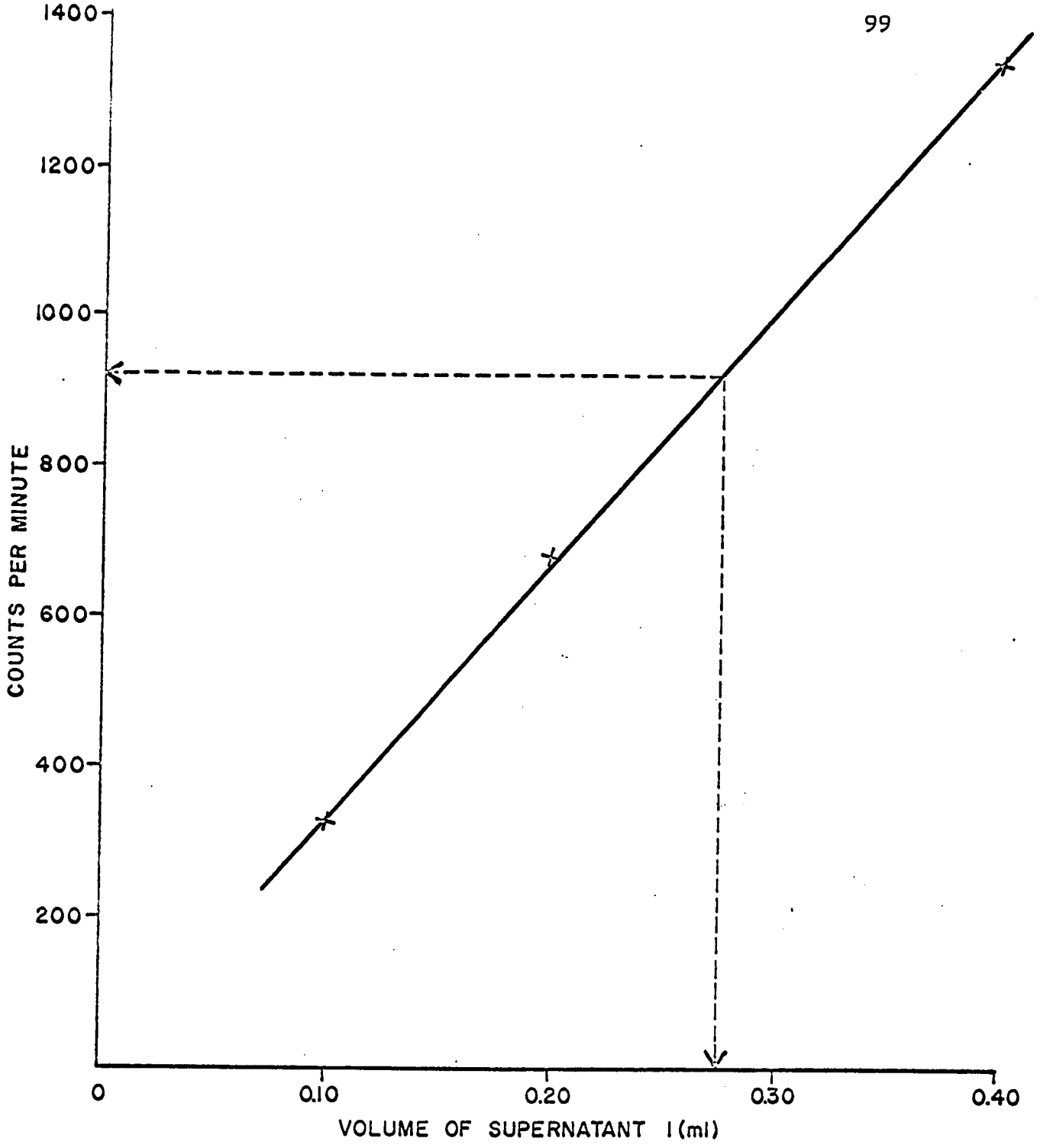
The cells were grown, harvested and washed as previously described. The final cell suspension gave 40-50 mg. dry weight of cells per ml. Each experiment was carried

out in a series of previously weighed Spinco centrifuge tubes, each tube being set up in duplicate. To each tube 4 ml. of the washed cell suspension was added. A 0.3-ml. volume of a solution containing 0.01 m curies inulin - C^{14} was added to the first two tubes and unlabelled inulin at the same concentration to the others. Finally, 4 ml. of glass-distilled water, or of various concentrations of NaCl or KCl were added to each tube and the contents were mixed. The tubes were incubated for 10 minutes at room temperature. The cells were then separated from the suspending fluid by centrifugation at 50,000 x G for 30 minutes in a Spinco ultracentrifuge. The supernatants (S_1) were collected, and the tubes containing the pellets were drained, dried with a swab and weighed. The cells containing the inulin - C^{14} were resuspended to the same volume in a suspending medium of the same composition as the original suspending medium. These cells were centrifuged again and a second supernatant (S_2) was collected. Both supernatant S_1 and S_2 were analysed for radioactivity and from that the extracellular fluid volume was calculated as described below. The packed cells and the supernatants S_1 and S_2 obtained from tubes not containing radioactive inulin were analysed for their content of Na^+, K^+ and Cl^-.

Measurement of Extracellular fluid volume (e.x.f.)

To determine the extracellular fluid volume of the packed cells, three aliquots of supernatant 1 (S_1) having volumes of 0.1, 0.2 and 0.4 ml., were diluted with the same volume of the same suspending medium as was used to resuspend the cells. A 0.1-ml. volume of these dilutions of S_1 and 0.1 ml. of the undiluted supernatant 2 (S_2) were spotted on circles of filter paper (Whitman No. 1) and dried with a hair dryer. Each dried filter paper was then placed in a liquid scintillation vial and covered with 5 ml. of a liquid scintillation mixture of the following composition: 0.5% 2,5-diphenyloxazole (PPO), 0.03% 1,4-bis-2(5-phenyl-oxazolyl)-benzene (POPOP), dissolved in toluene. The radio-activity in the samples was counted using a liquid scintillation spectrometer. A curve was constructed relating the radioactivity of the dilutions of S_1 to the volume of S_1 which had been diluted with the same volume of the same salt solution used to resuspend the cells (Fig. 1). From the C^{14} activity of the aliquot of S_2, the volume of S_1 which had been trapped in the cell pellet could be determined by reference to the curve. This was the extracellular fluid volume (e.c.f.) of the packed cell preparation. A typical curve is shown in Figure 1.

Figure 1. A typical curve relating the volume of supernatant 1 to the counts obtained when aliquots of supernatant 1 are diluted with the same volume of salt solutions as is used to resuspend the cells in obtaining supernatant 2.
The dotted line shows how counts obtained on an aliquot of supernatant 2 are used to determine the volume of supernatant 1 trapped in the cell preparation.


1400
1200
1000
800
600
400
200
COUNTS PER MINUTE
0
0.10
0.20
0.30
0.40
VOLUME OF SUPERNATANT I (ml)

Calculation of intracellular fluid volume (i.c.f.)

The intracellular fluid volume was calculated to be the difference between the total fluid volume and the extracellular fluid volume of the packed cell preparation. The total fluid volume was obtained by subtracting the dry weight of cells added to the original suspension from the weight of the packed cells. The total weight so obtained was converted to volume by assuming the specific gravity of the total fluid to be the same as that of the extracellular fluid.

Determination of Na^+ and K^+

The supernatant obtained after the cells were removed from the suspension by centrifugation was analysed directly for Na^+ and K^+ by flame photometry. The packed cells from the same suspension were transferred quantitatively to 30-ml. Vycor glass Kjeldahal flasks with the aid of 5 ml. concentrated HNO_3 and were heated until the HNO_3 had evaporated. All samples were then digested by a modification of the procedure of Sanui and Pace (1959). Perchloric acid in 3-ml. quantities was added to the Kjeldahl flask containing the yellow residue and the digestion was performed on an electrically heated micro-Kjeldahl digestor.

When the samples became colorless the perchloric acid was removed under partial vacuum according to the method of Gordon (1966). In this procedure one end of a Vycor glass tube was inserted into the neck of the flask and the other was immersed in water in a filter flask. The fumes from the flask were drawn through the water by attachment of the filter flask to an aspirator pump. The method effectively removed the perchloric acid in a short time and confined the fumes. Suitable dilutions of the residue were made with glass-distilled water. Analysis was performed using a flame photometer attachment for a Zeiss PMQ11 spectrophotometer. Reagent grade NaCl and KCl in glass-distilled water were used as standards and their emission read at the same time as the samples. Na^+ or K^+ in the samples was determined by reference to a plot relating Na^+ or K^+ concentration of the standard to per cent emission.

Determination of Cl^-

The supernatant obtained when the cells were centrifuged from the suspension was analysed for Cl^- directly by a volumetric method (A.O.A.C., 1960). The packed cells were transferred to a platinum dish and moistened with 10 ml. of 5% Na_2CO_3 solution. When the water had evaporated to dryness, the cells were ignited at 450° to 490°C (<500°C)

overnight. The ashes were then extracted with hot water, filtered through Whatman No. 42 filter paper and washed thoroughly. The residues were returned to the platinum dishes and ignited again at the same temperature overnight. The second ash was dissolved in diluted HNO_3, filtered and the filtrate was added to the water extract. The final volume was adjusted to 50 ml. The Cl^- content was determined on this solution and on aliquots of the supernatant volumetrically by adding an excess of a standard solution of 0.1 N $AgNO_3$. The excess of $AgNO_3$ was back titrated with 0.1 N KCNS in dilute HNO_3 using Fe $(NH_4)_2SO_4.12H_2O$ as indicator. One ml. nitrobenzene was added to minimize the reaction between thiocyanate and silver chloride and to ensure a good end point (Belcher and Nutten, 1960). The titration was carried out with the aid of a micrometer syringe. The quantity of Cl^- was calculated from the amount of $AgNO_3$ found necessary to precipitate it.

Dry weight determination

A known volume (e.g., 2 ml.) of cell suspension was placed in a weighing bottle and dried overnight at 95°C in an oven. When cooled, the bottle containing the cells was transferred to a dessicator and held there until it reached constant weight.

Source of materials

Inulin-C^{14} (0.1 m curies) was purchased from the New England Nuclear Corporation. Unlabelled inulin was obtained from Matheson Coleman and Bell Company Inc. The inorganic salts used were Reagent Grade chemicals.

Preparation of glassware

All glassware used to prepare dilutions of the cells for plating and to prepare samples for cation and anion determination were soaked in a mixture of concentrated nitric acid and sulfuric acid (2:1 v/v) for 3 hours. This treatment was followed by 3 rinses with tap water and finally by 10 rinses with glass-distilled water.

RESULTS

The effect of NaCl as freezing menstruum on the survival of Aerobacter aerogenes (Mac 112)

To test the effect of NaCl as a freezing menstruum on the viability of Aerobacter aerogenes (Mac 112) after freezing and immediate thawing and again after storage for 1 week at -20°C, the experiment was carried out by suspending cells for freezing at a concentration of about 2×10^{10} per ml. in glass-distilled water and in various concentrations of NaCl. This concentration of cells was used in the previous studies (Kuo, 1966; MacLeod et al., 1967). The results in Table 26 show that the survival of Aerobacter aerogenes (Mac 112) after freezing was best in glass-distilled water and in concentrations of NaCl lower than 0.01 M. There was, however, a drastic decrease in viability when the concentration of NaCl was increased to 0.05 M and above. It can be seen that there was about a 20% loss of viability when cells were suspended in glass-distilled water for freezing but no further change after storage for 1 week, whereas when cells were frozen in 0.2 M NaCl there was about a 70% loss of viability immediately and a 90% loss after storage at -20°C

TABLE 26

Effect of various concentrations of NaCl as suspending fluid for freezing on the viability of *Aerobacter aerogenes* (Mac 112) after freezing, and after freezing and storage

Suspending fluid	Treatment of cells					
	Unfrozen		Frozen and thawed immediately		Frozen and stored*	
	Count** 10^8 cells/ml	Survival %	Count 10^8 cells/ml	Survival %	Count 10^8 cells/ml	Survival %
Glass-distilled water	225 ± 5	100	173 ± 4	76.9	170 ± 5	75.7
0.001 M NaCl	219 ± 8	100	170 ± 6	77.7	174 ± 6	79.9
0.005 M NaCl	218 ± 8	100	164 ± 8	76.3	166 ± 4	76.0
0.01 M NaCl	221 ± 6	100	148 ± 5	66.8	142 ± 6	64.3
0.05 M NaCl	228 ± 3	100	89 ± 4	38.8	70 ± 2	30.7
0.1 M NaCl	228 ± 6	100	79 ± 5	34.3	55 ± 6	24.1
0.2 M NaCl	226 ± 7	100	70 ± 3	30.8	21 ± 2	9.4

*Frozen in liquid air (-196°C) and stored for 1 week at -20°C

**Plated on minimal medium plus 1% yeast extract

for 1 week. These observations are in agreement with previous findings in this laboratory (Kuo, 1966), and also with those recorded by Harrison (1956) and Clement (1961).

The lethal effect of NaCl on the viability of Aerobacter aerogenes (Mac 112), however, can be eliminated by the addition of some organic hydrophilic non-electrolytes to the freezing menstruum as demonstrated in Table 27. As in the previous results (Table 26) the cells suspended in glass-distilled water for freezing showed only about 20% loss of viability after freezing and storage for 1 week at -20°C. On the other hand, 80% of cells that were suspended in 0.05 M NaCl for freezing were killed after being frozen and stored for 1 week. Cells suspended in 0.05 M NaCl plus 0.55 M glycerol, dimethyl sulfoxide (DMSO), or inositol for freezing lost only 15 to 20% of their viability, after being frozen and stored for 1 week at -20°C. These observations are similar to the results obtained by previous workers (Hallander et al., 1954; Tanguay, 1959; Postgate and Hunter, 1961; Farrant, 1965).

TABLE 27

Capacity of glycerol, DMSO (Dimethylsulfoxide), or Inositol to protect against the loss of viability caused by the presence of NaCl in the freezing suspension

Suspending fluid	Treatment of cells					
	Unfrozen		Frozen and thawed immediately		Frozen and stored*	
	10^8 Count cells/ml	Survival %	10^8 Count cells/ml	Survival %	10^8 Count cells/ml	Survival %
Glass-distilled water	231 ± 5	100	187 ± 5	80.8	180 ± 3	77.9
0.05 M NaCl	224 ± 3	100	60 ± 4	29.3	45 ± 7	20.0
0.05 M NaCl + 0.55 M glycerol**	230 ± 3	100	197 ± 4	86.0	194 ± 4	84.5
0.05 M NaCl + 0.55 M DMSO	226 ± 4	100	196 ± 5	86.7	198 ± 4	87.5
0.05 M NaCl + 0.55 M Inositol	225 ± 5	100	186 ± 5	83.5	182 ± 4	80.8
0.05 M NaCl + 0.275 M Inositol	227 ± 2	100	181 ± 5	79.4	179 ± 4	78.7

*See Table 26

**0.55 M Glycerol ≏ 5%
0.55 M Inositol ≏ 10%
0.55 M DMSO ≏ 4.3%

The effect of various inorganic salts as freezing menstruum on the viability of Aerobacter aerogenes (Mac 112)

After determining how various concentrations of NaCl in the freezing menstruum affected the viability of Aerobacter aerogenes (Mac 112), an investigation was carried out to determine whether or not ionic specificity was a factor involved in the death of the cells under freezing conditions. Studies were conducted to compare the effect of various inorganic compounds in the freezing menstrua on the survival of Aerobacter aerogenes (Mac 112). The results are recorded in Table 28. Na_2SO_4 at concentrations providing the same level of Na^+ ion as the NaCl tested, caused much less loss of viability than NaCl, suggesting that the Cl^- ion might be responsible for the difference in the toxicity during freezing and storage. This is supported by the observation that KCl or $MgCl_2$ had the same toxicity for the cells during the freezing of Aerobacter aerogenes (Mac 112) as NaCl. $MgSO_4$, on the other hand, was much less toxic for Aerobacter aerogenes (Mac 112) during freezing and storage. It is, therefore, most likely that the anions rather than the cations of these compounds were responsible for the decrease of viability of the cells of Aerobacter aerogenes (Mac 112) observed after freezing. It is of great interest, however, in connection with the question of the relation of freezing

TABLE 28

Effect of various inorganic salts as suspending fluid for freezing on the viability of Aerobacter aerogenes (Mac 112) after freezing, and after freezing and storage

Suspending fluid	Treatment of cells					
	Unfrozen		Frozen and thawed immediately		Frozen and stored*	
	Count** 10^8 cells/ml	Survival %	Count 10^8 cells/ml	Survival %	Count 10^8 cells/ml	Survival %
Glass-distilled water	186 ± 5	100	150 ± 10	80.2	147 ± 5	78.9
0.05 M NaCl	180 ± 8	100	50 ± 4	27.7	35 ± 5	19.1
0.05 M KCl	179 ± 10	100	48 ± 3	26.4	29 ± 3	15.6
0.025 M $MgCl_2$	196 ± 5	100	94 ± 7	47.9	36 ± 4	16.2
0.05 M $MgCl_2$	196 ± 5	100	88 ± 8	44.8	20 ± 3	9.9
0.025 M $MgSO_4$	193 ± 8	100	162 ± 5	84.0	155 ± 5	80.2
0.05 M $MgSO_4$	192 ± 7	100	129 ± 7	66.8	117 ± 3	60.6
0.025 M Na_2SO_4	186 ± 6	100	159 ± 3	85.1	144 ± 3	77.0
0.05 M Na_2SO_4	195 ± 8	100	154 ± 7	78.8	137 ± 5	70.1

*Frozen in liquid air (-196°C) and stored for 1 week at -20°C

**Plated on minimal medium plus 1% yeast extract

stress to the stress imposed by aerosolization that Webb (1960) reported that inorganic salts in the solutions used to suspend bacterial cells for aerosolization were all extremely toxic to the airborne cells. Furthermore, he found that all chlorides tested affected the aerosol behaviour in the same way and concluded that the effects of NaCl were due to Cl^- ion.

The results in Table 28 also indicated that NaCl and KCl when tested at the same concentration were almost equally effective in producing loss of viability when present in the suspending medium during freezing. It seemed probable, however, that the external and internal concentrations of Na^+, K^+ and Cl^- might be different. If this was so, then the similarity in the effects of the two salts, at the same concentrations outside the cell, would suggest that the primary lesion causing cellular death occurred externally. Accordingly, the internal Na^+, K^+ and Cl^- ion concentrations of cells of Aerobacter aerogenes (Mac 112) were determined for various external concentrations of the two salts. A relatively thick suspension of cells containing about 1×10^{11} cells per ml. was suspended in either glass-distilled water, 0.1 M KCl, 0.2 M KCl, 0.1 M NaCl or 0.2 M NaCl. The intracellular concentrations of Na^+, K^+ and Cl^- were determined in each suspension. The results are recorded in Table 29.

TABLE 29

Intracellular Na^+, K^+ and Cl^- ion concentrations in Aerobacter aerogenes (Mac 112) suspended in various concentrations of NaCl or KCl, and its effects on the viability of Aerobacter aerogenes after freezing and thawing

Suspending fluid	Extracellular			Intracellular			Viability after freezing** %
	K^+	Na^+	Cl^-	K^+	Na^+	Cl^-	
	(mM)*						
Glass-distilled water	0.9±0.0	0.8±0.0	2.4±0.1	65±2.0	9.0±1.3	8.8±4.5	94.3
0.1 M NaCl	2.6±0.0	115±5.0	110±0.2	80±3.6	88±6.5	74±10.9	62.3
0.2 M NaCl	3.2±0.1	220±0.0	213±0.2	80±3.5	135±7.3	145±1.0	58.6
Glass-distilled water	0.9±0.0	0.6±0.0	1.7±0.1	78±2.5	8.4±0.2	14±3.7	92.9
0.1 M KCl	103±1.0	1.4±0.1	109±2.0	199±2.1	4.0±0.1	66±0.1	68.1
0.2 M KCl	204±0.0	1.5±0.0	215±1.0	255±10.5	2.9±0.5	159±0.5	44.9

*Based on the average of duplicate determinations

**Cell density at a concentration of 1×10^{11} per ml. was frozen at -196°C and thawed immediately; plated on minimal medium plus 1% yeast extract

In the absence of added NaCl or KCl, there was a small amount of Na^+ present as a contaminant in the suspending medium. Under these circumstances there was more Na^+ or K^+ in the cells than in the medium with K^+ predominating. When NaCl was added to the suspension at a concentration of 100 mM or 200 mM, the intracellular Na^+ concentration was found to be about 60-75% of the outside concentration. On the other hand, when KCl was added to the suspension at the same concentration as the NaCl tested, the intracellular K^+ ion concentration was found to be always higher than that of the medium. The intracellular Cl^- ion concentration was approximately only 60-70% of the Cl^- ion content of the suspending medium at the 100 and 200 mM level of either NaCl or KCl. It can also be seen that although Na^+ and K^+ inside the cell varied appreciably depending on the salt in the suspending medium, the total cation concentration as represented by the sum of the Na^+ and K^+ ion concentrations was very nearly the same in both systems at the corresponding salt concentration in the suspending medium. The loss in viability of Aerobacter aerogenes (Mac 112) after freezing and thawing was proportional to the concentration of the salts in which the cells were suspended, but was not dependent on whether NaCl or KCl was used. Loss in viability of the cells of Aerobacter aerogenes (Mac 112) paralleled more closely the

changes in internal Cl^- concentrations than any of the internal cation concentrations.

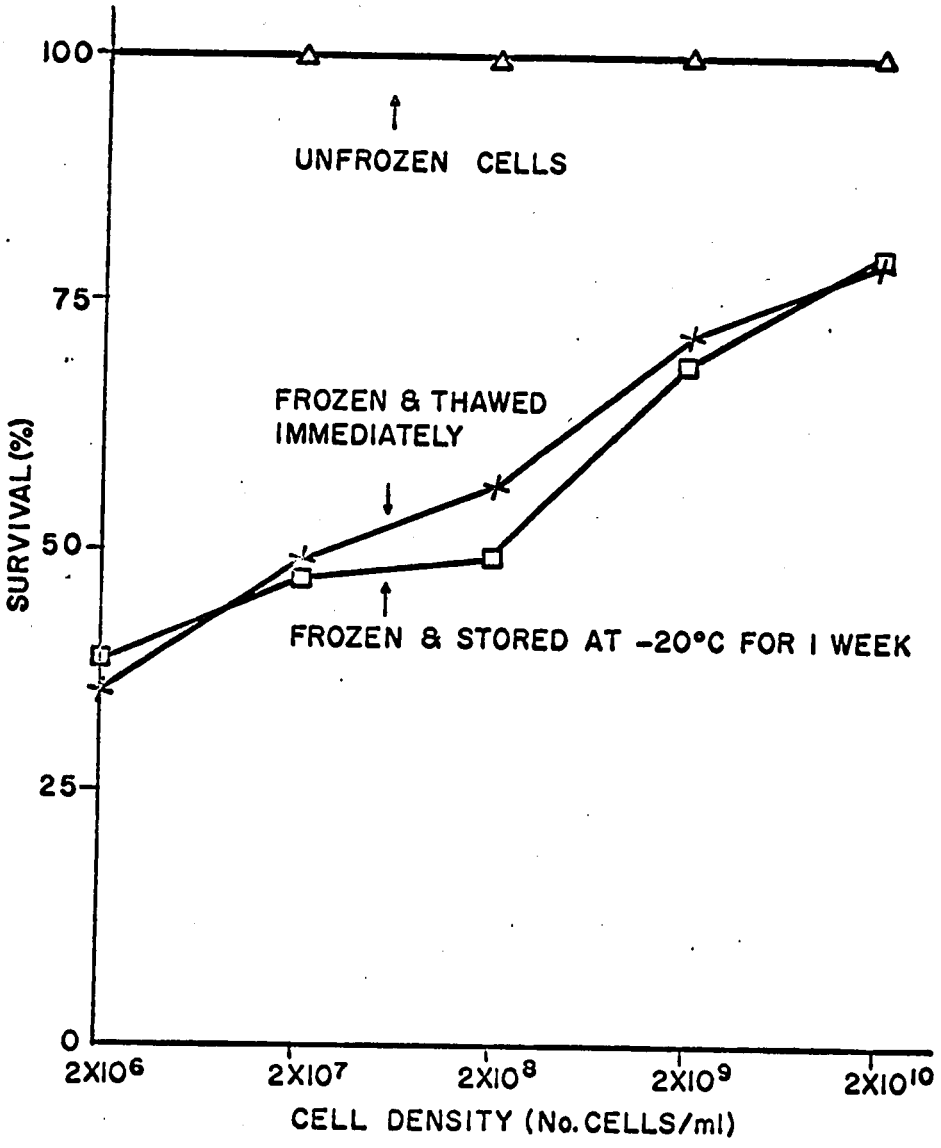
The effect of cell density and of glass-distilled water as a freezing menstruum on the survival of Aerobacter aerogenes (Mac 112)

Previous results have demonstrated that only a small loss in viability occurred when cells of Aerobacter aerogenes (Mac 112) at concentrations of 2×10^{10} per ml. were frozen in glass-distilled water. These findings are in agreement with those recorded by Harrison (1956), Clement (1961) and Kuo (1966) who found that distilled water was a relatively innocuous environment for the freezing of bacterial cells. Postgate and Hunter (1961), and Arpai (1962), on the other hand, observed that a considerable kill of cells occurred on freezing when distilled water was used as a freezing menstruum. The contradictory observations may be due to a number of factors, such as the use of a different species, a different growth phase of the culture, freezing temperature or to the fact that where a considerable kill occurred in distilled water, the water contained toxic trace elements from a defective still. Another likely possibility is that a different cell density was employed. For instance, Arpai (1962), studying Escherichia coli and Pseudomonas fluorescens,

used cultures containing only 5×10^5 cells per ml. for freezing, whereas 2×10^{10} cells per ml. of Aerobacter aerogenes (Mac 112) were used in the study of Kuo (1966) as well as in the present study. Clement's study (1961) used 2×10^9 cells per ml. of Escherichia coli for freezing. It was of interest to see the effect of population density on the survival of Aerobacter aerogenes (Mac 112) during freezing when cells were suspended in glass-distilled water. Five suspensions containing different concentrations ranging from 2×10^6 to 2×10^{10} cells per ml. of Aerobacter aerogenes (Mac 112) in glass-distilled water were tested. The results, Fig. 2, show that loss in viability of frozen cells was inversely proportional to cell density. Suspensions of 2×10^6, 2×10^8 and 2×10^{10} cells per ml. had viabilities of 35, 55 and 80% respectively, after being frozen in liquid air ($-196^{\circ}C$) and thawed immediately. Similar results were obtained for cells after freezing and storage for 1 week.

To explore whether the protection at high cell densities during freezing was due to the solutes that escape from frozen cells, supernatants were prepared from both unfrozen, and frozen and thawed cell suspensions containing 1×10^{10} cells of Aerobacter aerogenes (Mac 112) per ml. in glass-distilled water, by removing the cells by centrifugation and filtering the supernatant through a sterile

Figure 2. Effect of cell density on the survival of Aerobacter aerogenes (Mac 112) after freezing, and freezing and storage when glass-distilled water was used as freezing menstruum. Per cent survival calculated for each suspension taking the bacterial count on the unfrozen suspension as 100%, based on the average of four plates. Plated on minimal medium plus 1% yeast extract.


100
75
50
25
0
SURVIVAL (%)
UNFROZEN CELLS
FROZEN & THAWED IMMEDIATELY
FROZEN & STORED AT -20°C FOR I WEEK
2X10⁶
2X10⁷
2X10⁸
2X10⁹
2X10¹⁰
CELL DENSITY (No. CELLS/ml)

millipore filter. Cells were then suspended in these supernatants at concentrations of 1×10^8 and 1×10^6 cells per ml. for freezing to compare with similar suspensions in glass-distilled water. A suspension of 1×10^{10} cells per ml. in glass-distilled water was employed as a control. In Figure 3 and Table 30, a 1×10^6 cells per ml. of the same organism when frozen in the supernatants shows a survival of 62% (in the supernatant from unfrozen cells), 88% (in supernatant from cells after freezing) as against a value of about 40% for the same cell density of Aerobacter aerogenes (Mac 112) frozen in glass-distilled water only. In fact, the suspension containing 1×10^6 cells per ml. prepared with the supernatant from frozen cells showed the same survival as that of suspension which before freezing contained a 1×10^{10} cells per ml.

Since glass-distilled water was used as the plating diluent in the above experiments, it was of interest to see whether the supernatant from frozen cells when used as the plating diluent would enhance the recovery of cells after freezing. The results (Table 31) show that the recovery of frozen cells in any cell density when the sterile supernatant from a frozen cell suspension was used as the plating diluent was no better than when glass-distilled water served

Figure 3. Comparative effect of glass-distilled water and the supernatant from unfrozen or frozen cell suspensions as freezing menstruum on the viability of Aerobacter aerogenes (Mac 112) at various cell densities.
Cells after freezing in liquid air (-196°C) and storing at -20°C for 1 week:
Curve 1 - unfrozen cells suspended in glass-distilled water;
Curve 2 - per cent survival after freezing of cells suspended in glass-distilled water;
Curve 3 - per cent survival after freezing of cells suspended in the supernatant from a suspension of unfrozen cells;
Curve 4 - per cent survival after freezing of cells suspended in the supernatant from a suspension of frozen cells.

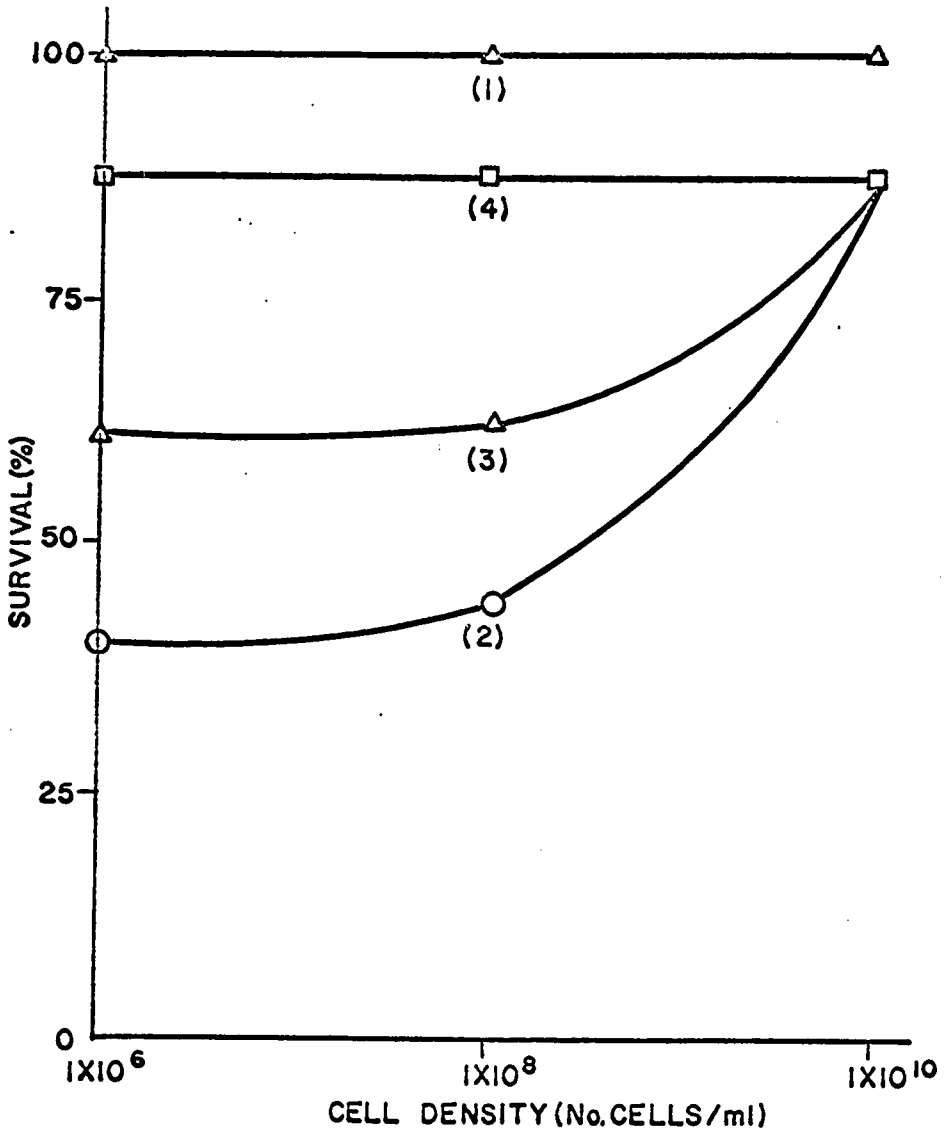

100
75
50
25
0
SURVIVAL (%)
(1)
(4)
(3)
(2)
1X10^6
1X10^8
1X10^10
CELL DENSITY (No. CELLS/ml)

TABLE 30

Comparative effect of glass-distilled water and supernatants from unfrozen, or frozen cell suspensions as freezing menstrua on the viability of Aerobacter aerogenes (Mac 112) at various cell densities

Cell density (cells/ml)	Freezing menstruum	Treatment of cells					
		Unfrozen		Frozen & thawed immediately		Frozen & stored*	
		Count**	Survival %	Count	Survival %	Count	Survival %
$\times 10^8$	Glass-distilled water	128 ± 4	100	103 ± 6	81.2	113 ± 6	88.2
$\times 10^6$	Glass-distilled water	131 ± 3	100	78 ± 3	59.6	59 ± 4	44.5
	Supernatant A***	124 ± 5	100	79 ± 5	63.9	90 ± 9	64.9
	Supernatant B	129 ± 3	100	99 ± 4	76.9	114 ± 8	88.1
$\times 10^4$	Glass-distilled water	135 ± 4	100	56 ± 5	41.7	54 ± 5	40.3
	Supernatant A	132 ± 4	100	72 ± 4	54.7	83 ± 6	62.8
	Supernatant B	126 ± 3	100	105 ± 3	82.8	112 ± 4	88.7

*Frozen in liquid air (-196°C) and stored at -20°C for 1 week

**Plated on minimal medium plus 1% yeast extract

***Supernatant A was prepared from a suspension before freezing containing 1×10^{10} cells per ml.; Supernatant B was prepared from an aliquot of the same suspension after freezing

TABLE 31

Comparative effect of glass-distilled water and supernatant from frozen cell suspension as plating diluents on the viability of Aerobacter aerogenes (Mac 112) after freezing and storage

Cell density cells/ml	Freezing menstruum	Plating diluent	Treatment of cells			
			Unfrozen		Frozen & stored*	
			Count**	Survival %	Count	Survival %
$\times 10^8$	Glass-distilled water	Glass-distilled water	128 ± 4	100	102 ± 7	80.8
		Supernatant B***			107 ± 3	83.4
$\times 10^4$	Glass-distilled water	Glass-distilled water	135 ± 4	100	51 ± 4	37.3
		Supernatant B			60 ± 3	43.6
	Supernatant B	Glass-distilled water	126 ± 4	100	106 ± 7	84.0
		Supernatant B			104 ± 6	82.6

*See Table 30.
**See Table 30.
***See Table 30. Supernatant B was prepared from a suspension after freezing and thawing.

as the plating diluent. It is evident, therefore, that the supernatants exert their protective effect during freezing and thawing, rather than during subsequent dilutions.

The effect of NaCl, $NaNO_3$ or Na_2SO_4 as freezing menstruum on the viability of Aerobacter aerogenes (Mac 112) at low cell concentrations

Previous results confirmed the findings of many workers that the presence of NaCl in the suspending medium during freezing increased the percentage loss of viable cells. It was also observed that a freezing menstruum containing $MgSO_4$ or Na_2SO_4, on the other hand, had no more destructive effect on Aerobacter aerogenes (Mac 112) than glass-distilled water. It was also found that cell suspensions, in glass-distilled water suffered 50% loss of viability following freezing if the cell density was only 2×10^7 cells per ml. Under the same conditions only a 20% loss of viability was observed for cell density of 2×10^{10} cells per ml. Tran (1960) demonstrated that certain inorganic salts if present at a sufficiently low concentration, protected mouse bone marrow cells against freezing. Of these salts, $NaNO_3$ was claimed to be the most effective, although NaI, NaBr, Na_2SO_4 and NaSCN also had protective activity. To see if it might be possible to protect cells of

Aerobacter aerogenes (Mac 112) containing 2×10^7 cells per ml. from loss of viability on freezing, the effect of suspending cells in glass-distilled water, and in different low concentrations of NaCl, $NaNO_3$ and Na_2SO_4 was compared. The results (Fig. 4) show that up to a concentration of 0.01 M of NaCl, some protection of viability from freezing over cells suspended in glass-distilled water was observed. A concentration of NaCl higher than 0.01 M increased the loss of viability. There was, however, further loss of viability after the cells were frozen and stored at $-20^{\circ}C$ for 1 week.

The results obtained with various concentrations of $NaNO_3$ and Na_2SO_4 are recorded in Figures 5 and 6 respectively. It is evident that $NaNO_3$ and Na_2SO_4 were much more protective against the loss in viability of Aerobacter aerogenes (Mac 112) on freezing than NaCl or glass-distilled water. However, for cells frozen and stored at $-20^{\circ}C$ for 1 week, somewhat different results were obtained. Figure 5 shows that the presence of $NaNO_3$ in the freezing menstruum had no protective effect for cells which had been frozen and stored at $-20^{\circ}C$ for 1 week, although quite significant protection was observed in the cells frozen and thawed immediately. The presence of Na_2SO_4 in the freezing menstruum at a concentration of 0.001 to 0.1 N tested was much more

Figure 4. Effect of different concentrations of NaCl in the freezing menstruum on the per cent viability of Aerobacter aerogenes (Mac 112) at a concentration of 2 x 10^7 cells per ml. after freezing, and freezing and storage.

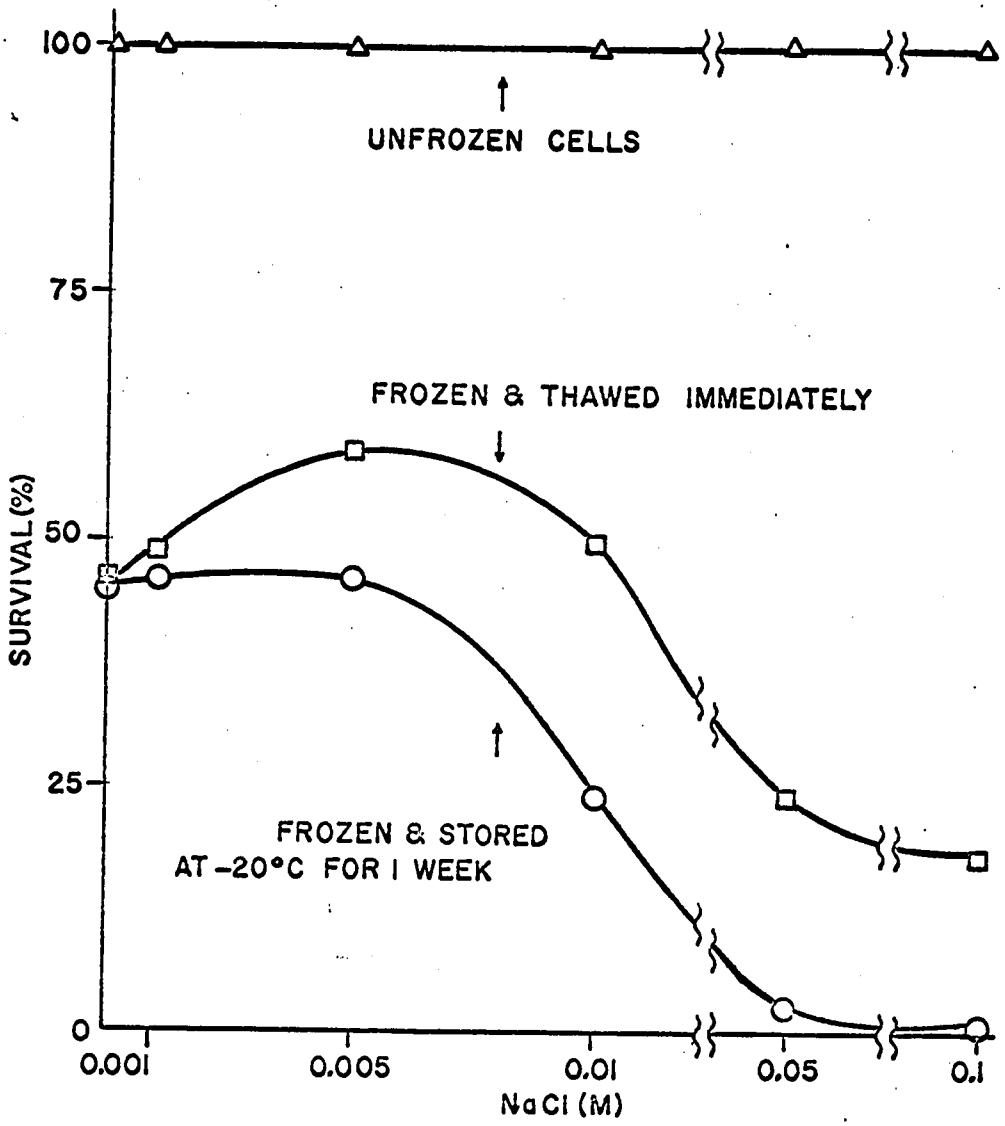

100
75
50
25
0
SURVIVAL (%)
UNFROZEN CELLS
FROZEN & THAWED IMMEDIATELY
FROZEN & STORED
AT -20°C FOR I WEEK
0.001
0.005
0.01
0.05
0.1
NaCl (M)

Figure 5. Effect of different concentrations of $NaNO_3$ in the freezing menstruum on the per cent viability of Aerobacter aerogenes (Mac 112) at a concentration of 2×10^7 cells per ml. after freezing, and freezing and storage.

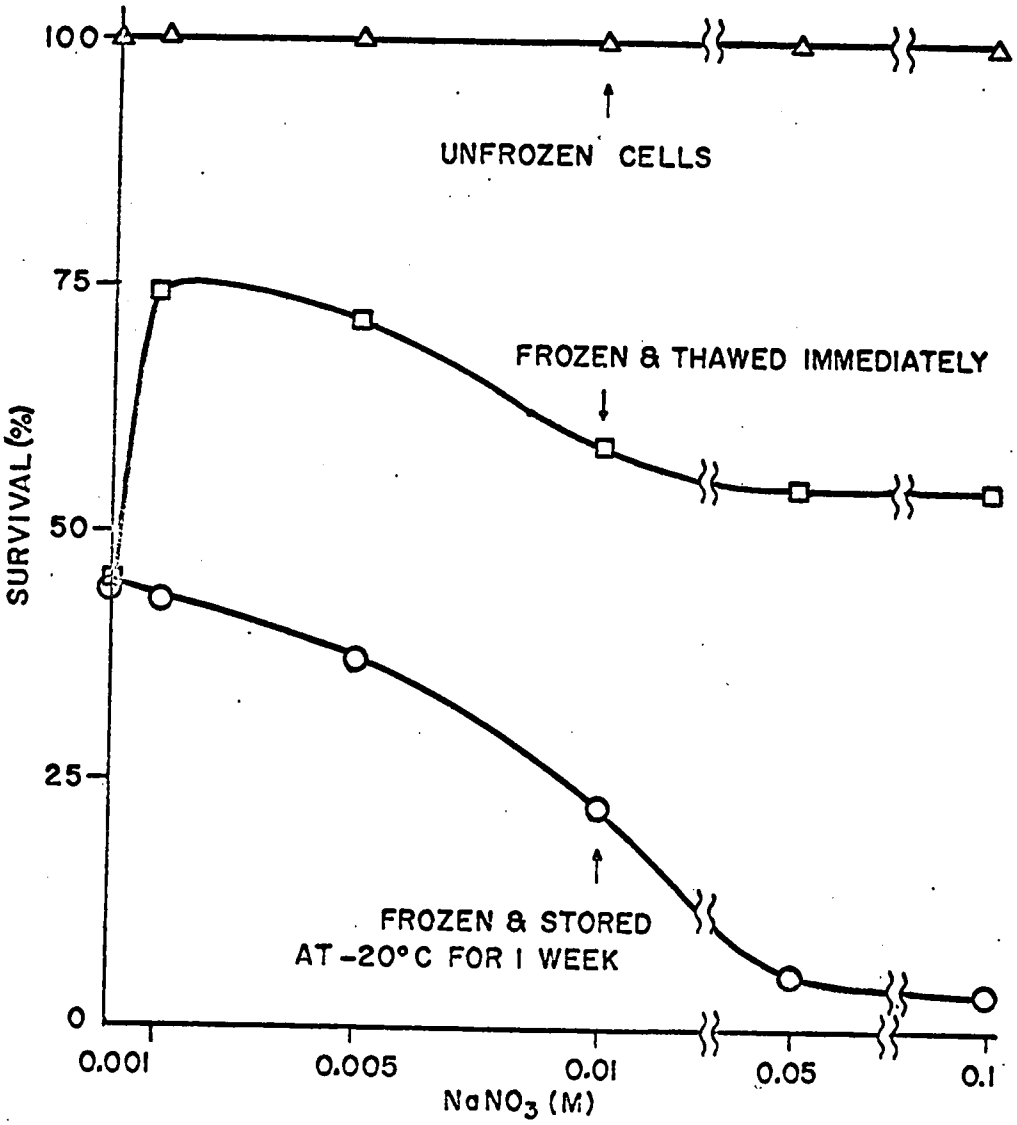
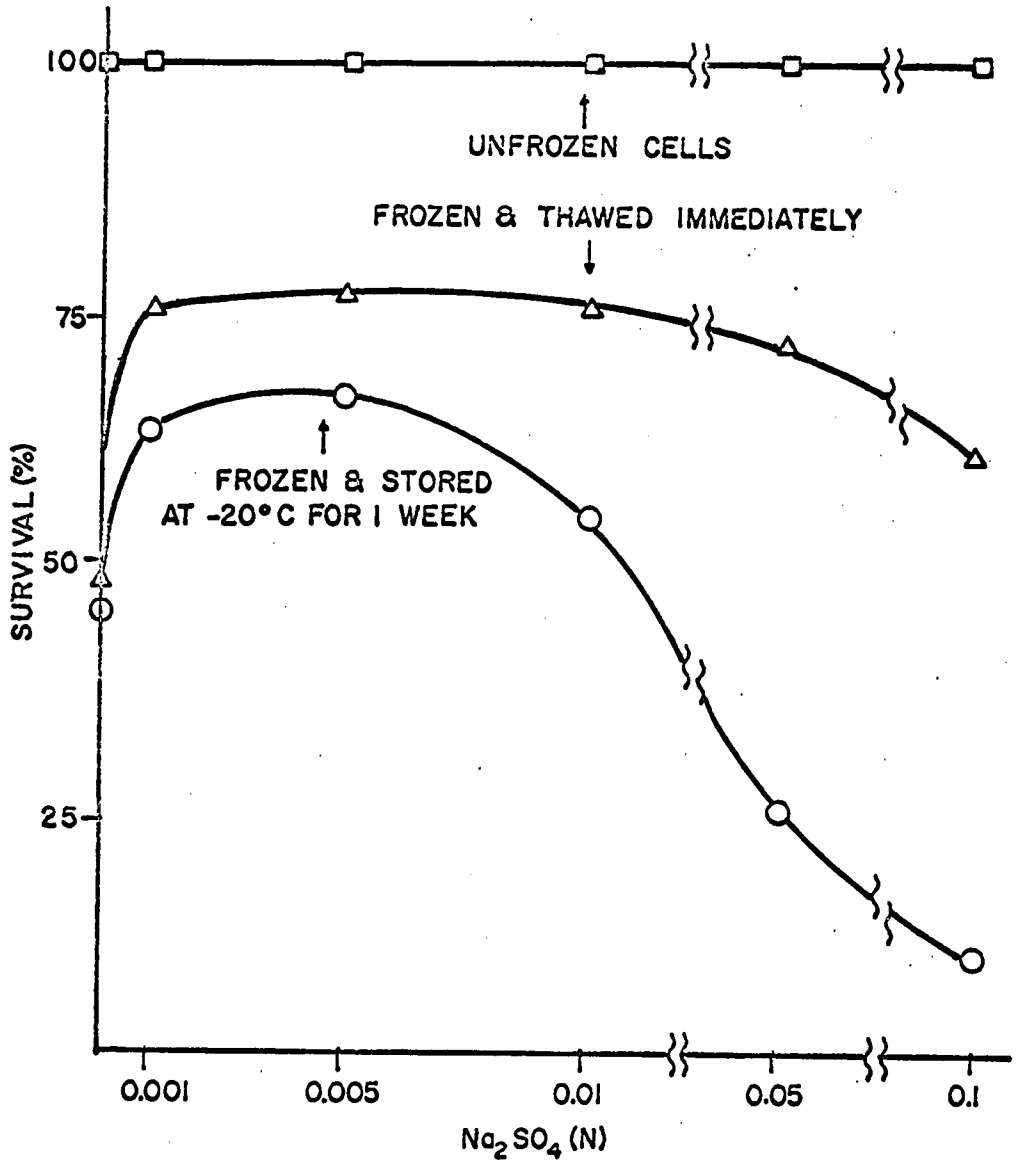
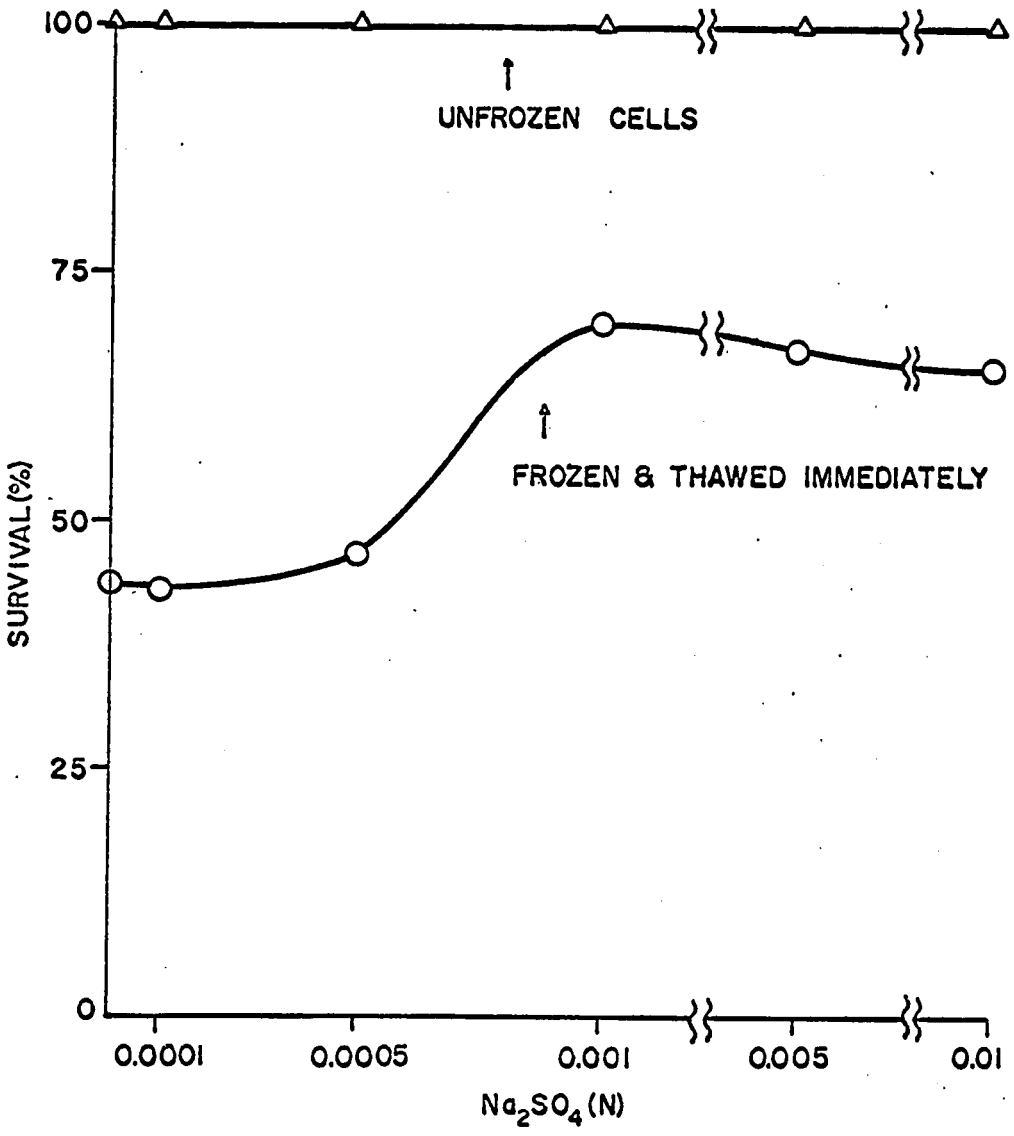

100
75
50
25
0
SURVIVAL (%)
UNFROZEN CELLS
FROZEN & THAWED IMMEDIATELY
FROZEN & STORED
AT -20°C FOR 1 WEEK
0.001
0.005
0.01
0.05
0.1
NaNO3 (M)

Figure 6. Effect of different concentrations of Na_2SO_4 in the freezing menstruum on the per cent viability of Aerobacter aerogenes (Mac 112) at a concentration of 2 x 10^7 cells per ml. after freezing, and freezing and storage. (I)


100
75
50
25
SURVIVAL (%)
UNFROZEN CELLS
FROZEN & THAWED IMMEDIATELY
FROZEN & STORED
AT -20°C FOR 1 WEEK
0.001
0.005
0.01
0.05
0.1
Na2 SO4 (N)

effective than glass-distilled water in overcoming the loss in viability of cells of Aerobacter aerogenes (Mac 112) frozen and thawed immediately, but had also less protective effect for cells which had been frozen and stored at -20°C for 1 week. Cells suspended in glass-distilled water and frozen showed no further loss of viability on storage. A striking feature of these results was that $NaNO_3$ or Na_2SO_4 at concentrations as low as 0.001 N were effective in protecting against the loss of viability of Aerobacter aerogenes on freezing. A further experiment was performed to determine the lowest concentration of Na_2SO_4 which exerted a protective action. The results in Figure 7 show that Na_2SO_4 at a concentration of 0.0005 N had little effect, and 0.001 N was about optimal.

Figure 7. Effect of different concentrations of Na_2SO_4 in freezing menstruum on the per cent viability of Aerobacter aerogenes (Mac 112) at a concentration of 2×10^7 cells per ml. after freezing (II).


100
75
50
25
0
SURVIVAL (%)
UNFROZEN CELLS
FROZEN & THAWED IMMEDIATELY
0.0001
0.0005
0.001
0.005
0.01
Na_2SO_4 (N)

DISCUSSION

There are a number of published reports that suggest the involvement of concentrated solute in causing freezing damage to cells. Harrison (1956), studying Escherichia coli and other strains of Gram negative organisms, thought that the rise in the concentration of electrolytes, which occurred when the suspending medium was frozen, might be the fundamental cause of damage to bacteria during freezing and subsequent thawing. Experiments were carried out to determine if reducing the concentration of NaCl in the freezing menstruum would improve the survival of bacteria frozen and stored at $-22^{o}C$. He found that the survival of these organisms was improved by diluting the solute, and the proportion of survivors was highest when the bacteria were suspended in distilled water. His results confirmed those of Lovelock (1953a, 1953b) who proposed that the most important cause of damage to red blood cells during freezing and thawing was the rise in concentration of electrolyte in the medium and within the cell when water separated out as ice. In the present study with Aerobacter aerogenes (Mac 112) it was found that NaCl was toxic when a concentration higher than 0.01 M was used as the freezing menstruum.

However, some protection against damage of frozen cells was observed if the concentration of NaCl was low enough and low cell density was employed for freezing.

If concentrated solute(s) is a major factor in the death of frozen- thawed cells as speculated by previous workers, damage should occur regardless of whether or not the freezing medium contains salts other than NaCl. The data in the present study indicate that all chlorides tested such as NaCl, KCl or $MgCl_2$ were toxic. However, Na_2SO_4 and $MgSO_4$, tested at the same concentrations as NaCl, were non-toxic. Thus, the cations (Na^+, K^+ or Mg^{++}) were not found to be the factors involved in the damage to the cells due to freezing. On the basis of these observations, it would appear that the lethal effect of solutes on freezing cells is not due simply to the consequence of concentrated solutes in or outside the cells as proposed by other investigators, but rather is due to the effect of particular anions on the organisms. Phosphate anion, used as freezing menstruum for Escherichia coli frozen and stored at $-9^{\circ}C$, has been shown to be non-toxic (Squires and Hartsell, 1955).

When cells of Aerobacter aerogenes (Mac 112) were suspended in various concentrations of NaCl, up to 200 mM, the intracellular Na^+ ion was found to be dependent on the

external Na^+ concentration and its concentration was always less than that of the external medium. On the contrary, when KCl was added as suspending medium, at a concentration the same as the NaCl tested, intracellular K^+ ion was always concentrated in the cells against a steep gradient. The results with Aerobacter aerogenes (Mac 112) in this study were similar to previous findings for the intracellular K^+ and Na^+ distributions in a number of halophilic and non-halophilic bacteria (Christian and Waltho, 1961, 1962; Schultz et al., 1963). The uptake of K^+ ion was not accompanied by the movement of an equal amount of the anion Cl^-. When either NaCl or KCl was used as the suspending medium, the Cl^- concentration in the cells was about 60-70% of the extracellular Cl^- content of the suspending medium. The loss in viability of the cells of Aerobacter aerogenes after freezing and thawing was proportional to the total salt concentration of the solution used to suspend the cells for freezing, but was irrespective of whether the salt was KCl or NaCl. Loss of viability due to freezing and thawing seemed to parallel more closely changes in the internal Cl^- concentration than changes in either the internal K^+ or Na^+ concentration.

In this study, when cells of Aerobacter aerogenes (Mac 112) were suspended in glass-distilled water, frozen in

liquid air (-196°C) and thawed immediately, or frozen and stored for 1 week, it was found that a well-defined relationship existed between the initial concentration of microorganisms in the suspensions before freezing and the percentage of survivors recovered after freezing and thawing. The more dilute the suspension for freezing, the lower the percentage of organisms surviving. Contradictory observations by previous workers (Major, 1955; Clement, 1961; Kuo, 1966; Postgate and Hunter, 1961; Arpei, 1962) on the survival of cells frozen in distilled water, can thus be explained by the fact that a different cell population was employed in their studies, although other factors might be responsible.

The population effect has been explained as being due to the release of the protoplasmic content of some of the cells into the suspending solutions, and the remaining cells being protected by the compounds leaking from neighboring cells. The greater the cell density, the greater the protection to the cells which remain. Bergman et al. (1957) studied the freezing-drying of Br. abortus and extracted a lecithin from the organism. This substance, which they named "the viability protective factor," protected cells of Br. abortus when the cells were freeze-dried in tryptose suspensions. Bretz and Ambrosini (1966) demonstrated that

extracts from heavy suspensions of Escherichia coli prepared by alternate freezing and thawing, or by heating, contained a protective factor that increased the survival of test cells frozen at -9°C. They observed that considerable protective activity was present in the frozen cell extract (FCE) but only after two, four, or eight cycles of alternate freezing (-75°C) and thawing (37°C) a culture. A single freezing treatment killed many cells and released low molecular weight substances which were not protective. They proposed that the protective factor appeared to be carbohydrate in nature. On the other hand, Packer et al. (1965) provided evidence indicating that the higher sensitivity of a diluted cell suspension of Escherichia coli on multiple freezing was due, not to the reduction in the number of cells, but to the dilution of substances in the spent growth medium which protected the cells. They found that sensitivity to freezing and thawing was constant at cell concentrations from 10^4 to 10^9 cells per ml. if the dilutions were made with cell-free spent medium. In the experiments conducted by Packer et al. (1965), the unwashed cells of Escherichia coli from the growth medium were frozen and thawed directly in spent growth medium, and then diluted in basal medium in the absence of glucose, or spent growth medium.

In the present study, cells of Aerobacter aerogenes

(Mac 112) were washed three times with glass-distilled water before being subjected to freezing (-196°C). The results obtained indicate that the population effect observed in this study cannot be accounted for either by a factor in spent growth medium, or by the presence of supernatant from frozen cells in the plating diluent. It was found that the supernatant fluid prepared from frozen cells protected a diluted cell suspension from the lethal effect of freezing as effectively as the protection afforded by high cell density. The supernatant fluid from unfrozen cells was also found to be quite effective. It is therefore evident that the organisms release a substance or substances into the suspending medium which even in very low concentrations has a remarkable capacity to protect cells from damage caused by freezing.

Although Bergmann et al. (1957) and Bretz and Ambrosini (1966) have demonstrated the nature of two factors from bacterial cell-free extracts able to protect cells from freezing, there is as yet no information on the nature of the component or components released by unfrozen cells which are active in protecting cells against freezing damage. Moreover, results in this study demonstrated that a single freezing and thawing of a suspension of Aerobacter aerogenes in glass-distilled water released substances very active in

protecting a subsequent suspension of cells against freezing; alternate freezing and thawing as proposed by Bretz and Ambrosini (1965) were not needed to demonstrate a protective effect. The nature of the protective substance is, therefore, still obscure.

It has long been known that glycerol protects mammalian and bacterial cells against damage from freezing and thawing (Lovelock, 1953; Hallander and Nell, 1954; Squires and Hartsell, 1955; Harrison, 1956; Haward, 1956; Postgate and Hunter, 1961). The present study has demonstrated that glycerol, dimethyl sulfoxide (DMSO), or inosital at 0.5 M concentrations effectively counteracted the toxic effect of 0.05 M NaCl as freezing menstruum on Aerobacter aerogenes (Mac 112). Although the protective effects produced by glycerol or DMSO have been attributed to reducing injury from concentrated solutes (Lovelock, 1953a, 1953b; Harrison, 1956; Farrant, 1965), the effect of inositol on freezing bacterial cells has not been reported. Studying air-borne bacterial cells, Webb (1960) observed that inositol was very effective in protecting cells against damage in aerosols. Inositol was assumed to replace water molecules in protein structure during dessication and to retain their natural biological stereochemical configuration.

The chemical structure of the organic compounds which protect against damage due to freezing seem to be characterized by the presence of hydrogen-bonding groups (OH-, NH_2-, etc.) (Nash, 1962; Doebbler and Rinfret, 1962; Morichi et al. 1963). If the presence of hydrogen-bonding group(s) in a compound is essentially for its protective activity, inorganic salts should not protect against injury during the freezing of cells. However, low concentration of certain inorganic salts has been demonstrated to protect mouse bone marrow cells against freezing and thawing (Tran, 1960). NaCl was found to afford partial protection in the case of Saccharomyces cerevisial (Mazur, 1963). In the present study, cells of Aerobacter aerogenes (Mac 112) were suspended in glass-distilled water and various concentrations of NaCl, $NaNO_3$ or Na_2SO_4 were used as freezing menstrua to determine the protective effect of these inorganic salts as compared with glass-distilled water. In order to eliminate or to decrease the protective effect of heavy suspensions of Aerobacter aerogenes when cells were suspended in glass-distilled water for freezing, cell suspensions at a concentration of 2×10^7 cells per ml. instead of 2×10^{10} per ml. were used in this study. It was observed that there was some protection against freezing by NaCl at concentrations ranging from 0.005 to 0.01 M. In the case of solution of $NaNO_3$ or Na_2SO_4 used as

freezing menstrua, much more significant protective effects were observed. These salts at concentrations as low as 0.001 N were very effective. It seems not unlikely, therefore, that the substance(s) released from unfrozen cells which protect cells against freezing when added to a diluted cell suspension could in part be inorganic salts.

For cells frozen and stored at -20°C for 1 week, it was found that there was no significant protective effect by any of the salts. In fact, they were found to be quite toxic as freezing menstrua at concentrations up to 0.01 N. Since additives such as Na_2SO_4 or $NaNO_3$ only prevent "immediate" freezing damage but not "storage" injury, it strongly suggests that two stresses are distinguishable.

The protective concentrations of these inorganic salts seemed to have a relatively narrow range, particularly in the case of NaCl as compared with organic additives such as glycerol or DMSO. The differences in behaviour between organic compounds and inorganic salts would suggest a different mode of action. Unfortunately, little information is available on protection of bacterial cells against freezing and thawing by low concentrations of inorganic salts. To clarify the mechanism of protective effect afforded by some inorganic salts during freezing, further investigation is necessary.

PART III

EFFECT OF FREEZING AND STORAGE ON LEAKAGE OF INTRACELLULAR SOLUTES FROM AND LOSS OF ENZYMATIC ACTIVITIES IN *AEROBACTER AEROGENES* (MAC 112)

INTRODUCTION

When bacterial cells are frozen the percentage of the population which dies is very much dependent on the nature of the solutes present. As shown in the previous results in this text, salts, particularly chlorides, have a marked lethal effect. Since far more cells die than are reversibly damaged, the problem of the mechanism of death under these circumstances is a more challenging one than metabolic injury. Although the problem has been studied extensively, the precise mechanisms by which freezing causes the death of bacterial cells are still unknown (Mazur, 1966). It is believed that high salt concentration causes protein denaturation. It is known that membrane damage occurs on freezing and that cells leak intracellular solutes. It has not been shown that this leakage is the cause of death. Similarly, some enzymes are sensitive to freezing while others are not. No one has correlated the loss of activity of a key enzyme on freezing with the extent of death of bacterial cells due to freezing. It is the purpose of this study to pinpoint the cause of death of bacterial cells due to freezing.

MATERIALS AND METHODS

Test organisms

Aerobacter aerogenes (Mac 112) was used throughout in these experiments.

Media: Preparation of cell suspensions and determination of viability

The procedures were the same as described in Part II.

Freezing procedure and storage

Prior to freezing, the washed bacterial cells were suspended either in glass-distilled water, or in a solution of NaCl or KCl depending upon the experiment. Nine ml. aliquots of this suspension containing 1×10^{11} cells per ml. were dispersed into polypropylene tubes, except where otherwise indicated. The tubes were then placed in liquid air ($-196^{\circ}C$) until the suspensions were frozen solid (about 5 minutes). The frozen suspensions were then transferred to a commercial deep freeze unit and stored at a temperature of $-20^{\circ}C$ until required.

Thawing Procedure

The procedures were the same as described in Part I.

Preparation of cell-free extracts

Sonication of unfrozen, or frozen and thawed cell suspension was performed in an MSE Ultrasonic Disintegrator at 0 to 4°C for 20, 40, 60 or 90 minutes, respectively. The total sonication time was made up of a series of short exposures for 5 minutes followed by cooling for 3 minutes to prevent heat denaturation. The cell debris was removed by centrifugation at 10,000 x G in a refrigerated centrifuge for 20 minutes. This supernatant is designated as the crude enzyme preparation and was used to check enzyme activities.

Preparation of a sterile supernatant solution from a suspension of frozen cells

The cells of Aerobacter aerogenes (Mac 112) were grown and harvested as previously described and washed three times with glass-distilled water. The washed cells collected from 4 liters of medium were then suspended in a 200-ml. volume of 0.05 M KCl. This heavy cell suspension containing 2×10^{11} per ml. was dispensed in 50-ml.

aliquots in 250-ml. screw-capped polypropylene bottles. The bottles were then placed in liquid air (-196°C) until the suspension froze solid. The frozen suspensions were thawed by placing the polypropylene bottles in a beaker of water at 25°C. The cells were centrifuged off and the supernatant was sterilized by filtration through a 0.45 micron HA millipore filter.

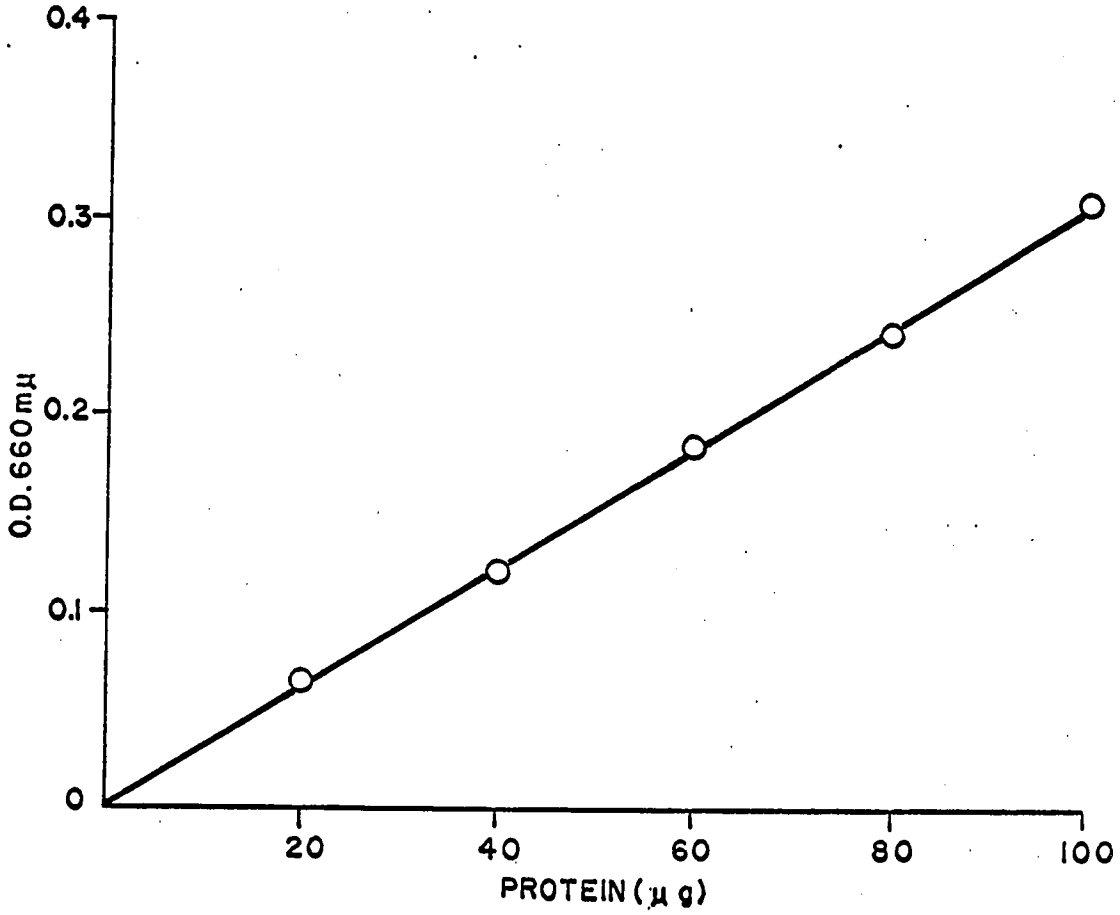
Protein determination

The protein determination was conducted according to the method described by Lowry et al. (1951). Bovine serum albumin (twice crystallized, Sigma Chemical Co.) was used as a standard. The absorbancy was measured at 660 mu with a Coleman Junior Spectrophotometer. A standard curve for protein determination is presented in Figure 8.

Determination of K^+

The supernatant obtained after the cells were removed from the suspension by centrifugation was analysed directly for K^+ by flame photometry. The procedure was the same as described in Part II.

Figure 8. Standard curve for protein determination.


0.4
0.3
0.2
0.1
0
O.D. 660mμ
20
40
60
80
100
PROTEIN (μg)

Assay of reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase

The reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase was assayed by measuring the decrease in absorbancy at 340 mμ which occurs as a result of the oxidation of $NADH_2$. The reaction mixture prepared in a quartz curvette (2-ml. volume, 0.5 cm. light path) contained in order of addition, 100 mM phosphate buffer (pH 7.5), 0.7 ml.; 1.0 μM $NADH_2$, 0.35 ml.; and glass-distilled water to give a total volume of 1.3 ml. Finally, 0.1 ml. of cell-free extract containing 0.5-1.0 mg. protein was added after the rest of the ingredients had been mixed. The absorbancy was recorded for up to 3 or 4 minutes, in a Zeiss PMQII spectrophotometer. All enzyme assays were conducted at room temperature.

Assay of malic acid dehydrogenase

Malic dehydrogenase was determined spectrophotometrically by following the reduction of nicotinamide adenine dinucleotide phosphate (NADP) at 340 mμ. The reaction mixture contained: 250 mM Tris HCl (pH 8.0), 0.5 ml.; 50 mM L-Malic acid, 0.2 ml.; 1.5 mM NADP, 0.2 ml.; and added glass-distilled water to give a total volume of 1.2 ml. Lastly, 0.2 ml. of an appropriate dilution of the cell-free extract was added to start the reaction.

In experiments where cell suspension was suspended in glass-distilled water alone, a 200 mM concentration of KCl (0.1 ml.) was added in the reaction mixture.

Specific activity of enzyme is expressed as the amount of which causes a decrease in optical density of 0.001 per minute, per mg. of protein.

Assay of adenosine triphosphatase (ATPase)

ATPase activity was determined by measuring the rate of release of inorganic phosphate from ATP. The reaction mixture prepared in a 10 x 75 mm. test tube contained 0.5 M Tris HCl (pH 9.0), 0.1 ml.; 0.05 M $MgSO_4$, 0.1 ml.; 0.05 M ATP, 0.1 ml.; and 0.1 ml. of cell-free extract containing about 0.5-1.0 mg. protein in a final volume of 1.0 ml.

All experiments were performed at 25°C. After a 5-minute temperature equilibration period, the reaction was started by the addition of enzyme and the mixture was incubated for 20 minutes. The reaction was stopped by the addition of 1.0 ml. of ice-cold 10% trichloroacetic acid. The precipitated protein was removed by centrifugation for 10 minutes at 10,000 x G. Aliquots of 0.1 ml. deproteinized supernatant were removed for estimation of inorganic

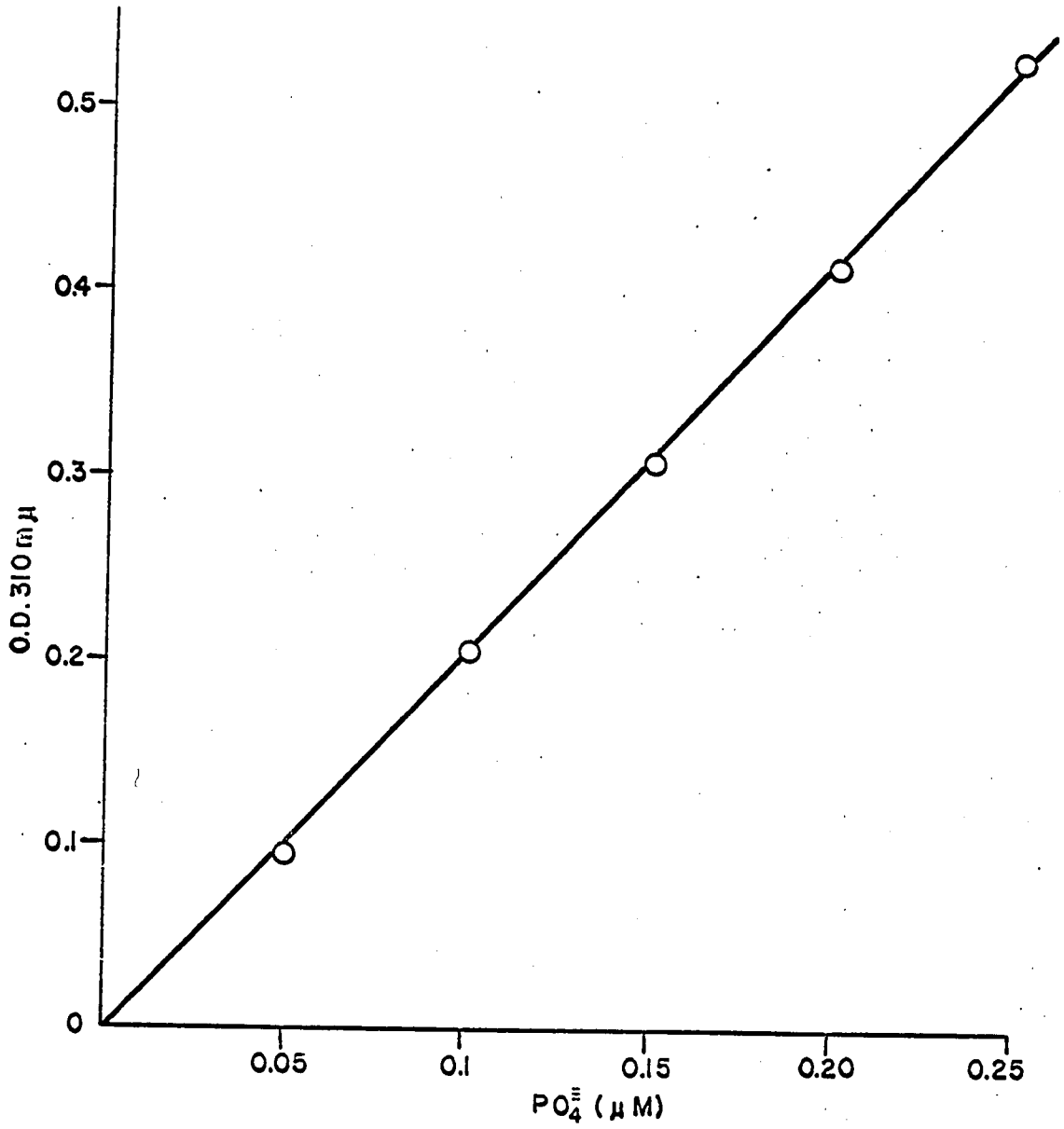
phosphate by the simplified procedure of Marsh (1959). Corrections were applied for blanks containing substrate without enzyme and enzyme without substrate. The enzyme activity is expressed in terms of micromoles of substrates hydrolyzed in 20 minutes, under conditions given above.

Determination of inorganic phosphate (orthophosphate PO_4)

This method was developed by Marsh (1959) to determine inorganic phosphate in the presence of adenosine triphosphate (ATP).

The sample, containing up to 0.2 uM of Pi, was transferred to a centrifuge tube calibrated at 12 ml., and diluted with glass-distilled water to 5.0 ml. Five ml. of butanol was added, followed by 0.5 ml. of 4% molybdic acid and 1 ml. of 20% citrate (pH 7.0). The tube was then shaken vigorously for 5-10 seconds. The volume was increased to 12 ml. with glass-distilled water and the tube was again shaken for 5-10 seconds. Following the second shaking, the tube was centrifuged for a few seconds to remove the last droplets of water from the butanol layer, and part of the upper phase was transferred by pipette to a quartz curvette. Two drops of methanol were added to remove a turbidity which occasionally developed,

Figure 9. Calibration curve for (Pi) inorganic phosphate determination.


0.5
0.4
0.3
0.2
0.1
0
O.D. 310 mμ
0.05
0.1
0.15
0.20
0.25
$PO_4^{\equiv}$ (μM)

and the curvette was inverted to mix the contents. Absorbance was measured at 310 mμ (hydrogen lamp) with a Zeiss PMQ11 spectrophotometer against a blank prepared as above but containing no phosphate.

Osmotic shock procedure

Osmotic shock to cells was carried out according to the procedure described by Neu and Heppel (1965). The cells of Aerobacter aerogenes (Mac 112) were grown and harvested as previously described and washed three times with glass-distilled water. The washed cells collected from 250 ml. of growth medium were then suspended in 50 ml. of 0.03 M Tris HCl (pH 8.0) containing 20% sucrose and 0.5 ml. of 0.01 M EDTA (pH 8.0) to give a final EDTA concentration of 1×10^{-3} M. The cell suspension was shaken on a rotary shaker for 10 minutes at room temperature and centrifuged at 10,000 x G for 10 minutes at 4^{o}C. The supernatant fluid was removed, and the cell pellet was rapidly dispersed in a 50-ml. volume of cold glass-distilled water. The suspension was mixed in an ice bath on a rotary shaker for 10 minutes, centrifuged, and the supernatant removed.

RESULTS

Relation of leakage to loss of viability due to freezing

The lethal effect of freezing and thawing on bacterial cells may be due to interference with bacterial permeability control mechanisms. Evidence cited to support this view is that, after freezing and thawing, cells release normal cellular constituents into the suspending medium (Lindeberg and Lode, 1963; Moss and Speck, 1966b; Bretz and Ambrosini, 1966) and are more penetrable than unfrozen cells to such extracellular solutes as toxic trace elements (MacLeod, Kuo and Gelinas, 1967). The metabolic injury studies (Parts I and II) suggest that cells become reversibly damaged because they lose low molecular weight compounds. It seemed possible that irreversible damage might result if the membrane became so permeable that critical macromolecules such as enzymes were lost from the cells. An intracellular enzyme, malic dehydrogenase, was chosen to test whether the extent of loss of the enzyme activity could be correlated with the per cent loss of viability of the culture. Initially, it

was necessary to establish if intracellular malic dehydrogenase would survive freezing, or freezing and storage as well as to determine if any was released into the suspending medium after thawing. A washed cell suspension at a concentration of 1×10^{11} cells per ml. was divided into two parts. One part was suspended in glass-distilled water, and the other in 0.1 M KCl. Nine ml. aliquots of each suspension were dispensed into 10 ml. polypropylene tubes and left unfrozen, or frozen and stored for 1 day, and for 3 weeks respectively. Both the unfrozen suspension and the suspension after freezing and thawing were treated in the same way. After centrifugation of the cell suspensions, the supernatant fluids were checked for malic dehydrogenase activity. The cells were resuspended in glass-distilled water or 0.1 M KCl and cell-free extracts were prepared with the aid of a sonic oscillator. After the removal of cell debris by centrifugation, malic dehydrogenase activity in the clear supernatant liquid was determined. The results (Table 32) show that freezing with liquid air ($-196^{o}C$) and storage at $-20^{o}C$ did little to inactivate malic dehydrogenase in the cells. Appreciable amounts of malic dehydrogenase were released from cells which were suspended in 0.1 M KCl for freezing and storage. The extent of leakage was essentially as

TABLE 32

Effect of freezing and freezing and storage on malic dehydrogenase activity of cells and supernatants of Aerobacter aerogenes (Mac 112)

Treatment of suspension	Malic dehydrogenase ****		Viability**	
	Cells***	Supernate	10^9 Count cells/ml.	%
Glass-distilled water suspended for freezing				
Unfrozen	119.2	0	89 ± 4	100
Frozen and stored for 1 day*	102.9	0	83 ± 5	93.2
Frozen and stored for 3 weeks	94.3	0	70 ± 2	78.6
0.1 M KCl suspended for freezing				
Unfrozen	110.2	0	89 ± 5	100
Frozen and stored for 1 day	102.4	118.6	49 ± 4	49.4
Frozen and stored for 3 weeks	103.4	121.2	42 ± 3	34.9

*Frozen in liquid air (-196°C) and stored for 1 day at -20°C.
**Plated on minimal medium plus 1% yeast extract.
***The cell suspension was sonicated for 20 minutes in an MSE.Ultrasonic Disintegrator and cell debris was removed by centrifugation at 10,000 x G for 20 minutes.
****Enzyme activity expressed as the amount of enzyme which causes a decrease in optical density of 0.001 per minute, per mg. of protein.

great after storage for 1 day as for 3 weeks. When cells were suspended in glass-distilled water for freezing, however, malic dehydrogenase appeared not to be released even after 3 weeks storage at -20°C. A viability test indicated that there was a 7.0% and a 21% loss of viability for cells suspended in glass-distilled water after freezing and storage for 1 day, and storage for 3 weeks, respectively; for cells frozen in 0.1 M KCl for 1 day and 3 weeks, a 50% and 65% loss of viability were noted. It thus appeared that a good relationship did not exist between the loss of viability and the release of malic dehydrogenase.

Chaplin et al. (1957) observed that the potassium leaked from red blood cells after freezing and storage. More recently, Hancock (1964), studying the early effect of streptomycin on Bacillus megatherium noted that streptomycin led to an increased efflux of potassium and specific inhibition of protein synthesis. It was considered likely that the inhibition of protein synthesis was due to the loss of potassium. It was of interest to see whether freezing and thawing caused the release of potassium from the cells of Aerobacter aerogenes (Mac 112). The results (Table 33) show that there was a leakage of potassium from the cells into the suspending medium after freezing and thawing. A good relationship appears to exist between the

TABLE 33

Effect of freezing, and freezing and storage on the loss of viability and release of K^+ by Aerobacter aerogenes (Mac 112) suspended in glass-distilled water, or 0.05 M NaCl as freezing menstruum

Treatment of suspension	K^+ release***	Viability**
	mM	%
Glass-distilled water suspended for freezing		
Unfrozen	0	100
Frozen and thawed immediately*	0.21	95.0
Frozen and stored for 3 weeks	0.46	91.0
0.05 M NaCl suspended for freezing		
Unfrozen	0	100
Frozen and thawed immediately	0.90	62.0
Frozen and stored for 3 weeks	2.26	11.0

*See Table 32
**See Table 32
***Extracellular K^+ concentration of after freezing substract that of before freezing

extent of loss of viability and the amount of K^+ released. However, it cannot be decided from these results whether death is due to loss of K^+, or whether K^+ loss is due to the extent of death. Potassium, an activator of some enzyme systems (Dixon and Webb, 1964), is required for the growth of micro-organisms (Lester, 1958). More recently, Dicks and Tempest (1966) proposed that a precise relationship between magnesium and potassium was necessary to maintain the ribosomal structures in a functional configuration in Aerobacter aerogenes.

If potassium loss from the cells was the cause of death in the present study and since potassium is maintained inside normal cells at a level exceeding that in the suspending medium, it was considered possible that a critically high K^+ level might have to be present in the cells at all times to maintain viability. To test this possibility a suspension of cells frozen in 0.05 M KCl was overlaid with an equal volume of either glass-distilled water, 1.0 M KCl, 2.0 M sucrose, or 1.0 M KCl plus 2.0 M sucrose. The cells were then thawed and mixed carefully during the process of thawing to ensure that they were, at all times, in contact with the solutes in the suspending medium. The sucrose was included in the experiment to check the possibility that osmotically sensitive forms of

the cells were being formed as a result of the stress imposed by freezing. The cells were diluted for plating with the solution used to overlay the frozen suspensions. The suspensions were then plated on minimal medium containing the solutes used for dilution. The results (Table 34) show that none of these suspending fluids or plating media was appreciably more effective in increasing the viable count than glass-distilled water or minimal medium. Sucrose, at the level tested (1.0 M), proved to be toxic when added to the plating medium.

It is well known that magnesium stabilizes isolated protoplast membranes (Weibull, 1956), spheroplasts (Lederberg, 1956), ribosomes (McQuillen, 1962) and cell envelope of Gram negative bacteria (MacLeod, 1965). It was of interest to determine whether the frozen suspension overlaid and diluted with $MgSO_4$ would increase or restore the viability of the cells after freezing and storage. The results are recorded in Table 35. $MgSO_4$ in the diluting fluid over the concentration range 0.05 M to 0.3 M had no capacity to increase the number of cells in the suspension able to grow and form colonies.

Since many intracellular solutes besides potassium could be leaking from the cells, the possibility of some

TABLE 34

Effect of maintaining thawed cells in contact with a high level of K^+ or sucrose on the recovery of viable cells from a frozen suspension of Aerobacter aerogenes (Mac 112)*

Diluting fluid	Plating medium	Count (10^8 cells/ml.)	
		Unfrozen cells	Frozen cells**
Glass-distilled water	Minimal	149 ± 5	43 ± 5
KCl (0.5 M)	Minimal	153 ± 7	48 ± 5
	Minimal + KCl	151 ± 6	37 ± 3
Sucrose (1.0 M)	Minimal	140 ± 8	37 ± 7
	Minimal + Sucrose	0	0
KCl + Sucrose	Minimal	151 ± 4	57 ± 5
	Minimal + KCl + Sucrose	0	0

*0.05 M KCl was used as freezing menstruum

**Frozen in liquid air (-196°C) and thawed immediately

TABLE 35

Effect of maintaining thawed cells in contact with various concentrations of $MgSO_4$ on the recovery of viable cells from a frozen suspension of Aerobacter aerogenes

Suspending medium for freezing	Diluting fluid	Count (10^8 cells/ml.)*		
		Unfrozen cells	Frozen and thawed** immediately	Frozen and stored for 1 week
Glass-distilled water	Glass-distilled water	175 ± 3	144 ± 3	130 ± 3
	0.05 M $MgSO_4$	188 ± 3	135 ± 9	141 ± 6
	0.1 M $MgSO_4$	177 ± 6	141 ± 7	130 ± 1
	0.2 M $MgSO_4$	175 ± 7	136 ± 2	143 ± 4
	0.3 M $MgSO_4$	175 ± 5	136 ± 3	134 ± 2
0.05 M KCl	Glass-distilled water	176 ± 11	53 ± 3	50 ± 2
	0.05 M $MgSO_4$	166 ± 6	60 ± 2	56 ± 2
	0.1 M $MgSO_4$	171 ± 2	60 ± 5	54 ± 1
	0.2 M $MgSO_4$	169 ± 7	60 ± 5	53 ± 1
	0.3 M $MgSO_4$	183 ± 5	56 ± 4	50 ± 3

*Plated on minimal medium plus 1% yeast extract.

**See Table 34.

other critical solutes being lost as a result of freezing was considered. A concentrated, sterile supernatant was prepared by growing Aerobacter aerogenes (Mac 112) in 4 liters of growth medium. The cells were harvested, washed and suspended in 200 ml. of 0.05 M KCl. The suspension was frozen, thawed, centrifuged and the supernatant liquid filtered through a millipore filter. This concentrated supernatant was used to overlay a frozen suspension of Aerobacter aerogenes during the thawing process, to dilute the cells for plating and also to supplement the plating medium. The results (Table 36) show that the concentrated supernatant had no capacity to improve the recovery of viable cells after freezing. The results were confirmed using other preparations of cell supernatants.

Correlation between loss of viability and enzyme activity after freezing and thawing

Further attempts were made to determine the specific metabolic function or functions that were being destroyed by freezing. Since freezing in the presence of salts is known to cause damage to the cytoplasmic membrane of cells, it seemed possible that one or more membrane bound enzymes might be affected by the freezing stress. If a key

TABLE 36

Effect of maintaining thawed cells in contact with a concentrated cell supernate on the recovery of viable cells from a frozen suspension of Aerobacter aerogenes *

Diluting fluid	Plating medium	Count (10^8 cells/ml)***	
		Unfrozen cells	Frozen cells**
Glass-distilled water	Minimal	202 ± 4	52 ± 2
	Minimal + Supernate	210 ± 10	55 ± 2
Supernatant	Minimal	200 ± 9	56 ± 3
	Minimal + Supernate	205 ± 10	59 ± 3

*0.05 M KCl was used as freezing menstruum

**Frozen in liquid air (-196°C) and thawed immediately

***Plated on minimal medium plus 1% yeast extract

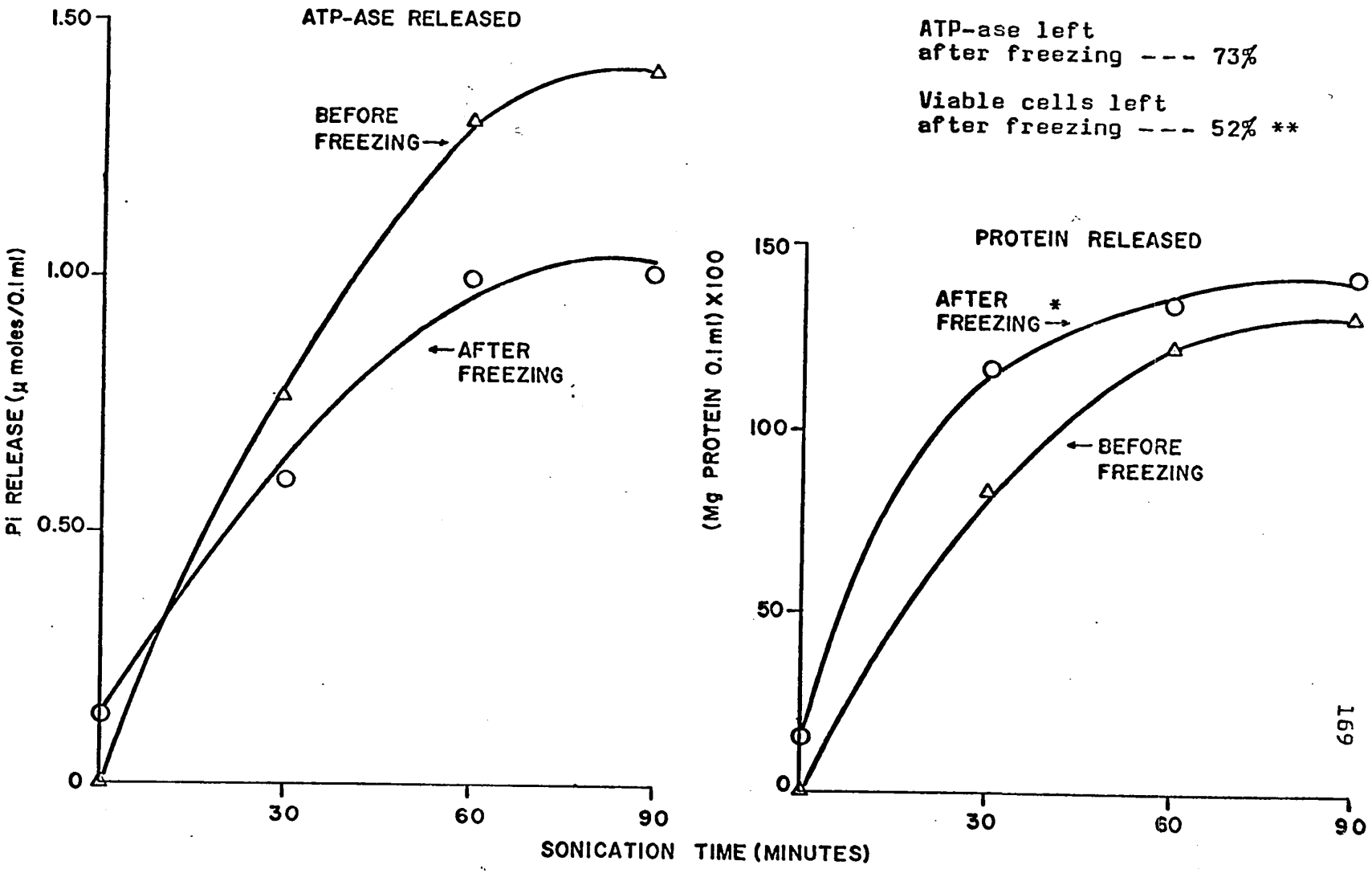
enzyme was involved, damage to it might be responsible for the loss of viability of the cells. Three enzymes were studied: malic dehydrogenase, an intracellular enzyme; reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase, a membrane bound enzyme; and adenosine triphosphatase (ATPase), an enzyme found both in the cytoplasm and associated with the cell membrane (Campbell et al., 1962; Abrams, 1965; Pangborn et al., 1962; Drapeau and MacLeod, 1963). In these experiments cell-free extracts were prepared by sonication in an M.S.E. ultrasonic disintegrator. Since this instrument had a smaller power output than a Raytheon 10 Kc sonic oscillator often used for studies such as these, longer times of exposure to sonication were required to effect cell breakage. It was desirable to test the effect of sonication time on recovery of the enzymes. The purpose of these experiments was to take a suspension of cells and assay the total activity of a given enzyme in the cells using the cell-free extract prepared from an aliquot of the suspension. To obtain a measure of total enzyme activity in a suspension the cells should be sonicated until all cells are ruptured but should not be exposed so long that enzyme denaturation occurs. Washed cells of Aerobacter aerogenes (Mac 112) were suspended in 0.1 M KCl. Nine ml. aliquots of the suspension, at a concentration

of 1×10^{11} cells per ml., were dispensed in polypropylene tubes. Some samples were left unfrozen, and others were frozen and stored for one day. Both the unfrozen suspension and the suspension after freezing and thawing were treated in the same way. Cell-free extracts of the cells were prepared after 0, 30, 60 or 90 minutes sonication, and tested for enzyme activities. The loss in enzyme activity after freezing was then compared with the loss of cell viability.

Adenosine triphosphatase (ATPase)

The results (Figure 10) show that ATPase was released into the supernatant after the cells had been frozen and stored for 1 day. Maximum release of ATPase activity from frozen cells occurred after exposure of the cells to sonication for 90 minutes. In these experiments the total sonication time was made up of a series of short exposures followed by a period of cooling to prevent heat denaturation of proteins. In the case of unfrozen cells, some 90 minutes were required before full release of enzyme had occurred. The release of enzyme correlated with the extent of solubilization of protein, as is also shown in Figure 10. It is evident from the results that cells, after freezing and thawing, are more readily ruptured than before freezing.

Figure 10. Effect of sonication time on the rate of release of ATPase and on the total ATPase activity of cells of Aerobacter aerogenes (Mac 112) before and after freezing. Protein release and cell viability before and after freezing are also recorded.
*Cells frozen (-196°C) and stored (-20°C) for 1 day.
**Plated on minimal medium plus 1% yeast extract.


ATP-ASE RELEASED
1.50
1.00
0.50
0
Pi RELEASE (μ moles/0.1 ml)
BEFORE FREEZING
AFTER FREEZING
30
60
90
SONICATION TIME (MINUTES)
ATP-ase left after freezing --- 73%
Viable cells left after freezing --- 52% **
PROTEIN RELEASED
150
100
50
0
(Mg PROTEIN 0.1 ml) X100
AFTER FREEZING *
BEFORE FREEZING
30
60
90

Total ATPase activity after freezing was 73% of that before freezing. Cell viability after freezing was only 52% of that before freezing. There was thus not a very close correlation between loss of ATPase activity and loss of viability after freezing.

Malic dehydrogenase

The experiments conducted were similar to those described for ATPase. The results in Figure 11 confirm the previous observation that cells after freezing are more easily disrupted by sonic oscillation and release cellular enzymes more readily. Longer periods of sonication caused maximum release of malic dehydrogenase from frozen cells after 60 minutes. Release of the enzyme, however, was still occurring after 90 minutes in the case of unfrozen cells. Since total malic dehydrogenase was not measured in unfrozen cells it can only be concluded that less than 63% remained after freezing. The corresponding viability after freezing was found to be 57%.

Reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase

The results in Figure 12 show that no $NADH_2$ oxidase activity was obtained from the supernatant of cells after

Figure 11. Effect of sonication time on the rate of release of malic dehydrogenase and on the total malic dehydrogenase activity of cells of Aerobacter aerogenes (Mac 112) before and after freezing.
*See Fig. 10.

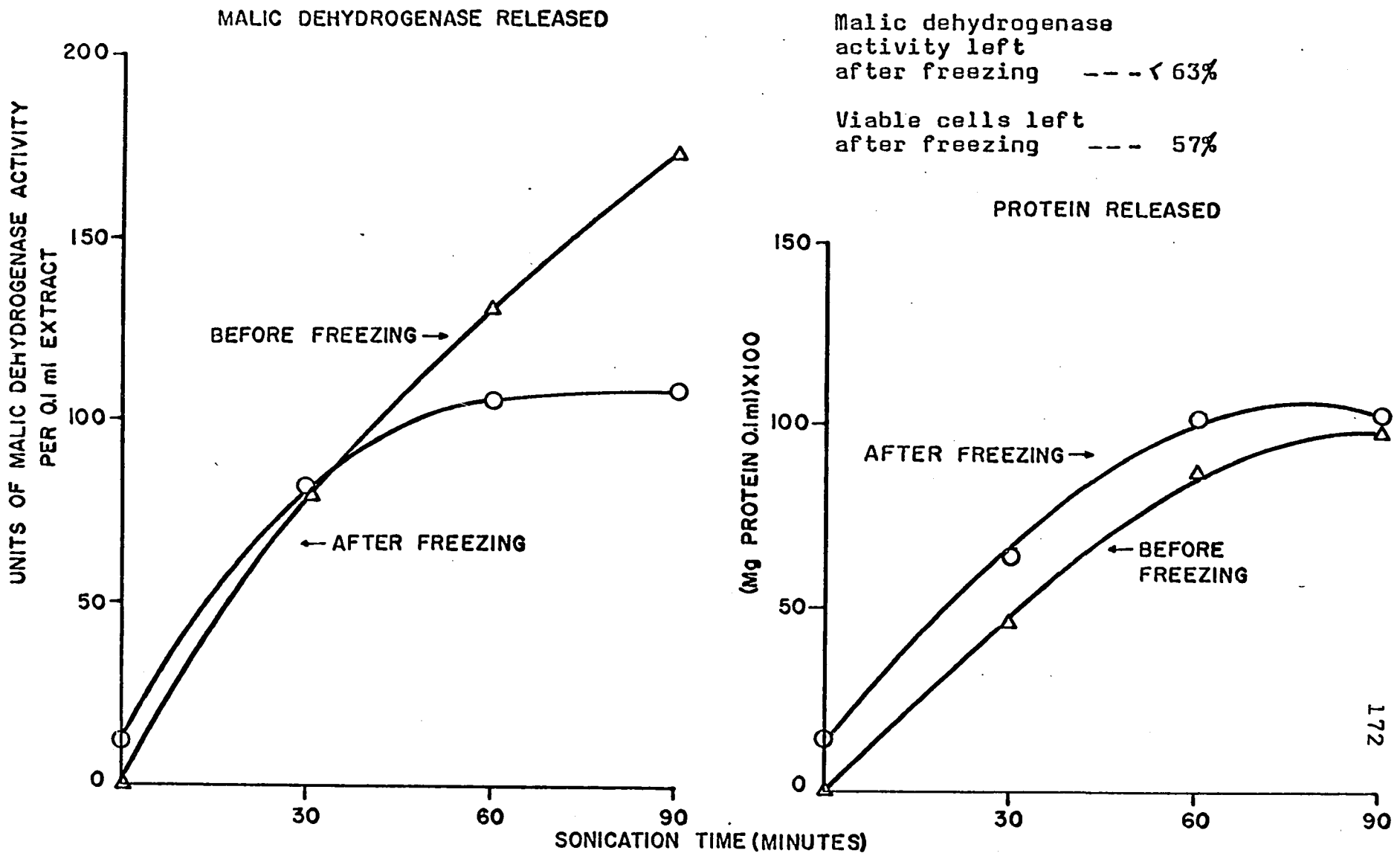
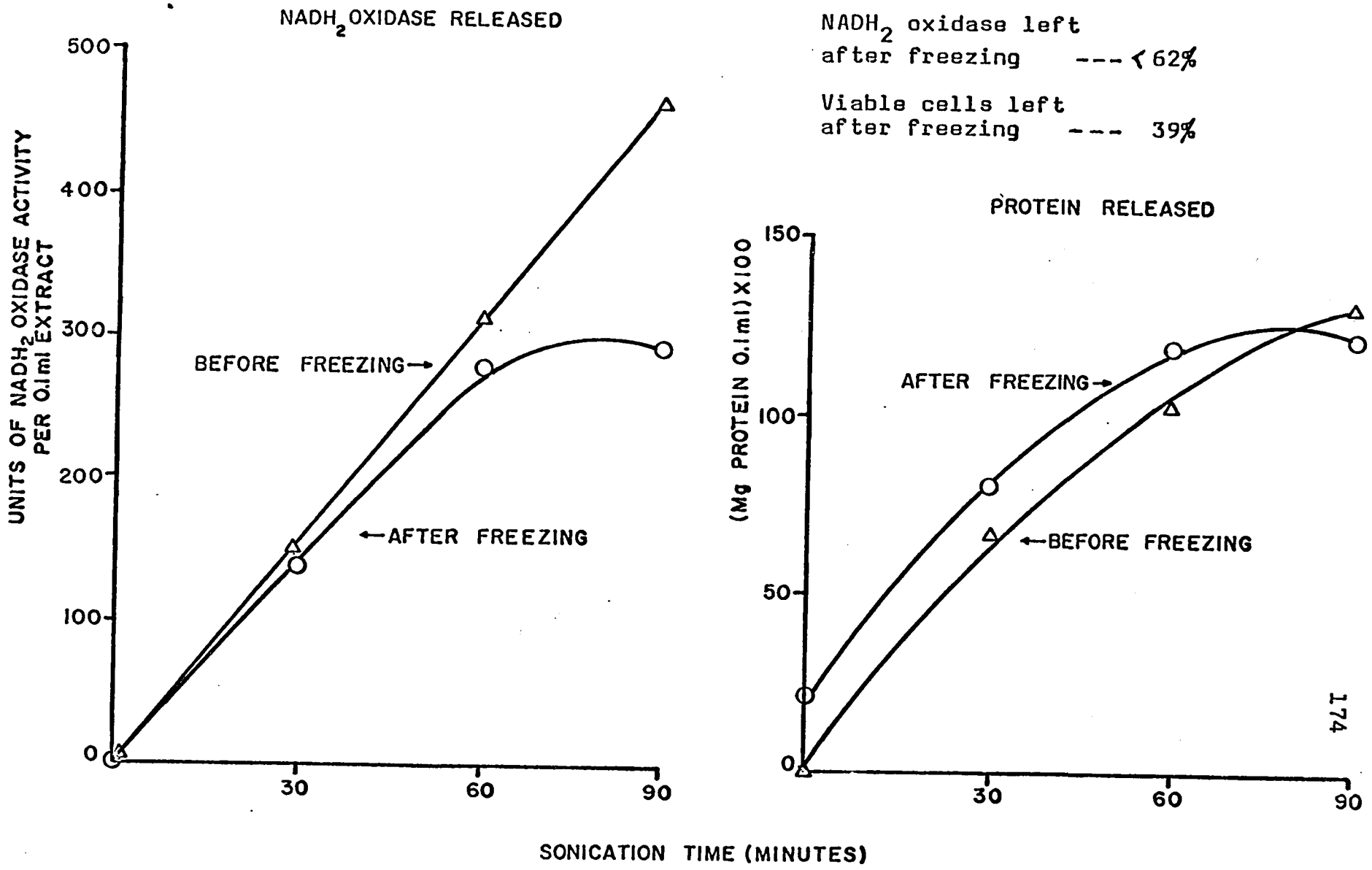

MALIC DEHYDROGENASE RELEASED
UNITS OF MALIC DEHYDROGENASE ACTIVITY PER 0.1 ml EXTRACT
200
150
100
50
0
BEFORE FREEZING
AFTER FREEZING
30
60
90
SONICATION TIME (MINUTES)
Malic dehydrogenase activity left after freezing --- 63%
Viable cells left after freezing --- 57%
PROTEIN RELEASED
(Mg PROTEIN 0.1ml) X100
150
100
50
0
AFTER FREEZING
BEFORE FREEZING
30
60
90

Figure 12. Effect of sonication time on the rate of release of DPNH-oxidase and on total DPNH-oxidase activity of cells of Aerobacter aerogenes (Mac 112) before and after freezing.
*See Fig. 10.


NADH2 OXIDASE RELEASED
UNITS OF NADH2 OXIDASE ACTIVITY PER 0.1ml EXTRACT
500
400
300
200
100
0
BEFORE FREEZING→
←AFTER FREEZING
30
60
90
NADH2 oxidase left after freezing --- < 62%
Viable cells left after freezing --- 39%
PROTEIN RELEASED
(Mg PROTEIN 0.1ml) X100
150
100
50
0
AFTER FREEZING→
←BEFORE FREEZING
30
60
90
SONICATION TIME (MINUTES)

freezing and storage. The complete release of $NADH_2$ oxidase from frozen cells was achieved after 60 minutes of sonication, while, in the case of unfrozen cells the enzyme activity in the extract was still increasing linearly after 90 minutes. Although no values for total enzyme activity in unfrozen cells were obtained, the results after 90 minutes of sonication indicate that less than 62% $NADH_2$ oxidase activity was retained by the cell population. The viability of the cells after freezing was 39%.

The effect of osmotic shock on release of enzymes and loss of viability

Previous results indicated that some intracellular enzymes such as malic dehydrogenase and ATPase were released into the suspending medium after the cells had been frozen and thawed. It was of interest to see if these enzymes would be released extracellularly when the suspension of cells was subjected to osmotic shock, since osmotic effect as a factor involved in cell injury during freezing and thawing has been proposed by several workers (Meryman, 1966, 1968; Bretz and Hartsell, 1959). A washed cell suspension of Aerobacter aerogenes (Mac 112) at a concentration of 1×10^{11} cells per ml. was suspended in 50 ml. of 0.03 M Tris HCl containing 20% sucrose, and

0.5 ml. of 0.01 M EDTA (pH 8.0). After 10 minutes, the supernatant fluid was removed by centrifugation and the cell pellet was rapidly dispersed in a 50 ml. volume of cold glass-distilled water. This suspension was mixed for 10 minutes on a rotary shaker and centrifuged. The supernatant fluid was removed to determine the enzyme activities of malic dehydrogenase, ATPase and $NADH_2$ oxidase as described previously. Viability was also determined both before and after osmotic shock. The results in Table 37 show that of three enzymes examined only ATPase showed any capacity to be released during osmotic shock. It can also be seen that much less protein and no enzyme activities were found in the suspending medium when treatment with EDTA (10^{-3} M) was omitted. In agreement with previous findings with Escherichia coli (Neu and Heppel, 1965), it was observed that osmotic shock treatment did not reduce cell viability (Table 38).

TABLE 37

Effect of osmotic shock on release of enzymes in suspending medium in the presence and absence of EDTA

EDTA (1×10^{-3} M)	Total Protein (mg/ml)	ATPase*	Malic dehydrogenase	$NADH_2$ oxidase
		Specific activity (units/mg)		
-	0.128	0	0	0
+	0.640	3.50	0	0

*Enzyme activity expressed as micromoles of Pi released in 20 minutes per mg. protein

TABLE 38

Effect of osmotic shock on the viability in a suspension of Aerobacter aerogenes (Mac 112)

Plating medium	Before osmotic shock	After osmotic shock*
	Count (10^9 cells/ml)	
Minimal	108 ± 4	95 ± 10
Minimal + 1% yeast extract	104 ± 4	105 ± 9

*Samples were removed for measurement of viability immediately before centrifugation of shock suspension.

DISCUSSION

The results obtained in the present investigations indicate that after storage at freezing temperature, Aerobacter aerogenes (Mac 112) released intracellular substances into the supernatants. The quantity of materials leaked into the suspending medium was dependent upon the nature of the intracellular substances and the composition of the freezing menstruum. It was found that there was leakage of an intracellular enzyme, malic dehydrogenase, and of adenosine triphosphatase (ATPase) after suspension of the cells in 0.1 M KCl for freezing and storage at -20°C for one day. However, the membrane bound enzyme, reduced nicotinamide adenine dinucleotide ($NADH_2$) oxidase was found not to be released. No malic dehydrogenase, and only a small amount of K^+ ion was released from cells which were suspended in glass-distilled water for freezing compared with cells suspended in 0.1 M KCl.

Bretz and Hartsell (1959) found that suspensions of Escherichia coli, after freezing and thawing, gave greater recoveries of viable cells when diluted with 20% sucrose

solution for plating than when diluted in ordinary buffer solutions. They concluded that the cells had developed osmotic sensitivity after freezing. The results in the present study are similar to the findings of Postgate and Hunter (1963) in that they did not demonstrate any increase in the viability of Aerobacter aerogenes after freezing and thawing when the cells were diluted in hypertonic sucrose as compared with dilution with glass-distilled water or ordinary diluting fluids. This observation was incompatible with the hypothesis that the lethal effects of freezing were connected with osmotic shock.

It was found that a release of ATPase from cells of Aerobacter aerogenes (Mac 112) occurred after osmotic shock, but no $NADH_2$ oxidase or malic dehydrogenase was detected in the suspending medium. Such osmotic shock treatment did not cause loss of viability. Similar results have been observed during studies conducted with osmotic shock on cells of Escherichia coli (Nassal and Heppel, 1966; Neu and Heppel, 1965). They suggested that the effect on bacterial cells of "osmotic shock" specifically releases superficially located enzymes. This is in contrast to the results obtained with frozen and thawed cells of Aerobacter aerogenes which lead to the release of the intracellular enzyme, malic dehydrogenase. This might be due to a

general increase in permeability of the cells. Direct cell counts of frozen cells have demonstrated that the material released was not a result of cell lysis (Meynell, 1958; Moss and Speck, 1966b).

Freezing caused the release of intracellular materials from the cells of Aerobacter aerogenes. The amount of leakage materials such as malic dehydrogenase or K^+ ion seemed to be proportional to the loss in viability of the cells after freezing and thawing. The greater lethal effect of KCl solution as a freezing menstruum probably caused more damage to the cytoplasmic membrane of the cells during freezing and thawing, and was accompanied by greater release of intracellular materials from the cells than in the case of distilled water as a freezing menstruum. However, on the basis of the present study, it is not known whether the leakage of these materials precedes or follows the loss of viability.

Lindeberg and Lode (1963) found that the amount of ultraviolet absorbing material appearing in the extracellular fluid of frozen and thawed suspensions of Escherichia coli correlated with the loss of viability. Moss and Speck (1966b) also observed that leakage material from frozen and thawed cells of Escherichia coli in phosphate buffer

contained peptides which in the supernatant fraction, paralleled loss of viability. Morichi et al. (1967) studied the death of freeze-dried Lactobacillus bulgaricus following rehydration and observed that although cellular ribonucleotides leaked out from the freeze-dried cells, there was no correlation between the viability of the cells and the amount of substances which were released from the cells.

In this study, an attempt was made during the thawing, diluting and plating process to maintain intracellular solutes and K^+ or Mg^{++} at a level which might permit cells of Aerobacter aerogenes (Mac 112) to function and thereby repair any mechanical damage due to freezing. It was found that none of these precautions increased the recovery of viable cells in a suspension after freezing and thawing. This finding suggests that the release of these substances may not be the cause of death. Rotman (1958) reported that the cells of Escherichia coli can lose as much as 80% of the total RNA content of the cells as breakdown products in the supernatant and still remain viable.

The results obtained with all three enzymes, malic dehydrogenase, $NADH_2$ oxidase, and ATPase, were consistent

with the conclusion that freezing brings about a general denaturation of proteins. Cells were ruptured more readily by sonication after freezing and proteins were more completely solubilized. Hanson and Nossal (1955) found that disintegration of yeast cells by means of a high-speed shaker solubilized a number of dehydrogenases. Freezing enhanced the degree of solubilization.

In the present study, the activities of these enzymes did not seem to be affected by sonic treatment, since the reduction of enzyme activity was not observed after 90 minutes of sonication. It has been reported that some enzymes are readily inactivated by sonic treatment. Following sonication of isolated liver mitochondria, Hogeboom and Schneider (1950) observed that succinoxidase was largely destroyed and octanoic acid oxidase was completely inactivated.

Some enzymes are known to be readily denatured by freezing, others are quite cryoresistant (Levitt, 1966). It has also long been suspected that the freezing injury of bacterial cells is associated with protein denaturation. Haine (1938) observed that the isolated protein of Pseudomonas aeruginosa was rapidly precipitated when stored at $-2^{o}C$, but little or no precipitate occurred at -20^{o} or $-70^{o}C$.

He also found that the death rate of this organism was maximum at -1^{o} or $-2^{o}C$, and therefore suggested that the death of bacterial cells at freezing temperature was related to the flocculation of cellular protein. Little or no information, however, is available about the relationship between the loss of enzyme activity and loss of viability. In the present study, all three enzymes show a loss in activity after the cells had been frozen and thawed. Although exact correlations between the extent of loss in enzyme activities and viability loss on suspension of the cells were not obtained, it might suggest that in some cells in the population, proteins are more readily denatured than in others. The loss of viability in a particular cell seems to be due not to loss of a particular enzyme or the integrity of a membrane protein, but to a general denaturation of functional proteins.

SUMMARY

SUMMARY

Suspensions of bacterial cells which gave the same plate count on minimal and enriched agar medium before freezing gave a higher count on the enriched medium afterwards. Those cells that are capable of growing on the enriched medium but not on the minimal medium have been described as "metabolically injured." In the course of the present study, it was found that when precautions were taken to remove toxic trace elements from the plating diluents, suspensions of Escherichia coli 451B but not Aerobacter aerogenes (Mac 112), following freezing and thawing, still gave higher counts when Trypticase (2%) or yeast extract (1%) was added to the minimal medium. Cysteine or some other metal binding agents when present in the plating medium was unable to increase the count on suspensions of Escherichia coli 451B. It was therefore evident that the capacity of enriching supplements such as Trypticase or yeast extract to increase the count on suspensions of this organism after freezing was due to more than their metal binding action.

Hydrolysis with HCl or H_2SO_4 partially inactivated

the Trypticase. However, the H_2SO_4 hydrolysate of Trypticase after further treatment with Dowex 50 x 8 (H^+) resin was found to be as effective as Trypticase as a supplement to the minimal medium. A mixture of amino acids approximating the composition of casein, when added at a level equivalent to 0.1% casein, also markedly increased the count, but at 2% was found to be inhibitory. Tests of sub-groups of amino acids in the mixture and of individual amino acids revealed that aspartic acid, at a level of 2.5 mM, could replace Trypticase completely as a supplement to the minimal medium. Glutamic acid and alanine were also active. The addition of 1.0 mM or higher concentration of serine to the minimal medium depressed the plate count. None of the amino acids or other supplements tested affected the count on the suspensions of the cells before freezing.

Using the replica plating technique, it was shown that the requirement for aspartic acid by a portion of the Escherichia coli 451B population after freezing was not due to the formation of stable auxotrophic mutants.

A number of strains of Escherichia coli, Aerobacter aerogenes, Serratia marcescens and two species of Pseudomonas were tested to determine their response to aspartic

acid, cysteine or Trypticase after freezing. None of these supplements was found to have a generalized capacity to increase the count on suspensions of the different strains and species after freezing. The results of these studies indicated that after freezing the response of the organisms to supplements in the plating medium was unique for each organism.

The effect of glass-distilled water, and solutions of various inorganic salts as freezing menstrua on the survival of Aerobacter aerogenes (Mac 112) was investigated. It was found that only a small proportion of bacterial cells in suspension lost their viability on freezing if the freezing medium was glass-distilled water or NaCl at a concentration lower than 0.01 M, provided the high cell population containing 1×10^{10} cells per ml. was employed for freezing. All chloride salts tested at a concentration of 0.05 M or higher were found to be toxic. However, Na_2SO_4 and $MgSO_4$ at the same concentration as NaCl were non-toxic. The lethal effect of NaCl on viability of Aerobacter aerogenes was effectively neutralized by glycerol, dimethyl sulfoxide or inositol.

The internal concentration of Na^+, K^+ and Cl^- ion in Aerobacter aerogenes (Mac 112) was demonstrated at

various levels of NaCl or KCl in the suspending medium. Na^+ and K^+ ion inside the cell varied appreciably depending on the salt and its concentration in the suspending medium. Intracellular Na^+ concentration was found to be lower than that of the outside concentration when a concentration of 100 or 200 mM NaCl was added to the suspending medium. On the other hand, when KCl was added to the suspension at the same concentration as NaCl, the intracellular K^+ concentration was always higher than that of the medium. The intracellular Cl^- ion concentration was approximately 60 to 70% of the Cl^- content of the suspending medium at the 100 and 200 mM level of either NaCl or KCl. The loss of viability of the cells after freezing was proportional to the concentration of the salts in which the cells were suspended, but was not dependent on whether NaCl or KCl was used. The loss of viability paralleled more closely the changes in internal Cl^- ion concentration than any of the cation concentration changes observed.

The extent of survival when cells of Aerobacter aerogenes (Mac 112) were suspended in glass-distilled water for freezing was markedly affected by the concentration of cells in the suspensions tested. As the concentration of cells in suspensions of this organism was

increased from 2×10^6 to 2×10^{10} per ml. the per cent viability after freezing rose from 35 to 80%. It was found that exudates from the cells before and during freezing could be responsible for the protection afforded by high cell density. The supernatants prepared from unfrozen cells had a remarkable capacity to protect a suspension of the cells from damage by freezing and thawing.

Cells of Aerobacter aerogenes (Mac 112) at a density of 2×10^7 cells per ml. were suspended in glass-distilled water and in varying concentrations of either NaCl, $NaNO_3$ or Na_2SO_4 and their viability tested after freezing. The results indicated that up to a concentration of 0.01 M of NaCl, some protection of viability from freezing and thawing over cells suspended in glass-distilled water was obtained. $NaNO_3$ and Na_2SO_4 at the same low concentrations were found to be much more protective than NaCl or glass-distilled water under the conditions studied. The protective effect of these inorganic salts was markedly decreased after the cells had been frozen and stored for 1 week.

Cells of Aerobacter aerogenes (Mac 112) after freezing and thawing leaked malic dehydrogenase, ATPase, and K^+ ion into the suspending medium. The amount of

released substances from the cells after freezing increased when the cells were suspended in 0.05 M KCl compared with glass-distilled water as freezing menstruum. Concentrated cell supernatants and various concentrations of KCl and $MgSO_4$ maintained in contact with the cells at all times during thawing and diluting did not increase the recovery of viable cells after freezing, leading to the conclusion that loss of intracellular solutes per se did not cause loss of viability.

Suspensions of Aerobacter aerogenes (Mac 112) after freezing and thawing, did not give greater recoveries of viable cells when the cells were diluted with sucrose (1M) solution than with glass-distilled water. It therefore seems unlikely that an osmotic effect is involved in bringing about the killing action of freezing and thawing. A release of ATPase but not $NADH_2$ oxidase and malic dehydrogenase from the cells of this organism occurred after "osmotic shock," and no loss of viability was obtained with such a treatment.

The relationship between the loss of viability and the loss in enzyme activity of the cells after freezing and thawing has been investigated. The results revealed that cells after freezing and thawing were ruptured more

readily by sonication and proteins were more completely solubilized. All three enzymes, ATPase, malic dehydrogenase and $NADH_2$ oxidase, tested were partially inactivated after the cells had been frozen and thawed, but no exact correlation was obtained between the extent of loss of the enzyme activity and loss of viability. It was concluded that a generalized denaturation of functional proteins was responsible for the loss of viability of cells on freezing.

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