REPAIR OF INJURY IN *ESCHERICHIA COLI* AFTER FREEZING

by

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REPAIR OF FREEZE-INJURY IN *ESCHERICHIA COLI*

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ABSTRACT

Ph.D. James Newman Bunch Microbiology

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The recovery of cells of *Escherichia coli* 451B damaged by freezing has been examined. After freezing and thawing, a proportion of cells demonstrated an increased sensitivity to 0.55 M NaCl in the plating medium, were unable to form colonies in a minimal glucose-salts plating medium, and were permeable to actinomycin D. These conditions were overcome by preincubation of the cells in a recovery medium consisting of PO_4^{-3}, K^+ and Mg^{++}, at pH 7.0

Mg^{++} was the most important component of this medium and its substitution by other divalent cations resulted only in a partial recovery of the cell population. Recovery was not affected by chloramphenicol, iodoacetate or cyanide, and the rate of recovery was retarded but not inhibited by a decreased incubation temperature. These data suggested that Mg^{++}-mediated recovery did not have an energy requirement and resulted in the repair of membrane permeability.

The addition of aspartic acid to the recovery medium resulted in cell multiplication, but only after a lag during which repair was effected. Repair was not appreciably enhanced by aspartic acid. A similar growth response was obtained with various other amino acids and citric acid cycle intermediates, but the addition of glucose or serine resulted in a considerably longer growth lag. Glucose did not participate in the recovery process.

RESUME

Ph.D. James Newman Bunch Microbiologie

REPARATION DU DOMMAGE DANS *ESCHERICHIA COLI* APRES LA CONGELATION

La récupération de cellules *Escherichia coli* 451B endommagées par le gel a été examinée. Après être gelées et dégelées, une proportion des cellules ont démontré une sensibilité accrue à 0.55 M NaCl dans le milieu de culture, et ne purent former de colonies dans un milieu contenant un minimal de sel de glucose, et furent perméables à l'actinomicine D. Ces conditions furent surmontées en préincubant les cellules dans un milieu de PO_4^{-3}, K^+ et Mg^{++}, au pH 7.0.

Mg^{++} est le composant le plus important de ce milieu et le substituer par d'autre cations divalent ne résulte qu'en une récupération partielle de la population de cellules. La récupération n'est pas affectée par le chloramphenicol, l'acétate iodé ou le cyanure, et le rythme de récupération est retardé, mais non empêché en décroissant la température d'incubation. Ces données suggèrent que la récupération par Mg^{++} ne requière aucune énergie et il en résulte la réparation de la perméabilité de la membrane.

L'addition d'acide aspartique au milieu de récupération, résulte en une multiplication des cellules, mais seulement après un lapse de temps, pendant lequel la réparation se fait. Cette réparation ne fut rehaussée appréciablement par l'acide aspartique. Une croissance semblable fut obtenue avec plusieurs autres acides aminés et les intermédiaires du cycle d'acide citrique, mais avec l'addition de glucose ou de sérine l'on prolonge considérablement ce lapse de croissance.

TABLE OF CONTENTS

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LIST OF FIGURES

CLAIM OF CONTRIBUTION TO KNOWLEDGE

1. After freezing and thawing a suspension of *Escherichia coli* 451B, a rapid decline in viability of a proportion of the population was overcome by the addition of Mg^{++} to the suspended cells.

2. The presence of Mg^{++} in a solution of phosphate and potassium salts rendered the viable cells impermeable to 0.55 M NaCl and actinomycin D during incubation in this medium.

3. Other divalent and monovalent cations tested could not substitute for Mg^{++} in the recovery of salt tolerance by a frozen and thawed suspension of cells.

4. Mg^{++}-mediated recovery of salt tolerance of a frozen and thawed suspension of cells did not require metabolic energy or protein synthesis.

5. Neither aspartic acid nor glucose enhanced the recovery of tolerance to NaCl of the viable cells in a frozen and thawed suspension of cells.

6. Aspartic acid but not glucose was shown to be a more suitable metabolic substrate to initiate the multiplication and division of both fresh, unfrozen cells of *Escherichia coli* 451B and the viable cells remaining after freezing and thawing.

INTRODUCTION

INTRODUCTION

Cellular damage to bacteria has been induced by a variety of stresses. These have included freezing and thawing (Straka and Stokes, 1959; Moss and Speck, 1966; Kuo and MacLeod, 1969; Ray and Speck, 1972), sublethal heat treatment (Iandolo and Ordal, 1966) and freeze-drying (Sinskey and Silverman, 1970; Ray, Jezeski and Busta, 1971).

Freezing damage to bacterial cells has been found to result in death or injury characterized by an increased nutritional requirement (Straka and Stokes, 1959; Moss and Speck, 1966). Kuo and MacLeod (1969) increased the count of a suspension of frozen and thawed cells of *Escherichia coli* 451B in a minimal glucose-salts medium by supplementing it with aspartic acid. They proposed that aspartate might be involved in the replenishment of the amino acid pool of freeze-damaged cells or in a specific repair of the damage caused by freezing.

Cellular damage has been characterized by sensitivity of injured cells to selective agents such as NaCl (Iandolo and Ordal, 1966) and sodium deoxycholate (Sinskey and Silverman, 1970).

In the present study, sensitivity of frozen and thawed cells of *Escherichia coli* 451B to NaCl was established as a criterion of cellular damage. Using this criterion, the roles of aspartate and components of

a minimal glucose-salts medium (Kuo and MacLeod, 1969) in the repair of freeze-injury were examined.

LITERATURE REVIEW

LITERATURE REVIEW

Nutritional Requirements of Frozen and Thawed Suspensions of Bacteria

It has long been recognized that the freezing of a bacterial suspension had an adverse effect on the viability of the population. Many microbial species demonstrating an increased nutritional requirement after freezing have been described in the literature and include: *Escherichia coli*, Straka and Stokes, 1959; Arpai, 1962; Moss and Speck, 1966; MacLeod *et al.*, 1966, 1967; Kocka and Bretz, 1967, 1969; Kuo and MacLeod, 1969; *Aerobacter aerogenes*, Postgate and Hunter, 1963; MacLeod *et al.*, 1966, 1967; *Streptococcus lactis*, Moss and Speck, 1963; *Shigella sonnei*, Nakamura and Dawson, 1962; *Pseudomonas spp.*, Straka and Stokes, 1959; Kuo and MacLeod, 1969; *Salmonella spp.*, Sorrells, Speck and Warren, 1970; Ray, Janssen and Busta, 1972; *Serratia marcescens*, Kuo and MacLeod, 1969; *Vibrio succinogenes*, Wolin, 1966; *Saccharomyces cerevisiae*, Hansen and Nossal, 1955.

Straka and Stokes (1959), using strains of *Escherichia coli* and *Pseudomonas spp.*, first described the nonlethal effects of freezing damage to bacterial cells and employed the term "metabolic injury". This was used to describe an increased nutritional requirement of a proportion of a frozen and thawed population. Before exposure to freezing, a

suspension of cells was capable of growth on a minimal salts-glucose agar medium as well as on Trypticase soy agar (TSA). After freezing, the various states of the bacterial cells were defined as:

1) Injured cells: those which grew on TSA but not on minimal agar after exposure. They were determined quantitatively by the difference in plate counts on the two media.

2) Dead cells: those which failed to grow on TSA after exposure. The difference between the initial TSA count and that after exposure indicated the number of cells killed.

3) Unharmed cells: those which grew on minimal agar after exposure to low temperatures.

Straka and Stokes found that the addition of trypticase to the minimal agar increased the count to the level of that on TSA and it was suggested that the peptide components of trypticase, an enzymatic digest of casein, were responsible for the increased counts. These might be required by injured cells for resynthesis of essential proteins denatured by the subzero temperatures. Moss and Speck (1966a), using several strains of *Escherichia coli*, also demonstrated increased counts of a frozen and thawed population when incubated in a minimal salts-glucose agar medium supplemented with trypticase over those obtained in minimal agar in the absence of trypticase. As had been the case with Straka and Stokes, acid-hydrolyzed casein did not increase the counts. Sephadex gel chromatography of trypticase demonstrated five similar small-chain

peptides which were as active as trypticase in increasing counts of a frozen and thawed population and hydrolysis of these peptides resulted in a loss of this activity. Isolation of extracellular material from the freezing menstruum of a frozen and thawed population revealed similar peptides which were judged to be a result of intracellular leakage since no lysis of cells was observed. Appearance of peptide material in the supernatant fractions paralleled losses in cell viability and these peptides were found to possess activity in agar media for the recovery of cells which had been metabolically injured by freezing.

MacLeod, Smith and Gélinas (1966) supplemented the minimal medium of Straka and Stokes with Bacto-casamino acids and increased the count of frozen and thawed suspensions of both *Aerobacter aerogenes* and *Escherichia coli*. In these experiments, cysteine was found to be as effective as Casamino acids. They postulated that frozen and thawed cells, being more permeable than unfrozen cells as a result of membrane damage, were susceptible to toxic trace elements present in the medium. The increased nutritional requirement of a frozen and thawed population reflected the need for compounds such as cysteine to chelate toxic trace elements and allow injured cells to remain viable and grow in the plating medium. This was confirmed by MacLeod, Kuo and Gélinas (1967) who found a toxic level of Cu^{++} in the distilled water used in the preparation of the plating diluent. A degree of sensitivity of unfrozen cells to this concentration of Cu^{++} in the plating diluent was demonstrated by the

counts of suspensions of cells suspended in the diluent and then plated on minimal medium supplemented with cysteine. These counts were higher than those obtained in minimal medium without added cysteine. The difference in count between the two plating media disappeared, however, when Mg^{++} was added to the plating diluent or when this diluent was prepared in a manner which eliminated toxic Cu^{++}. The presence of Mg^{++} in the plating diluent did not increase the count of a frozen and thawed suspension of cells plated on minimal medium. The increased counts of suspensions of frozen and thawed cells on supplemented media over those obtained on unsupplemented media were ascribed to the capacity of the supplement, i.e. cysteine, to remove toxic elements which had become bound to the cells during suspension in the plating diluent. This binding was presumed to be at loci within the cell exposed as a result of membrane damage where Mg^{++} in the diluent would not act as an effective antagonist. When steps were taken to reduce the content of toxic trace elements in the plating diluent, the metabolic injury produced in a suspension of cells of *Aerobacter aerogenes* by freezing and storage, as evidenced by the development of differences in plate count on minimal and supplemented media, tended to disappear. In similar experiments with *Escherichia coli* 451B, a strain used by Straka and Stokes (1959) and Moss and Speck (1966a) in their studies of metabolic injury, Kuo and MacLeod (1969) demonstrated a degree of sensitivity of unfrozen cells to the levels of Cu^{++} employed in the plating diluent, and upon freezing and thawing, the degree of sensitivity was increased. The use of water

redistilled in glass did not alter the counts of suspensions of frozen and thawed cells on unsupplemented media compared to the counts obtained on supplemented media. It was speculated that since the basic lesions in cells injured by freezing and storage appeared to be membrane damage, leading to increased penetrability, changed sensitivity of the injured cells to medium components other than toxic trace elements might be expected. Further work by Kuo and MacLeod (1969) demonstrated the uniqueness of various strains of bacteria in their responses after freezing to minimal media enriched with various supplements. Frozen and thawed suspensions of cells of *Escherichia coli* 451B were found to have higher counts on minimal medium supplemented with trypticase or aspartate than in the absence of a supplement. However, the count of a frozen and thawed suspension of cells of *Escherichia coli* 451B was not increased when plated on minimal medium supplemented with cysteine. In contrast, several strains of *Aerobacter aerogenes* did not respond to aspartate as a supplement. It was concluded that if the freezing of an organism led to membrane damage, the nature of the response of the damaged cells to medium components varied from organism to organism.

Manifestations of Metabolic Injury in Frozen and Thawed Bacteria

Besides an increased nutritional requirement, a variety of other symptoms of injury have been noted as a result of freezing and thawing.

An increased growth lag has been noted in enriched media after freezing and thawing (Squires and Hartsell, 1955), (Postgate and Hunter, 1963). Kocka and Bretz (1969), using an enriched agar medium with a slide-culture technique, demonstrated that frozen and thawed cells of *Escherichia coli* took up to three times as long to undergo a first cell division as cells which had not been frozen.

Wolin (1966) found that *Vibrio succinogenes* had an increased sensitivity to lysozyme after freezing and thawing. He speculated that freezing and thawing destroyed the association between the non-mucopeptide and mucopeptide layers, making the mucopeptide susceptible to lysozyme. This phenomenon had previously been noted in *Escherichia coli* by Kohn and Szybalski (1959). They found, in addition, that incubation of a suspension of cells in nutrient broth for sixty minutes after freezing and thawing led to complete recovery of resistance to lysozyme. Bretz and Kocka (1967), demonstrated that *Escherichia coli* had a sensitivity to actinomycin D after freezing and thawing and equated this to the lysozyme sensitivity seen by Kohn and Szybalski (1959). Similar observations were made by Ray, Janssen, and Busta (1972), who concluded that freezing and thawing of *Salmonella anatum* resulted in an alteration of the lipopolysaccharide layer, rendering the cells sensitive to actinomycin D and lysozyme.

Ray and Speck (1972) reported an inability of a frozen and thawed suspension of *Escherichia coli* to form colonies on a medium

containing 0.1% sodium deoxycholate, a surface-active agent, but otherwise nutritionally adequate. Normal cells could do so. Ray, Janssen and Busta (1972) noted a similar finding with *Salmonella anatum* using 0.2% deoxycholate in the enriched plating medium and attributed this sensitivity to an alteration in the cell wall and cytoplasmic membrane. Attempts to relate this action directly to the lipoprotein of the membrane were inconclusive.

Evidence of increased permeability in frozen and thawed cells has been widely noted in the literature. Lindeberg and Lode (1963) noted the release of UV-absorbing materials from a suspension of frozen and thawed cells of *Escherichia coli*. This loss of material appeared to be proportional to the loss of viability of the cells in the suspension. Mazur (1963) found an increase in the concentration of solutes in the medium of a suspension of *Escherichia coli* after freezing which was proportional to the loss of viability. Moss and Speck (1966b) found that freezing and storage of a strain of *Escherichia coli* in 0.038 M phosphate buffer resulted in a loss of cell viability and a pronounced leakage of cellular material which had maximal absorption at 260 nm. This leakage material contained protein in the form of peptides of relatively small molecular weight. Direct cell counts indicated that the material was not a result of cell lysis. When frozen in distilled water, only small amounts of 260 nm absorbing material were detected, but the decline in viability was greater. Kuo (1969) demonstrated a release of K^+ and small amounts

of malic dehydrogenase when a suspension of *Aerobacter aerogenes* was frozen and thawed in 0.1 M KCl. There was no protein leakage and considerably less K^+ leakage when the suspending medium was distilled water. Ray and Speck (1972), after freezing and thawing a strain of *Escherichia coli* in distilled water, found leakage of material which absorbed at 260 and 280 nm. Direct microscopic counts discerned no evidence of lysis.

Kuo (1966) studied the effect of freezing and storage on the respiratory activity of *Aerobacter aerogenes*. When glucose served as the oxidizable substrate, respiratory activity correlated well with viability as determined by bacterial colony count on the glucose minimal medium of Straka and Stokes (1959). Where an amino acid mixture was employed as the respiratory substrate, freezing and storage appeared to decrease viability more than respiratory activity, although the glucose plating medium was again used to determine viability. Hansen and Nossal (1955) reported that the activity of the dehydrogenases of a number of substrates (succinate, lactate, citrate, malate, fumarate, and glutamate) in extracts of frozen and thawed cells were about the same as with extracts from unfrozen cells. Kuo (1969), on the other hand, found that freezing and thawing caused decreased activity of malic dehydrogenase, ATPase and $NADH_2$ oxidase in cells of *Aerobacter aerogenes*.

Shikama (1965) observed that the ultraviolet absorbance of DNA was not increased after freezing and thawing. He suggested that the double-stranded helical structure of DNA would not be broken down by

freezing and thawing. Employing the replicate plating technique, Kuo (1969) demonstrated that the apparent nutritional requirement for aspartate of a proportion of a frozen and thawed population of *Escherichia coli* 451B was not due to the formation of stable auxotrophic mutants. The inability of the freezing process to act as a mutagen was also demonstrated by Postgate and Hunter (1963) and MacLeod, Smith and Gélinas (1966). Arpai and Tomishava (1966) observed that freezing was able to enhance the induction of streptomycin resistance in *Escherichia coli* by chemical mutagens. This observation may be related to the findings of Sato and Takahashi (1970). They demonstrated that cold-shocked cells of *Escherichia coli* had more single-strand breaks in the DNA structure than unshocked cells and attributed this to an inability of DNA-ligase to make the structure continuous. The impairment of the enzyme was speculated to result from a loss of Mg^{++} from the shocked cells.

Sorrells, Speck and Warren (1970) noted that although freezing and thawing of *Salmonella gallinarum* resulted in metabolic injury as defined by Straka and Stokes (1959), this treatment did not affect the pathogenicity of the cells when inoculated into six week-old chicks.

Causes of Freezing Injury in Cells

Early studies into the causes of freezing death attributed it to the mechanical action of ice crystals compressing and piercing the cellular membrane.

Lovelock (1957) observed freezing of human erythrocytes with varying solutions of NaCl. Above 0.85 M, the quantities of lipid lost by the cells increased greatly and there was a selectively greater loss of phospholipid. He suggested that the high ionic strength of the surrounding medium, as a result of water freezing out, weakened phospholipid bound to the membrane by ionic forces and rendered the cell more permeable to cations.

Mazur (1970) has suggested that freezing injury to cells occurs in two ways: (1) solution effects are responsible for injury when cooling is slower than optimal, and (2) intracellular freezing is responsible for injury when cooling is faster than optimal. Optimum cooling rates vary widely among different types of cells. As cells are cooled, intracellular water becomes supercooled while extracellular water forms ice. Water begins to flow out of the cell in response to the vapour pressure gradient between the intracellular, supercooled water and the extracellular ice. The rate of flow of water is dependent upon the permeability of the membrane which also prevents entry of ice crystals above a particular temperature. If the temperature of the system is decreased further, equilibrium of the gradient is achieved by the formation of intracellular ice.

Employing liposome preparations, Siminovitch and Chapman (1971) clearly demonstrated the dependence of intracellular and extracellular ice formation on the rate of cooling of suspending media as well as the

effect of ice formation on liposomes. Various markers were incorporated into liposomes prepared from egg yolk lethicin. The liposomes were then frozen at a controlled rate and thawed. Light microscope observations of liposomes frozen at a slow rate (0.5°C/min) demonstrated extracellular formation of ice with simultaneous diminution of liposome volume until the liposomes were barely discernable among the ice crystals. Upon thawing, liposomes swelled visibly and reoccupied nearly the same area as before freezing. Liposomes which had been treated in this manner appeared to have lost some of the smooth and regular features of normal liposomes and there was a pronounced increase in the release of both ionic and nonionic markers trapped in the liposomes e.g. phosphate, chromate, glucose and glycine. Rapid freezing (20.0°C/min) resulted in formation of intracellular ice. Liposomes thus frozen gave no evidence of contraction and upon thawing, ice crystals inside and outside of the liposomes melted without any indication of swelling. Unfortunately, similar observations of marker release from rapidly frozen liposomes were not made.

Cells cooled very rapidly achieve equilibrium by intracellular ice formation with very little dehydration. Cells cooled slowly are subjected to dehydration and it is here that solution effects occur. Mazur (1970) listed these as: (1) concentration of intracellular and extracellular solutes, (2) decrease of cell volume, (3) precipitation of solutes as their solubilities are exceeded with accompanying alteration

of pH, and (4) precipitation of all solutes below the eutectic point of the system.

Zimmerman (1964) found that a large decline in the viability of a suspension of *Serratia marcescens* occurred when saline solutions suspending the cells were frozen below the eutectic point of the solutions. When cells suspended in NaCl solutions with concentrations up to 20% were frozen to a temperature which did not allow phase-transition of the solutions from liquid to solid, little effect on the viability of the suspended cells was observed. Similarly, cells suspended in saline solutions containing up to 20% NaCl showed little decline in viability in the absence of freezing. This suggested that freezing and subsequent phase-transition crystallization of saline solutions was required before injury would be manifested in suspended cells.

Besides solution effects and intracellular freezing, Mazur has speculated that the recrystallization of small intracellular ice crystals to larger ones during the thawing process also contributes to freezing injury.

Repair of Injury in Sublethally Stressed Cells

In defining metabolic injury as an increased nutritional requirement after freezing, previous investigators chose a minimal plating medium which would not support the growth of a proportion of a

frozen and thawed population (Straka and Stokes, 1959; Moss and Speck, 1966a; Kocka and Bretz, 1969; Kuo and MacLeod, 1969). This injured proportion of the population was supported by an enriched plating medium. Repair of the metabolic injury was effected in the enriched medium and resulted in an enhanced colony count. Injury has also been defined by an increased susceptibility to a concentration of salt, or a surface-active agent such as deoxycholate, which is otherwise ineffective. When a sublethally stressed population is added to an enriched plating medium containing such an otherwise innocuous agent, the count is depressed, thus revealing the proportion of the population which has been injured. By incubating an injured population in a suitable environment before plating, it has been possible to assess the nature of the injury and note recovery of the population by subsequent plating in an enriched medium with and without a selective agent to which injured cells are sensitive. This technique has been employed to study damage resulting from a variety of sublethal stresses including heat (Iandolo and Ordal, 1966), freeze-drying (Sinskey and Silverman, 1970; Ray, Jezeski, and Busta, 1971), and freezing (Ray, Janssen and Busta, 1972; Ray and Speck, 1972a, b).

Iandolo and Ordal (1966) exposed *Staphylococcus aureus* to sublethal heat stress and found an increased sensitivity to 7.5% NaCl in TSA plating medium. The heated menstruum contained K^+, free ribonucleotides, and amino acids, and this suggested an alteration of membrane permeability of the stressed cells. Incubation in a solution containing

glucose, amino acids and phosphate resulted in a return of salt tolerance during an extended lag period. The recovery was not affected by chloramphenicol but was completely inhibited by actinomycin D. The authors suggested that recovery from heat damage involved membrane repair followed by RNA synthesis and subsequent growth. This was substantiated by the rapid incorporation of labelled amino acids into RNA at a rate parallel to the return of salt tolerance. The fact that energy-requiring reactions were occurring in spite of the presence of 2,4-dinitrophenol was explained on the basis of substrate phosphorylation of glucose. The recovery of some enzymatic activity in the presence of chloramphenicol was thought to be a result of renaturation of heat-labile protein and not *de novo* synthesis. This view was substantiated by Allwood and Russell (1968) who demonstrated considerable RNA degradation and increased permeability in *Staphylococcus aureus* after sublethal heat treatment. In a continuing study of heat injury of *Staphylococcus aureus*, Sogin and Ordal (1967) demonstrated the site of lesion in RNA degradation to be ribosomal RNA. Rosenthal and Iandolo (1970) found that the 30s subunit of heated cells appeared to be selectively attacked. Analysis of RNA showed that 16s RNA was destroyed and that the secondary structure of 23s RNA was altered. Metabolic studies demonstrated that sublethal heat affected the glycolytic pathway of *Staphylococcus aureus* (Bluhm and Ordal, 1969) and severely inactivated TCA cycle dehydrogenases (Tomlins, Pierson and Ordal, 1971). In general, however, the overall metabolic activities of this organism were not severely affected.

In a study with a Gram-negative organism, techniques similar to those described above were employed to determine the effect of sublethal heat on a population of *Salmonella typhimurium* (Tomlins and Ordal, 1971). Heat-treated cells were plated on Levine Eosin Methylene Blue agar (EMB) with 2.0% NaCl or TSA + 0.25% sodium citrate. The difference in count represented the injured population. Treated cells incubated in a citrate-salts medium before plating demonstrated an increasing tolerance to EMB-NaCl plating medium and after four hours counts on both plates were the same (from an initial difference of about 15 fold). After four hours, the lag ended and cells multiplied in citrate-salts medium. Recovery was inhibited by 2,4-dinitrophenol indicating an energy requirement which only citrate could supply, and recovery was inhibited by chloramphenicol, in contrast to *Staphylococcus aureus*. There was little incorporation of labelled thymidine into recovering cells but labelled uracil was incorporated at a rapid rate in the first three hours after which time it plateaued. After three hours there was an incorporation of labelled leucine into protein. The later formation of protein was attributed to rRNA newly synthesized in the first three hours of recovery. This step was assumed to be inhibited by chloramphenicol.

By fractionation of rRNA, Tomlins and Ordal (1971) demonstrated that sublethal heat treatment of *Salmonella typhimurium* totally degraded the 16s RNA species and partially degraded the 23s species. Sucrose gradient analysis demonstrated that after heat injury, the 30s ribosomal

subunit was destroyed and the sedimentation coefficient of the 50s particle was decreased to 47s. During the recovery of thermally injured cells of *Salmonella typhimurium*, a steady-state accumulation of ribosomal precursor particles was observed. Recovering cells were initially unable to synthesize protein due to the depletion of 30s ribosomes during thermal injury. Chloramphenicol was found to effectively inhibit the maturation of 30s subunits and, unlike *Staphylococcus aureus*, the rate-limiting step in the recovery of *Salmonella typhimurium* from thermal injury was the protein synthesis involved in the maturation of the newly synthesized 30s subunits. In contrast, thermally injured *Staphylococcus aureus* employed preexisting ribosomal proteins in the maturation of rRNA and consequently, chloramphenicol was ineffective in inhibiting recovery.

Tomlins, Vaaler and Ordal (1972) reasoned that since cellular integrity was altered by heat treatment, it was possible that lipid synthesis occurred early during the recovery period as a competent membrane would be required for the concentration of essential intracellular pools required for other biosynthetic processes. The lipoprotein-lipopolysaccharide envelope in *Salmonella* contains the bulk of the cellular lipid and it was determined by isotope dilution assay that 26.6% of the lipid present in recovered cells of *Salmonella typhimurium* was synthesized during the recovery process. Since the heating menstruum yielded almost no lipid at all, disruption of the membrane during injury could have occurred without signification release of lipid.

The concentrations of phosphatidyl ethanolamine and phosphatidyl glycerol synthesized during growth or repair were similar; however, the amount of cardiolipin synthesized during recovery was greater than that obtained from normal cells. The fatty acid species synthesized during recovery were qualitatively similar but quantitatively dissimilar to the fatty acid profile of normal cells. The decreased concentrations of cyclopropane fatty acids with concomitant increases in their parent monoenoic acids gave presumptive evidence for the partial inactivation during injury of the enzyme cyclopropane fatty acid synthetase.

Sinskey and Silverman (1970) characterized injury incurred by *Escherichia coli* upon freeze-drying by means of enriched and minimal plating media and a selective plating medium containing deoxycholate. By adding gelatin to the freezing menstruum and controlling the freezing rate, injury as a result of freezing was eliminated and the majority of the injury demonstrated was attributed to the freeze-drying operation. An altered permeability was demonstrated by sensitivity to deoxycholate, actinomycin D and leakage of RNA. Rehydration and incubation of freeze-dried cells in a minimal glycerol-salts medium for five hours demonstrated that the cell population became insensitive to actinomycin D, regained a capacity to concentrate ^{14}C-labelled methylthiogalactoside, and began to incorporate ^{3}H-labelled uracil. After an additional 2.5 hours of incubation, protein synthesis began as measured by ^{14}C-labelled leucine incorporation. DNA synthesis, as measured by thymidine uptake, occurred

approximately 100 minutes after protein synthesis was initiated. In control cells, the same sequence of events occurred but the intervals of time between synthesis of RNA, protein and DNA were shorter.

Analysis of ribosomal extracts of rehydrated freeze-dried cells demonstrated the presence of appreciable degradation products. Freeze-dried cells had a lower concentration of 70s ribosomes, and the 50s and 30s fractions were present in reduced amounts. It was concluded that, in the first five hours of incubation, the lost RNA was replaced and damaged ribosomes were repaired. Synthesis of RNA and replenishment of the amino acid pools presumably took place before repair and resumption of growth.

In a similar study with freeze-dried *Salmonella anatum*, Ray, Jezeski and Busta (1971) found somewhat conflicting results to those of Sinskey and Silverman (1970). Repair of injury induced by freeze-drying in non-fat milk solids occurred rapidly after rehydration. Injury in surviving cells was defined as the inability to form colonies on a plating medium containing deoxycholate. Recovery of this ability occurred after rehydration and incubation in water for two hours. Recovery was reduced by lowering the temperature of incubation but appeared to be unaffected by the addition of actinomycin D or chloramphenicol to the recovery medium. This suggested to the authors that neither RNA or protein synthesis were involved in the recovery process. They concluded that differences in the composition of test solutions,

methods of determination of injury, and test organisms employed could account for the results of Sinskey and Silverman. Total inhibition of repair of injury by DNP and partial inhibition by CN^-, together with the temperature dependency of the repair rate, suggested that the recovery process was dependent upon the formation of ATP. When fresh cells of *Salmonella anatum* were treated with EDTA, the injury incurred was neutralized by the addition of Ca^{++}. This repair was also retarded by DNP. This observation, together with the sensitivity of freeze-dried cells to deoxycholate and dodecyl sulphate, led the authors to conclude that recovery of freeze-dried *Salmonella anatum* was an energy-dependent repair of the lipopolysaccharide component of the cell wall. This was in apparent agreement with the results of Bretz and Kocka (1967).

Ray, Janssen and Busta (1972) investigated the repair of injury induced by freezing and thawing *Salmonella anatum*. Fast freezing and slow thawing of cells suspended in water resulted in injury of more than 90% of the cells that survived the treatment. Injury of cells was defined as an inability to grow and form colonies on a selective medium consisting of xylose-lysine-peptone agar containing 0.2% deoxycholate, but capable of growth on this medium in the absence of deoxycholate. Incubation of a frozen and thawed population of cells in a minimal glucose-salts broth resulted in recovery of tolerance to deoxycholate plating medium within two hours. They further observed that nearly the same degree of recovery was achieved by using only potassium phosphate as the recovery medium at the same concentration (0.25%) as that used in

the minimal glucose-salts medium. The addition of 0.01% $MgSO_4$ to the phosphate recovery medium increased the extent of repair. The repair process in the presence of phosphate was not prevented by actinomycin D, chloramphenicol, or D-cycloserine, but was prevented by cyanide and 2,4-dinitrophenol. The greatest degree of recovery occurred at pH 8.0 and was substantially reduced at pH 5.0. It was concluded that the repair process might involve energy metabolism in the form of ATP but that RNA, protein and mucopeptide synthesis were not involved. Frozen and thawed cells of *Salmonella anatum* demonstrated a susceptibility to lysozyme which indicated to the authors that alteration of the lipopolysaccharide layer of the cell envelope had occurred. They proposed that phosphate-mediated recovery of damaged cells involved an ATP-dependent repair of the LPS layer and the enhancement by Mg^{++} resulted from stabilization of the cross-linkages of the LPS layer.

Ray and Speck (1972a) investigated the repair of injury induced by rapidly freezing and thawing *Escherichia coli* in distilled water. Inability of the frozen and thawed cells to form colonies on Trypticase soy agar containing 0.3% yeast extract was interpreted as death. The difference in count between this medium and the same medium supplemented with 0.1% deoxycholate was considered to be the proportion of the population which had been injured. Frozen and thawed cells incubated in a minimal glucose-salts broth at pH 7.0 before surface-plating demonstrated recovery of tolerance to deoxycholate in the plating medium within two

hours. Incubation in K_2HPO_4 (0.5%) or pyruvate (0.1%) demonstrated about half this level of recovery. Incubation in K_2HPO_4 + Mg^{++} resulted in a level of recovery equal to that seen with the minimal glucose-salts broth. No recovery was seen in the presence of glucose alone. In the presence of PO_4^{-3} alone, optimum recovery occurred at a pH of 8.5 and no recovery was seen at pH 5.5. The recovery of cells in PO_4^{-3} at pH 7.0 was temperature-dependent with no recovery occurring below 15°C and optimal recovery occurred in a temperature range between 25°C and 35°C. The authors concluded that phosphate was required for some essential enzymatic acitivity, and together with pyruvate, could be utilized by enzyme(s) for the synthesis of energy-rich compounds which might be required for the repair process. Mg^{++} might be necessary for the activity of enzymes involved in the repair process or for maintaining stability of the cell wall, or both. The inability of glucose to facilitate repair was attributed to damage of some components of the glucose transport system during the freezing process.

In a subsequent paper, Ray and Speck (1972b) further characterized the PO_4^{-3} repair mechanism with specific inhibitor studies. Repair of freeze-injured *Escherichia coli* cells incubated in 0.5% K_2HPO_4 at pH 7.0 and 25°C was unaffected by the addition of chloramphenicol, actinomycin D, penicillin, D-cycloserine or hydroxyurea. They concluded, therefore, that the repair mechanism might not involve the synthesis of protein, RNA, mucopeptide or DNA. Phosphate repair was inhibited by the addition of

DNP and cyanide and incubation of freeze-injured cells in 5.0 mM ATP resulted in repair of about half of the population of injured cells. Incubation of freeze-injured cells in lysozyme, sodium lauryl sulphate or sodium deoxycholate resulted in the loss of about half of the injured population, and with lysozyme, lysis of the population was evidenced by a loss of turbidity of the suspended cells. Incubation of freeze-injured cells in the phosphate recovery medium for one hour before exposure to these agents demonstrated a greatly reduced susceptibility. It was concluded that in the freeze-injured cells, the lipopolysaccharide layer probably undergoes some kind of change and allows lysozyme and the surface-active agents to come in contact with their respective substrates. This change in freeze-injured cells was reversible and could be repaired in K_2HPO_4, probably through the synthesis and utilization of ATP. Mg^{++}, it was felt, probably accelerated this process.

Involvement of Mg^{++} in the Integrity of Stressed Cells

Although the precise structural role of Mg^{++} remains a matter of speculation, its involvement in the envelopes, ribosomes and enzyme complexes of various Gram-negative and Gram-positive cells has been well documented. Weibull (1956) first noted that the integrity of *Bacillus megaterium* protoplasts was dependent on the presence of Mg^{++} ions. Recently, Wisdom and Welker (1973) have found a similar result with *Bacillus stearothermophilus*.

Eagon, Simmons and Carson (1965b) demonstrated the presence of Mg^{++} and Ca^{++} in the cell wall fraction of *Pseudomonas aeruginosa*. In previous work, (Eagon and Carson, 1965a), the ability of the chelating agent, EDTA, to lyse whole cells of *Pseudomonas aeruginosa* was established. The authors suggested that divalent cations formed cross-linkages between the mucopeptide and non-mucopeptide components of the cell wall and could be disrupted by chelation. McClure (1967) fractionated the envelope of a *Halobacterium* species and found a 1:1 ratio of Mg^{++} to the major lipid of the cytoplasmic membrane, the diether analogue of phosphatidyl glycerophosphate. He proposed a tentative model in which Mg^{++} was firmly bound between lipid and protein. In it, he suggested that a tetradentate inter-molecular chelate existed between groups on the protein, magnesium and lipid head groups. Gordon and MacLeod (1966) extracted lipids from the cell envelopes of *Pseudomonas aeruginosa* and a marine pseudomonad and found Mg^{++} associated with diphosphatidyl glycerol. They suggested that a bidentate chelate complex might be involved. In determining the amount of Mg^{++} present in native and reaggregated membranes of Mycoplasma, Kahane, Ne'eman and Razin (1973) found that practically all the membrane-bound Mg^{++} in *Acholeplasma laidlawii* remained so bound after the removal of over 80% of the protein by Pronase. They suggested that this might be due to the high proportion of phosphatidyl glycerol contained in the membranes. EDTA released the bulk of Mg^{++} bound to native and reaggregated membranes except for a small but fairly constant amount which was too tightly bound and unavailable

for chelation.

Forsberg, Costerton and MacLeod (1970) found that successive washings of a marine pseudomonad with 0.5 M NaCl released a loosely-bound outer layer by displacing the Mg^{++} present. They suggested that the components of this outer layer were cross-linked to each other and to the underlying layer by Mg^{++} bridges. Subsequent fragmentation of the outer double-track structure of the cell wall by successive washing in 0.5 M sucrose was hastened if the cells had been previously washed with 0.5 M NaCl. Again, it was suggested that the displacement of Mg^{++} from this layer increased its fragility on subsequent suspension of the cells in the absence of salts. Strange and Shon (1964) found that the washing of *Aerobacter aerogenes* in 0.85% NaCl resulted in a loss of adsorbed Mg^{++} on the surface of the cells and increased the death-rate of cells at 47°C. Addition of Mg^{++} to the heating diluent reversed this result. Tempest and Strange (1966) determined that up to 26% of the Mg^{++} of *Aerobacter aerogenes* was loosely adsorbed to the surface of the cells. Surface-adsorbed Mg^{++} stimulated polysaccharide synthesis and increased the resistance of bacteria to stresses including starvation, heat-accelerated and substrate-accelerated death, and cold shock.

More recently, Sato, Suzuki, Izaki and Takahashi (1971), without reference to the previous work of Strange, Shon and Tempest, have described a saline-sensitive phenomenon in *Escherichia coli*. When cells at the logarithmic phase of growth were washed in buffer and suspended

in 0.15 M NaCl buffer for a period of 15 minutes, 90% of the population were found to have lost their colony-forming ability on nutrient agar. Similar results were obtained when cells were suspended in LiCl, $Na_2SO_4 \cdot 10H_2O$, K_2SO_4 or KCl. The addition of $5x10^{-2}$ M magnesium acetate or magnesium sulphate to the suspending medium protected the cells and allowed almost 100% viability on the plating medium. It was determined that a five minute incubation in $5x10^{-2}$ M Mg^{++}, after suspension in buffered saline for sixty minutes, resulted in a complete return of viability. Some recovery was noted with Ca^{++} and Mn^{++}, but to a much lesser extent. Leakage of Mg^{++} from saline-treated cells was about three times more than the untreated cells. This suggested that the lethal effect of the sodium chloride treatment was related to the loss of Mg^{++} from the treated cells. In a subsequent paper, Sato, Izaki and Takahashi (1972) demonstrated that the Mg^{++}-mediated recovery from saline treatment was not inhibited by either chloramphenicol, 2,4-dinitrophenol or sodium azide. UV-absorbing materials (260 nm) were released progressively during incubation in NaCl, suggesting an injury to the membrane. When Mg^{++} was included in the incubation medium, the release of UV-absorbing materials was suppressed almost completely. This might indicate protection of the membrane from the injurious effect of NaCl. Although no analysis of released material was made by the authors, Tempest and Strange (1966) noted a similar release of material, particularly an amino acid fraction which they suggested came from the soluble amino acid pool of the cells.

Scheirer and Gerhardt (1973) noted that the presence of 0.01 M magnesium chloride caused a relatively small but significant reduction in the equivalent porosity of the protoplast membrane, but not in that of the cell wall, in intact *Bacillus megaterium* cells. Brock (1962) noted that Mg^{++} starvation in growing cells of *Escherichia coli* resulted in a marked filamentation of the cells, a loss in ability to stain with methylene blue, a drop in viability, and a marked increase in ability of whole cells to hydrolyze a galactoside for which the strain lacked a transport mechanism. These observations suggested an instability of the membrane resulting from a Mg^{++} deficiency. Fiil and Branton (1969) determined that a culture of *Escherichia coli* remained viable after twelve hours in a Mg^{++}-free medium but after 24 hours, only 2% of the population retained the ability to form colonies on an agar medium. The freeze-etching technique demonstrated that the membrane of Mg^{++}-supplemented, exponentially growing cells demonstrated a uniform pattern of particles in or on the membrane. The precise location of these particles within the membrane structure could not be ascertained because of uncertainty as to the preferred plane of fracture during cleavage. The function and composition of these particles remain unknown but it has been speculated that they are protein in nature and represent multi-enzyme complexes or possibly specialized sites associated with transport functions (Bayer and Remsen, 1970).

Fiil and Branton found that when exponentially growing cells

were transferred to a Mg^{++}-deficient medium, a paracrystalline pattern appeared on the membranes within three hours and covered increasingly large areas with prolonged starvation. Within twelve hours after the removal of magnesium, infoldings of the membranes and large areas devoid of particles appeared. This response of cells to Mg^{++} deficiency was irreversible and could not be observed when Ca^{++} was deficient. The authors speculated that Mg^{++} deprivation affected membrane structure indirectly, via other perturbances in cell metabolism, e.g. ribosome breakdown, since no decrease in the Mg^{++} content of the cell envelope *per se* was detected, even after 24 hours of Mg^{++} deprivation.

Mg^{++} has been demonstrated by several workers to prevent or repair the deleterious effects of cold shock. Farrell and Rose (1968) compared the effects of cold shock on a mesophile and a psychrophile. Cold shock in the psychrophile appeared to be similar in many respects to cold shock in the mesophile, particularly with regard to the kinetics of the process and the ability of Mg^{++} to protect the bacteria against loss of viability. Cells of both types were grown at 30°C, harvested and washed in 30 mM NaCl at 30°C. Dilution of these suspensions were rapidly chilled to -2°C and incubated at that temperature for 50 minutes after which approximately 50% of both types remained viable. The addition of 5.0 mM Mg^{++} to the incubating medium at -2°C resulted in 100% viability in both cases after fifty minutes. No correlation was found between loss of viability and leakage of UV-absorbing materials and

it was noted that the amounts of UV-absorbing compounds released during washing amounted to about 50% of those that remained in the psychrophile and about 75% of the amounts retained in the mesophile. The effect of Mg^{++} upon the release of UV-absorbing compounds was not reported, but the results obtained with saline washing appeared to be in agreement with those of Strange and Shon (1964).

Farrell and Rose noted that neither the psychrophile nor the mesophile, when grown at 10°C, showed a measurable decrease in viability when dilute suspensions were rapidly chilled to -2°C. This indicated that growth of the bacteria at this temperature caused certain changes in composition of the organisms such that they became insensitive to cold shock. The authors proposed that an increased content of double bonds in the fatty acids of the membrane phospholipids resulted in a decreased melting point of these lipid chains. It was suggested that upon freezing, channels were formed in the membrane which allowed the exit of intracellular low molecular weight solutes — a situation retarded by the presence of unsaturated fatty acids in the phospholipids. Since no correlation could be drawn between loss of viability and leakage of UV-absorbing compounds, it was postulated that loss of other intracellular solutes was more directly responsible for death of the organisms. Leder (1972), investigating cold shock of *Escherichia coli*, similarly speculated that it may be essential for the proper functioning of the cell membrane as a permeability barrier that the fatty acid chains be in a liquid-like state.

By causing rapid crystallization of the membranes through chilling, sudden isoosmotic shock would create hydrophilic channels, facilitating the escape of intracellular solutes.

Sato and Takahashi (1968) found that exponentially-growing *Escherichia coli* cells suffered 80-90% loss of viability when cold-shocked to 3°C in 10^{-2} M Tris buffer. The addition of 10^{-2} M Mg^{++} to the suspending medium protected the cells from cold shock. The addition of 10^{-1} M Mg^{++} two minutes after cold-shocking resulted in a rapid recovery of viability (up to 100%), but upon further incubation, the viability declined, possibly as a result of the high level of Mg^{++}. The possibility of Mg^{++}-mediated recovery gradually decreased with incubation of shocked cells in chilled buffer in the absence of Mg^{++} ion. After an incubation for 120 minutes, no significant increase in viability was observed by the addition of Mg^{++}. Loss of UV-absorbing material from shocked cells was not affected by the subsequent addition of Mg^{++}. Slow chilling of the cells in Tris buffer alone did not affect the viability — an observation in agreement with Leder's postulation. Sato and Takahashi (1969) reported that logarithmically-growing cells of *Escherichia coli*, cold-shocked by rapid chilling to 3°C in Tris buffer, resulted in about 60% loss of viability when measured in terms of colony formation on plates. The addition of Mg^{++} to the suspending medium before shocking prevented loss of viability and its addition ($5x10^{-3}$M) after shocking resulted in recovery of viability after 15 minutes at 30°C. Increased permeability

of shocked cells was determined by penetration of a fluorescent dye. Shocked cells demonstrated seven times as much fluorescence as untreated control cells. The authors concluded that the shocked cells lost their viability as a result of leakage of intracellular bivalent cations, at least in *Escherichia coli*. However, an involvement of another mechanism for the death of the cells was evident, since the ability of *Escherichia coli* to recover from cold shock gradually decreased on incubation in cold Mg^{++}-free Tris buffer. Mg^{++}-mediated recovery was inhibited by 2,4-dinitrophenol but not chloramphenicol, therefore suggesting an energy requirement but not protein synthesis. The inhibitory effect of DNP was overcome by NAD or ATP + nicotinamide but these agents were ineffective in the absence of Mg^{++}. The authors implicated DNA ligase as one of a few known enzymes requiring NAD as an energy source. In a subsequent paper referred to previously in this review (Sato and Takahashi, 1970), the authors suggested that logarithmically growing cells contain a number of nicks in the DNA strands which are normally joined by DNA ligase. In shocked cells, the absence of Mg^{++} as a required cofactor made DNA ligase inoperative.

MATERIALS AND METHODS

MATERIALS AND METHODS

Test Organism

The organism used throughout this investigation was *Escherichia coli* 451B (Mac 614). Cultures were initially lyophilized, and tubes were opened at various intervals throughout the course of the investigation. Slants of the organism were maintained on Trypticase soy agar (BBL) and were transferred weekly. After 3-4 weeks, a new lyophilized tube was opened.

Water Supply

Water distilled in a copper-lined Barnstead still was redistilled in a Corning AG-2 all-glass still. This water was used for the preparation of media and diluents, and the rinsing of glassware.

Preparation of Glassware

Where defined media or diluents were employed, glassware was first soaked in chromic acid, then in a concentrated sulphuric-nitric acid mixture (2:1) after which it was rinsed in tap, then glass-distilled water.

Preparation of Cells for Freezing

Using a modified procedure from Kuo (1969), cells were obtained from a 24-hour Trypticase soy agar slant and inoculated into 10 ml of Trypticase soy broth in a 50 ml flask. The broth contained 1.5% Trypticase (BBL), 0.5% Phytone (BBL), and 0.5% NaCl (Fisher Scientific). After 8 hours at 37°C, the contents of the flask were transferred to a 2.0 litre flask containing 250 ml of Trypticase soy broth at 37°C. The flask was then incubated for 15 hours. When the culture had entered the stationary phase at 15 hours, the culture was transferred to a 250 ml polypropylene centrifuge bottle and centrifuged at 9000 x g for five minutes in a Sorval RC2B refrigerated centrifuge at 4°C. The supernatant was decanted and the pellet washed and centrifuged three times with cold 0.85% NaCl. After the third centrifuging, the cells were resuspended to 50% transmission at 650 nm in a Coleman Jr. colourimeter and two ml of this suspension were added to 98 ml of 0.85% saline in a screwcap bottle. This dilution yielded approximately $2x10^7$ cells/ml. Where fresh cells were required for experimentation, 10 ml of this suspension were diluted to 100 ml in 0.85% saline and 0.5 ml of this dilution were employed immediately as an inoculum for the recovery media.

Procedure for Freezing and Thawing of Cells

Using a modified procedure from Kuo (1969), six ml aliquots of a $2x10^7$ cells/ml suspension were added to capped polypropylene tubes

(75x15 mm) and frozen to -20°C in a deep freeze. After 22-24 hours, a tube was removed, immersed in 175 ml of H_2O at 20°C in a 250 ml beaker and allowed to thaw. As the last trace of ice disappeared, the tube was agitated and 0.5 ml aliquots were immediately withdrawn to be used as inocula for the recovery media.

Recovery Media

The term 'recovery medium' was used to describe a 99.5 ml volume of nutrients contained in a 4 ounce screwcap bottle. Throughout the course of this study, all recovery media were dispensed in a similar fashion.

1) PKM recovery medium

This medium contained 0.005 M K_2SO_4, 0.01 M NaH_2PO_4, and 0.001 M $MgSO_4 \cdot 7H_2O$ to give the following final concentrations:

0.01 M PO_4^{-3}

0.01 M K^+

0.001 M Mg^{++}

The medium was prepared by mixing 0.348 g K_2SO_4 and 0.552 g NaH_2PO_4 in glass-distilled water. The solution was adjusted to pH 7.0 with 0.1 M NaOH and diluted to 200 ml, after which it was dispensed into two four ounce screwcap bottles. After autoclaving, a 50 ml aliquot of the solution was aseptically diluted to 100 ml with sterile 0.002 M $MgSO_4 \cdot 7H_2O$ to yield the PKM recovery medium.

Where other ions were substituted or added, the solutions were prepared in a similar fashion, using equimolar amounts of various ions. In the case of other divalent cations, the pH of the solution was adjusted to 6.5 before autoclaving.

Where heat-labile supplements were added, the PKM recovery medium was prepared and filter-sterilized with a 0.22μ Millipore filter (25 mm dia.).

Where other supplements were employed in addition to phosphate, magnesium and potassium, (e.g. aspartate or glucose) appropriate additions were made to a 0.002 M $MgSO_4$ solution, the pH was adjusted to 7.0, and the solution autoclaved before diluting with an equal volume of double-strength phosphate-potassium solution described above.

2) AKM recovery medium

In the first experiments, this medium contained 0.005 M aspartate (disodium salt - Calbiochem) and 0.001 M $MgSO_4 \cdot 7\ H_2O$ and was adjusted with KOH to pH 7.0. In later experiments, the medium was prepared by adding 0.134 g of aspartate and 0.174 g of K_2SO_4 to a solution of 0.001 M $MgSO_4 \cdot 7H_2O$. The solution was adjusted to a pH of 7.0 with NaOH, diluted to 200 ml with 0.001 M $MgSO_4 \cdot 7H_2O$, and autoclaved. This gave the following final concentrations:

0.005 M aspartate

0.01 M K^+

0.001 M Mg^{++}

Where other substances were substituted for aspartate, equimolar amounts were employed and the solutions prepared in a similar fashion.

3) Minimal recovery medium

The minimal recovery medium employed was the defined medium of Straka and Stokes (1959) and consisted (per litre) of:

basal salts;		
	K_2HPO_4	7.0 g
	KH_2PO_4	3.0 g
	$(NH_4)_2SO_4$	1.0 g
	sodium citrate	0.1 g
	$MgSO_4 \cdot 7H_2O$	0.1 g
glucose		2.0 g

The minimal medium was prepared by dilution of a ten times concentrated stock solution of the basal salts to double strength, and 100 ml aliquots were autoclaved in 100 ml screwcap bottles. Double strength glucose ±0.01 M aspartate, where required, was prepared in a similar fashion, with the pH adjusted to 7.0 with 1.0 M KOH. After cooling, 50 ml aliquots of glucose with and without aspartate were aseptically diluted to 100 ml with the double strength basal salts solution. Recovery media prepared in this manner were refrigerated until used.

Plating Media

1) Trypticase soy agar (TSA) plating medium

This medium, containing 5% Trypticase, 5% Phytone, 1.5% agar (all BBL products) and 0.5% NaCl was dissolved, dispensed in 10 ml aliquots into clean test tubes, autoclaved, and refrigerated until used. Before use, the tubed media were liquefied in an autoclave and cooled to 45°C.

2) Trypticase soy agar + salt (TSAS) plating medium

This medium was prepared in the same manner as TSA, but in suspending the medium, sufficient 3 M NaCl was added to ensure a final concentration of 0.55 M NaCl in the plating medium.

3) Minimal plating medium

The minimal plating medium employed consisted of the minimal recovery medium with 1.5% agar added. The medium was prepared by dilution of a ten times stock solution of the basal salts to double strength. This was dispensed in 5 ml aliquots into acid-washed test tubes, autoclaved, and refrigerated until used. A double strength glucose-agar mixture was prepared in a similar fashion.

Where 0.005 M aspartate was employed in the final medium, 0.665 g of sodium aspartate was added to the double strength glucose-agar solution, the pH was adjusted to 7.0 with 0.1 M KOH, and the medium was dispensed as before.

Where required, sufficient 3.0 M NaCl was added to the basal salts solution to ensure a final concentration in the plating medium of 0.3 M NaCl.

When plating, the content of a tube of basal salts solution was added to a Petri dish together with the content of a tube containing the organic components of the medium. This yielded the final concentrations of the minimal plating medium.

Plating Procedure

At the beginning of an experiment, 0.5 ml of fresh or frozen and thawed inoculum was added to 99.5 [redacted] a recovery medium held in ice, and shaken vigorously. One ml aliquots were withdrawn immediately, diluted to 100 ml with 0.002 M $MgSO_4 \cdot 7H_2O$, and one ml aliquots of this dilution were added to blank Petri dishes. In this manner, the count of the recovery culture was established for zero time. The remaining volume of each recovery culture was then agitated in a reciprocating waterbath shaker held at 37°C.

The one ml inocula in the Petri dishes were diluted with the appropriate plating medium and swirled to ensure even distribution of the inoculum. One ml aliquots of the recovery culture were routinely taken at 0.5, 1.0, 2.0, 3.0, 4.5, and six hours, diluted to 100 ml with 0.002 M magnesium dilution blanks held at 37°C and plated in quadruplicate. Inocula were prepared in such a way as to ensure a count of

100-200 colonies/plate in plates of TSA or minimal medium + aspartate. After setting, the plates were incubated in an air convection incubator at 37°C for three days.

After incubation, plates were counted on a New Brunswick colony counter, the values of quadruplicate plates were summed, and mean values were expressed as log number of cells/ml of recovery culture. All values were confirmed and the standard deviation of quadruplicate plates determined by means of a computer program (see Appendix A).

Respirometry

Respirometry was performed using a Gilson differential respirometer. For these experiments, 15 ml respirometer flasks with a centre well and one sac were acid-washed and dried. Two and one half ml of the appropriate media were added to the outer area of the flask and 0.5 ml of 20% KOH were added to the centre well which contained a folded strip of Whatman's No. 1 filter paper.

The flasks were held on ice while 0.5 ml of inoculum were added to each, and the flasks were then attached to the manometers and submerged in the water bath, where they were allowed to equilibrate to 37°C for 15 minutes. The inoculum was equilibrated in the presence of the substrate to ensure that its viability did not decrease as the temperature increased as would be the case if the cells were suspended in the freezing menstruum alone. After equilibration, the manometer

valves were closed and the experiment proceeded.

In these experiments, a heavier suspension of cells than was used in the plating experiments was employed. After washing the cells, a suspension was prepared which gave 28% transmission of light at 650 nm. From this suspension, 20 ml were added to 80 ml of sterile, cold 0.85% NaCl. This suspension yielded approximately $4x10^8$ cells/ml. This suspension was diluted ten fold for experiments with fresh cells, and after freezing, was used undiluted for experiments involving frozen and thawed cells.

At the same time, a normal suspension at 50% transmission was made from the same TSB culture and diluted in the usual manner. This diluted suspension was frozen at the same time, then thawed and used as inoculum in plating experiments which were run simultaneously with the manometric measurements.

EXPERIMENTAL AND RESULTS

EXPERIMENTAL AND RESULTS

Introduction

In an earlier investigation by Kuo and MacLeod (1969) of freezing and thawing damage to *Escherichia coli* 451B, it was reported that twice as many cells of a frozen and thawed suspension formed colonies when plated on a minimal medium supplemented with aspartic acid as on the minimal plating medium alone. Various other amino acids were found to have this capacity to increase the count of a suspension of cells after freezing and thawing, but not to the same degree as aspartate. Fresh (unfrozen) cells attained the same count on both the minimal medium and the aspartate-supplemented minimal medium. Aspartate was therefore judged to have a specific role in recovery from metabolic injury due to freezing and thawing.

If aspartate were actively involved in the repair of membrane damage, it seemed likely that this repair would be effected before growth and division. To explore this possibility, frozen and thawed cells were incubated in an aspartate recovery medium prior to plating.

Recovery in an Aspartate Medium

A suspension of cells was frozen using the standard procedure,

and upon thawing, an aliquot was transferred to AKM recovery medium. Samples were removed for plating immediately and at intervals to three hours. At each interval, samples were plated on minimal medium and on minimal medium containing aspartate. The results are seen in Figure 1B.

When initially plated, about twice as many colonies appeared on the plating medium containing aspartate. On subsequent incubation in the AKM recovery medium, counts obtained in the minimal plating medium increased until they were equal to those obtained with aliquots plated in the minimal medium + aspartate. In a similar experiment conducted with fresh, unfrozen cells (Figure 1A), no difference in count could be discerned in the presence and absence of aspartate in the plating medium.

The results obtained in the zero time counts of these experiments closely paralleled those obtained by Kuo and MacLeod. By incubating the frozen and thawed suspension in the AKM medium, the initial difference in plate counts noted by Kuo and MacLeod was overcome. During this period of incubation, the counts on minimal medium + aspartate (curve 3) remained stable while at the same time, those on minimal medium (curve 4) increased until they equalled those on minimal medium + aspartate.

In contrast, aliquots taken from a suspension of fresh, unfrozen cells incubated in AKM medium did not demonstrate an initial difference in counts on minimal medium with and without aspartate.

FIGURE 1

Figure 1

A. Incubation of a suspension of fresh cells of *Escherichia coli* 451B in AKM recovery medium.

Aliquots of the incubating suspension of cells were subsequently plated in minimal agar ± 0.005 M aspartic acid.

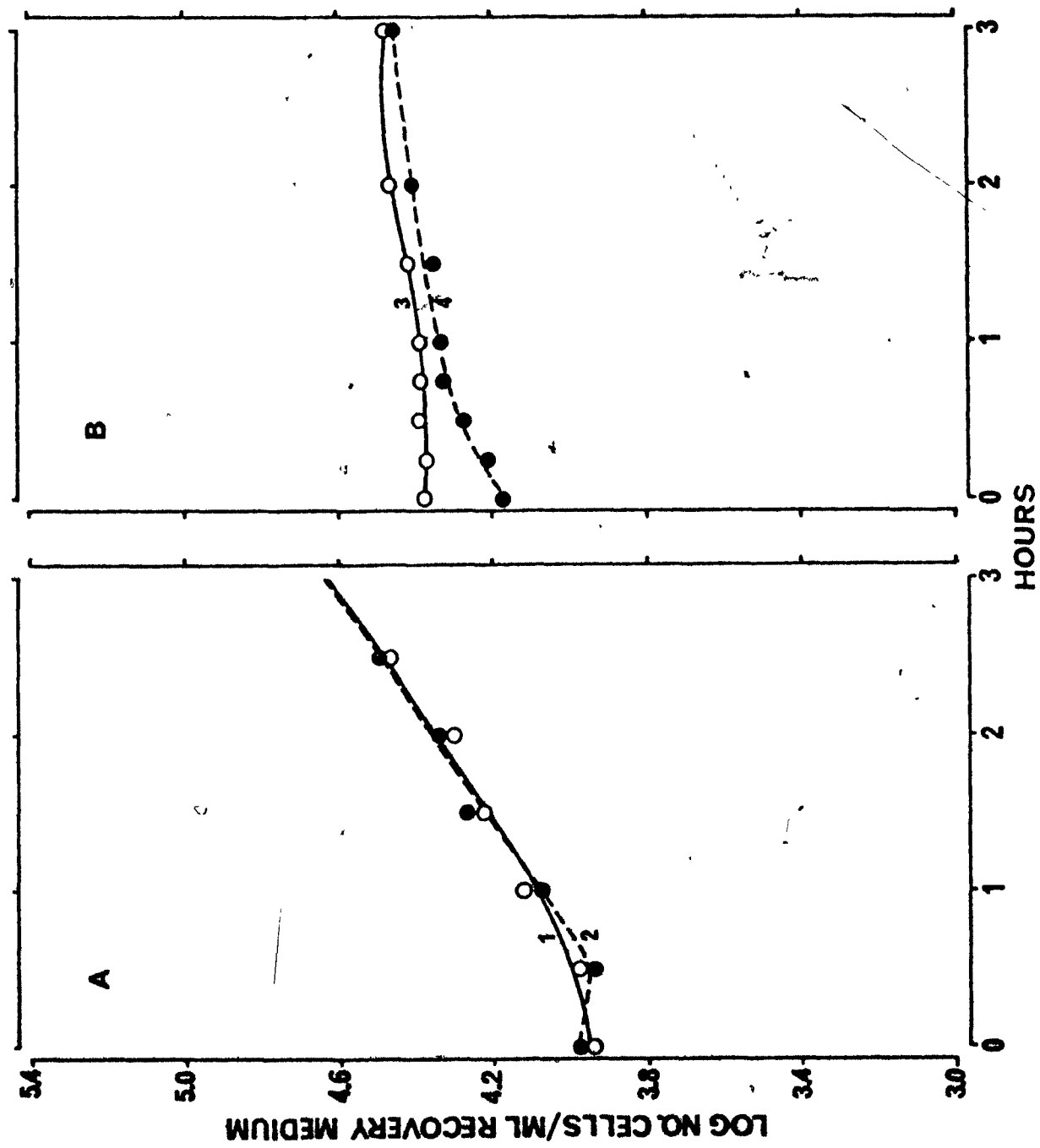
Curve 1. minimal agar + aspartate
Curve 2. minimal agar alone

B. Incubation of a suspension of frozen and thawed cells of *Escherichia coli* 451B in AKM recovery medium.

Aliquots of the incubating suspension of cells were subsequently plated in minimal agar ± 0.005 M aspartic acid.

Curve 3. minimal agar + aspartate
Curve 4. minimal agar alone

Counts of aliquots of the incubating suspensions of cells obtained on the plating media were plotted against the interval of incubation in the recovery media.


A
B
1
2
3
4
5.4
5.0
4.6
4.2
3.8
3.4
3.0
0
1
2
3
HOURS
LOG NO. CELLS/ML RECOVERY MEDIUM

After one-half hour, the counts on both plating media increased rapidly with subsequent samplings on the recovery culture.

Salt Tolerance as a Criterion of Metabolic Injury

It has been noted in previous work (MacLeod, Kuo and Gelinas, 1967) that one of the effects of freezing and thawing was increased permeability of the cell membrane. Kuo and MacLeod (1969) had noted the effectiveness of Trypticase as a supplement to the minimal medium and it was reasoned that the use of a complex plating medium would ensure the survival of a larger proportion of a suspension of frozen and thawed cells. The addition of NaCl to this medium would inhibit the growth of those cells with increased permeability and therefore serve as a criterion of metabolic damage. Following the procedure of Ordal (1966) with heat-damaged cells, Trypticase soy agar with and without NaCl was employed to enhance the difference in count of injured cells.

The most effective concentration of NaCl was determined by incubating frozen and thawed cells in the AKM recovery medium and plating aliquots of this suspension into TSA medium containing various concentrations of NaCl. The results are seen in Figure 2. The most effective concentration of NaCl was judged to be that which would initially demonstrate a substantially lower count than the TSA control plates, but which would approximate the TSA counts before the end of the lag phase in the recovery medium.

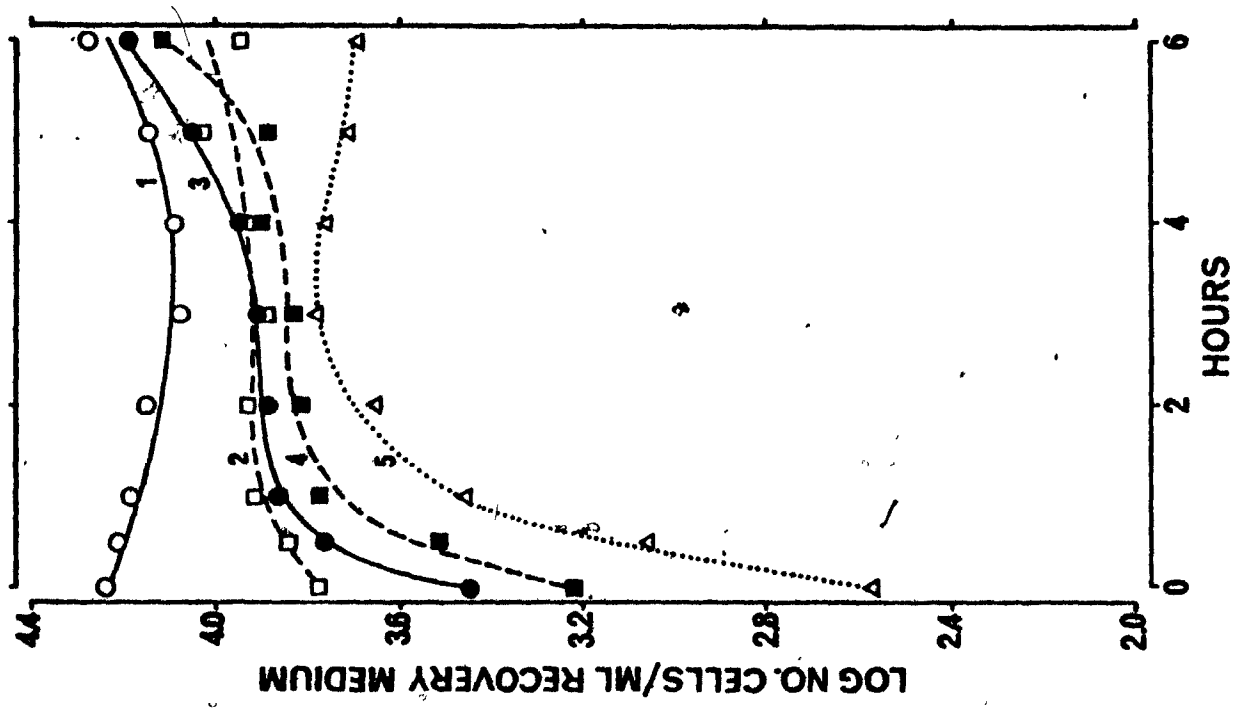
FIGURE 2

Figure 2

Effect of various concentrations of NaCl in TSA plating medium on colony formation by frozen and thawed cells.

Aliquots of suspensions of frozen and thawed cells were added to various plating media after incubation in AKM recovery medium.

Curve	Plating Medium
1	TSA
2	TSA + 0.5 M NaCl
3	TSA + 0.55 M NaCl
4	TSA + 0.6 M NaCl
5	TSA + 0.7 M NaCl


LOG NO. CELLS/ML RECOVERY MEDIUM
4.4
4.0
3.6
3.2
2.8
2.4
2.0
HOURS
0
2
4
6
1
2
3
4
5

With 0.5 M NaCl in the TSA plating medium (curve 2), counts were found to increase rapidly when frozen and thawed cells were incubated in AKM recovery medium. At 0.7 M NaCl (curve 5), counts never achieved the level of the TSA control plates up to six hours. The optimum concentration range was judged to be 0.55 to 0.6 M NaCl and these two concentrations were used in succeeding experiments. In an experiment with fresh, unfrozen cells, no difference was seen in the counts obtained on TSA or TSA + 0.55 M NaCl (TSAS) although the colonies on TSAS were somewhat smaller after three days of incubation. Incubation of frozen and thawed cells in AKM resulted in increased salt tolerance of this population and counts on the suspension were seen to increase about ten fold using TSAS as the plating medium until they approximated those on TSA. A similar result was obtained when a surface-plating procedure was employed rather than pour-plating.

Comparison of Sensitivity to Salt in TSA or Minimal Medium + Aspartate as a Criterion of Metabolic Injury

The effectiveness of TSA ± NaCl in establishing sensitivity to salt as a criterion of metabolic injury was demonstrated by comparing this medium to minimal medium + aspartate to which a final concentration of 0.3 M NaCl had been added — a concentration which had been found to be optimal for assessing injury in this medium.

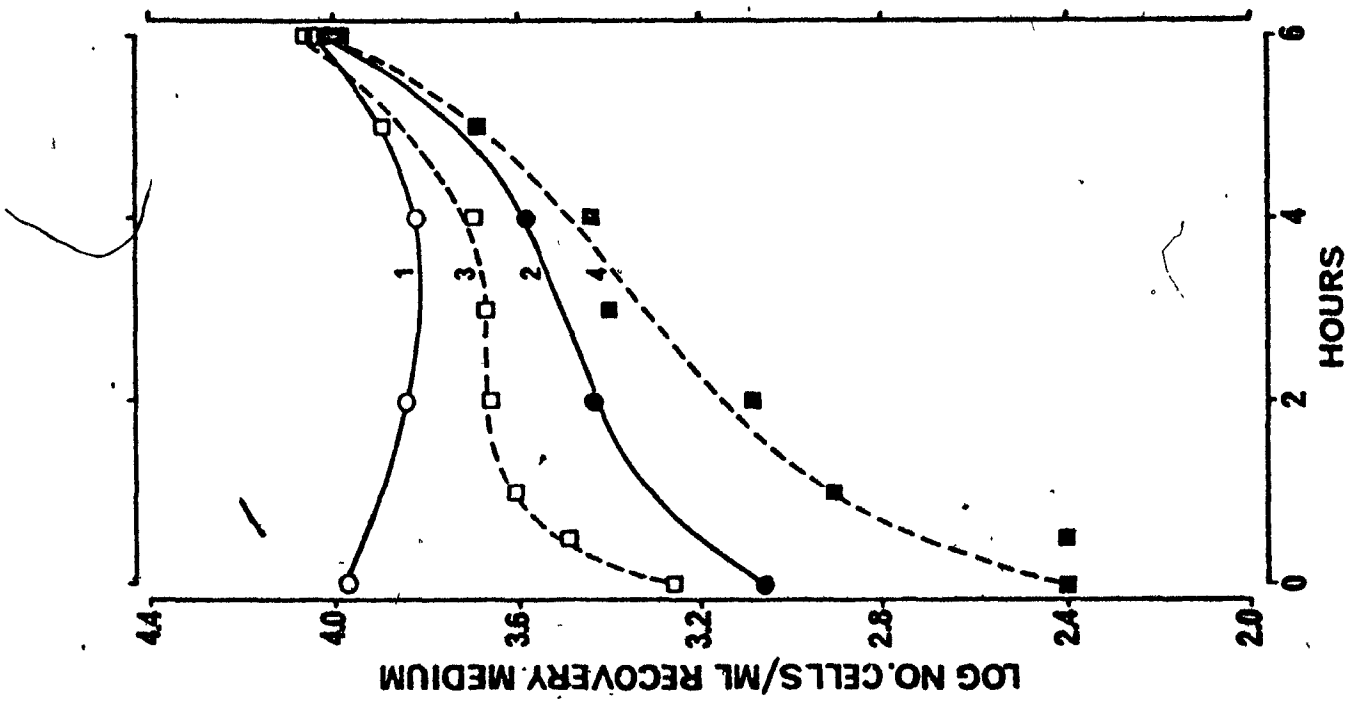
In Figure 3, the increase in salt tolerance after incubation

FIGURE 3

Figure 3

Comparison of counts obtained on TSA ± 0.55 M NaCl and minimal medium + aspartate ± 0.3 M NaCl after incubation of frozen and thawed cells in AKM recovery medium.

Curve	Plating Medium
1	TSA
2	TSA + 0.55 M NaCl (TSAS)
3	minimal medium + aspartate
4	minimal medium + aspartate + 0.3 M NaCl


LOG NO. CELLS/ML RECOVERY MEDIUM
4.4
4.0
3.6
3.2
2.8
2.4
2.0
HOURS
0
2
4
6
1
2
3
4

in AKM is seen in the counts of TSA + 0.55 M NaCl (curve 2) and minimal medium + aspartate + 0.3 M NaCl (curve 4). Although the counts on all plates were similar after six hours of incubation in the recovery medium, an initial difference was seen between TSA and minimal medium + aspartate. The increase in count seen in minimal medium + aspartate suggested an initial sensitivity to this plating medium which was overcome by preincubation in AKM recovery medium. TSA was seen to be a more stable control and to enhance the colony-forming capacity of the population in the recovery medium.

Substitution of Aspartate
in the AKM Medium by Glucose

It had been established in previous experiments that a proportion of a frozen and thawed suspension of cells were unable to form colonies in the minimal plating medium where glucose served as the sole carbon-energy source. It was of interest, therefore, to compare the effectiveness of aspartate and glucose in restoring salt tolerance to such a population. An equimolar amount of glucose was substituted for aspartate in the AKM recovery medium, and a recovery medium consisting of 0.001 M Mg^{++} alone, without a carbon source, served as a control. A frozen and thawed suspension was added to the media with subsequent plating into TSA and TSAS. The results are seen in Figure 4.

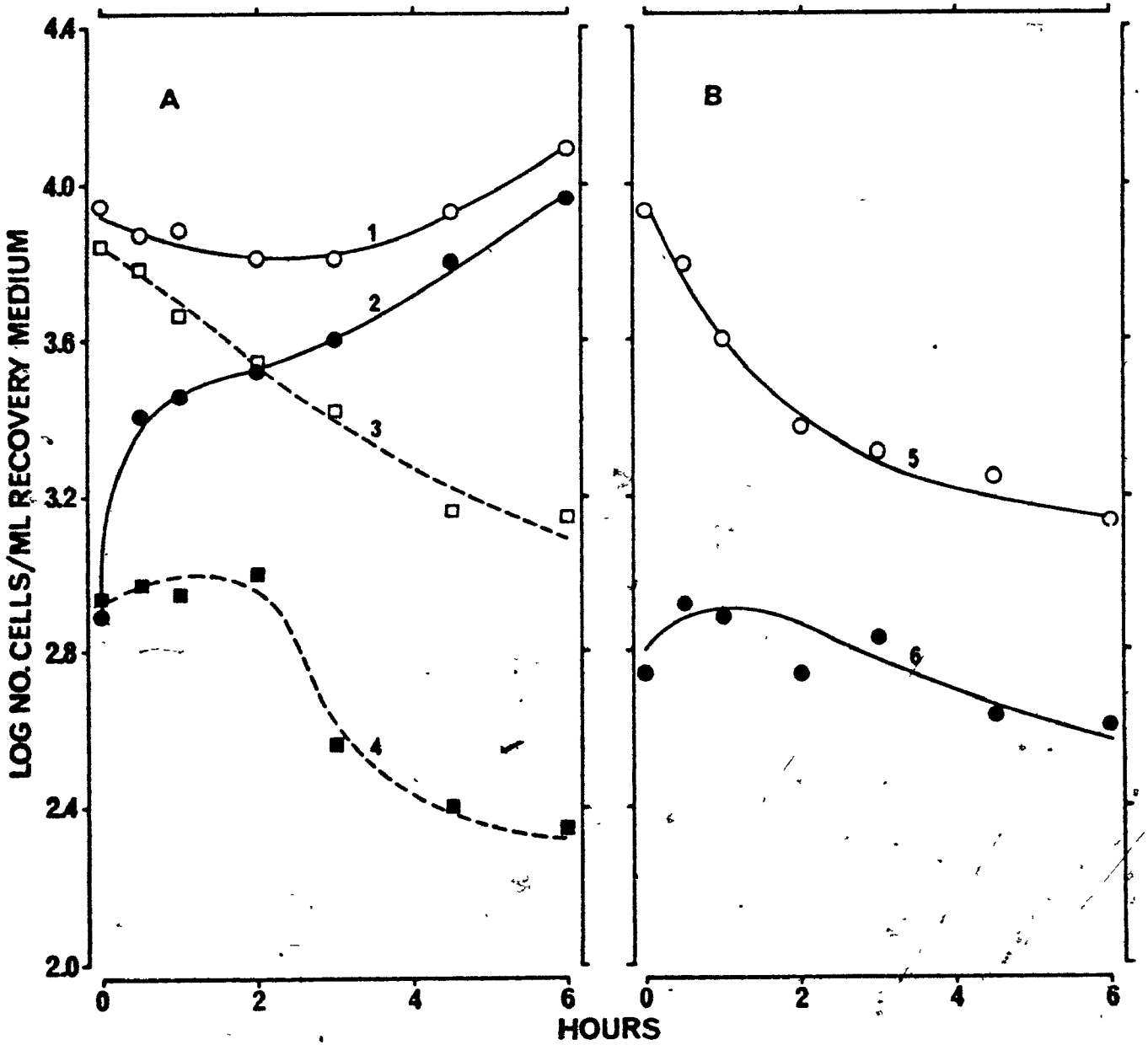
In the absence of aspartate, there was a gradual decline in the viability of the incubating population (curves 3, 5) and no

FIGURE 4

Figure 4

Effect of substitution of aspartate in the AKM recovery medium by glucose on recovery of salt tolerance by a suspension of frozen and thawed cells.

Curve	Plating Medium	Recovery Medium
1	TSA	AKM
2	TSAS	
3	TSA	0.005 M glucose + 0.01 M K_2SO_4 + 0.001 M $MgSO_4 \cdot 7H_2O$
4	TSAS	
5	TSA	0.001 M $MgSO_4 \cdot 7H_2O$
6	TSAS	


A
B
LOG NO. CELLS/ML RECOVERY MEDIUM
4.4
4.0
3.6
3.2
2.8
2.4
2.0
1
2
3
4
5
6
0
2
4
6
HOURS

appreciable degree of recovery was noted (curves 4, 6).

Substitution of Other Organic Substrates in the AKM Recovery Medium

Kuo and MacLeod (1969) had noted an enhanced plate count when the minimal plating medium was supplemented with other amino acids but not to the same degree as with aspartate. To test their effectiveness in restoring salt tolerance to a frozen and thawed population, a number of substrates were substituted for aspartate in the AKM recovery medium.

Equimolar concentrations of the sodium salts of various TCA intermediates were substituted for aspartate in the AKM recovery medium and aliquots of the recovery cultures were subsequently plated into TSA and TSAS at various intervals up to twelve hours. The results are seen in Figure 5. In most cases, values at twelve hours are estimated.

AKM medium served as a control and counts on TSA (curve 1) from this culture remained stable until cell multiplication began at 3-4 hours. Recovery of salt tolerance (curve 2) proceeded at a usual rate. Declining viability in the malate culture, as demonstrated by TSA counts (curve 3), resulted in a reduced count on TSAS (curve 4). However, recovery was seen and a growth rate approaching that of the aspartate medium was obtained. Results similar to malate were seen with succinate (curves 7, 8).

FIGURE 5

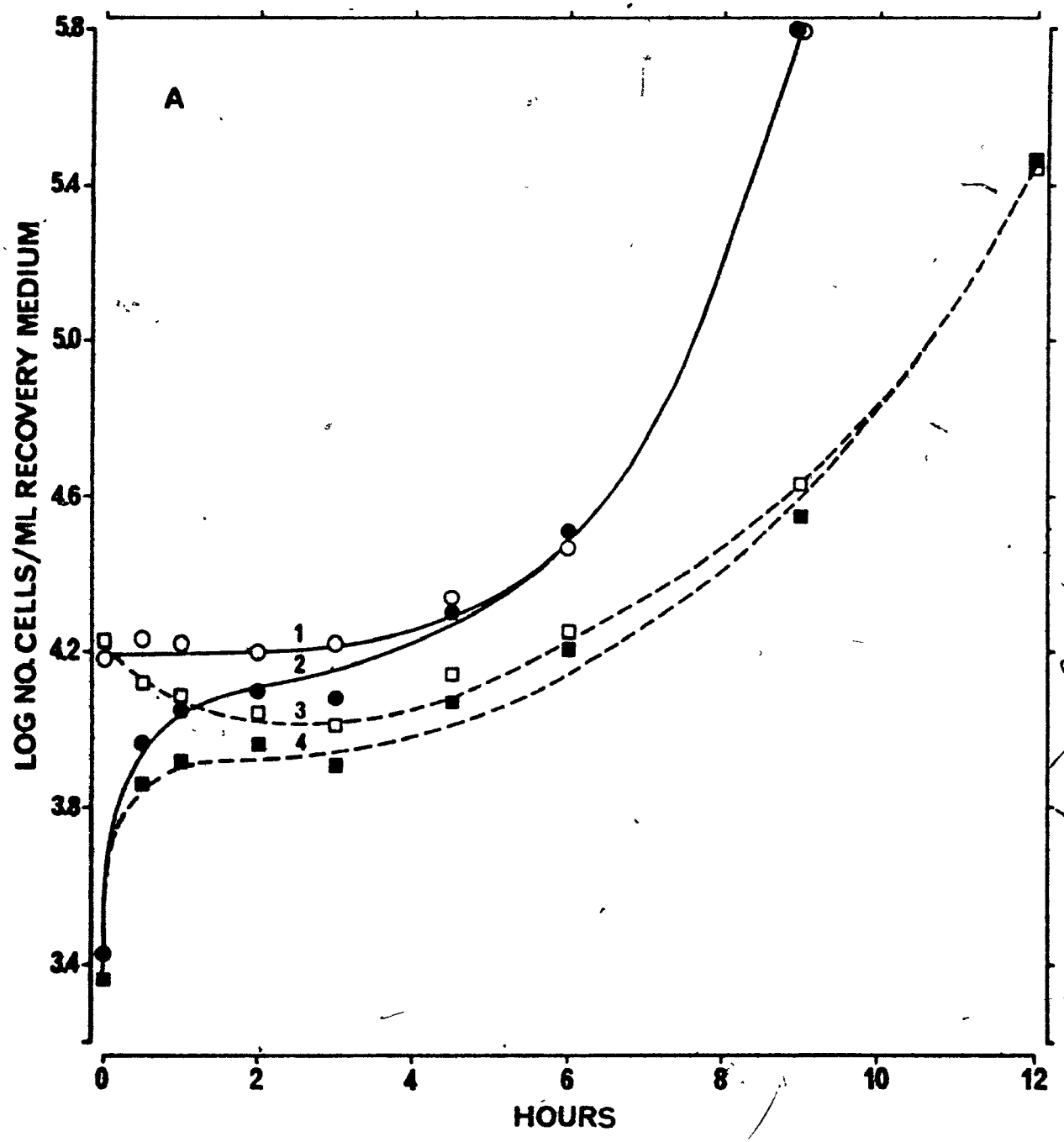
Figure 5 (A and B)

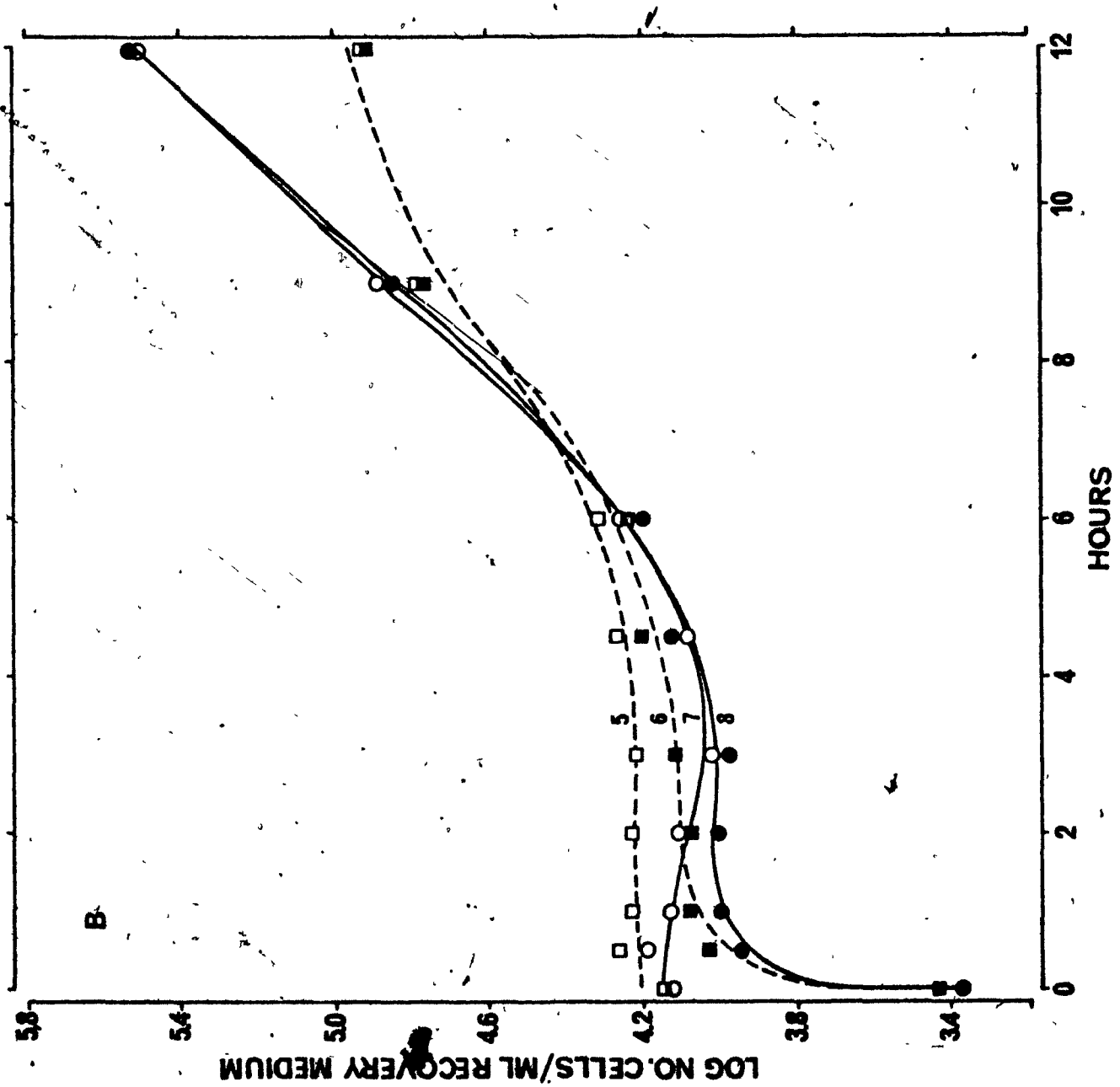
Effect of substitution of aspartate in the AKM recovery medium by citric acid cycle intermediates on the recovery of salt tolerance by a suspension of frozen and thawed cells.

Concentrations of K^+ and Mg^{++} in all recovery media were the same as in AKM.

Curve	Plating Medium	Recovery Medium
1	TSA	
2	TSAS	AKM (0.005 M aspartate + 0.01 M K^+ + 0.001 M Mg^{++}
3	TSA	
4	TSAS	0.005 M sodium malate + K^+ + Mg^{++}
5	TSA	
6	TSAS	0.005 M sodium citrate + K^+ + Mg^{++}
7	TSA	
8	TSAS	0.005 M sodium succinate + K^+ + Mg^{++}

The results shown in Figure 5A and 5B are from identical suspensions of cells incubated at the same time and are thus directly comparable.


A
LOG NO. CELLS/ML RECOVERY MEDIUM
5.8
5.4
5.0
4.6
4.2
3.8
3.4
0
2
4
6
8
10
12
HOURS
1
2
3
4


B
LOG NO. CELLS/ML RECOVERY MEDIUM
5.8
5.4
5.0
4.6
4.2
3.8
3.4
HOURS
0
2
4
6
8
10
12
5
6
7
8

Although the response to citrate was slower than that with succinate or malate (curves 5, 6), the result suggested utilization of citrate as a carbon-energy source — a result not seen with fresh, unfrozen cells.

In a similar experiment with equimolar concentrations of amino acids (Figure 6), proline (curves 7, 8), and to a lesser extent, alanine (curves 3, 4) were found to be as effective as aspartate. Recovery of salt tolerance was similar in all cases. Serine (5, 6), which had previously been reported to be inhibitory to the growth of a frozen and thawed population, and phenylalanine (9, 10), were found to initiate a much slower growth response. In the case of serine, some loss of viability was noted, but recovery of salt tolerance proceeded in this case and in the case of phenylalanine.

Results in these experiments suggested that a variety of organic substrates other than aspartate, when present in the incubation medium, could enhance the salt tolerance of a frozen and thawed population. It appeared, however, that aspartate was the most effective of the compounds tested in initiating division and multiplication.

Comparison of AKM and Minimal Medium + Aspartate as Recovery Media

It had previously been demonstrated that substitution of aspartate in the AKM recovery medium by glucose resulted in decreased viability of a frozen and thawed suspension of cells without any

FIGURE 6

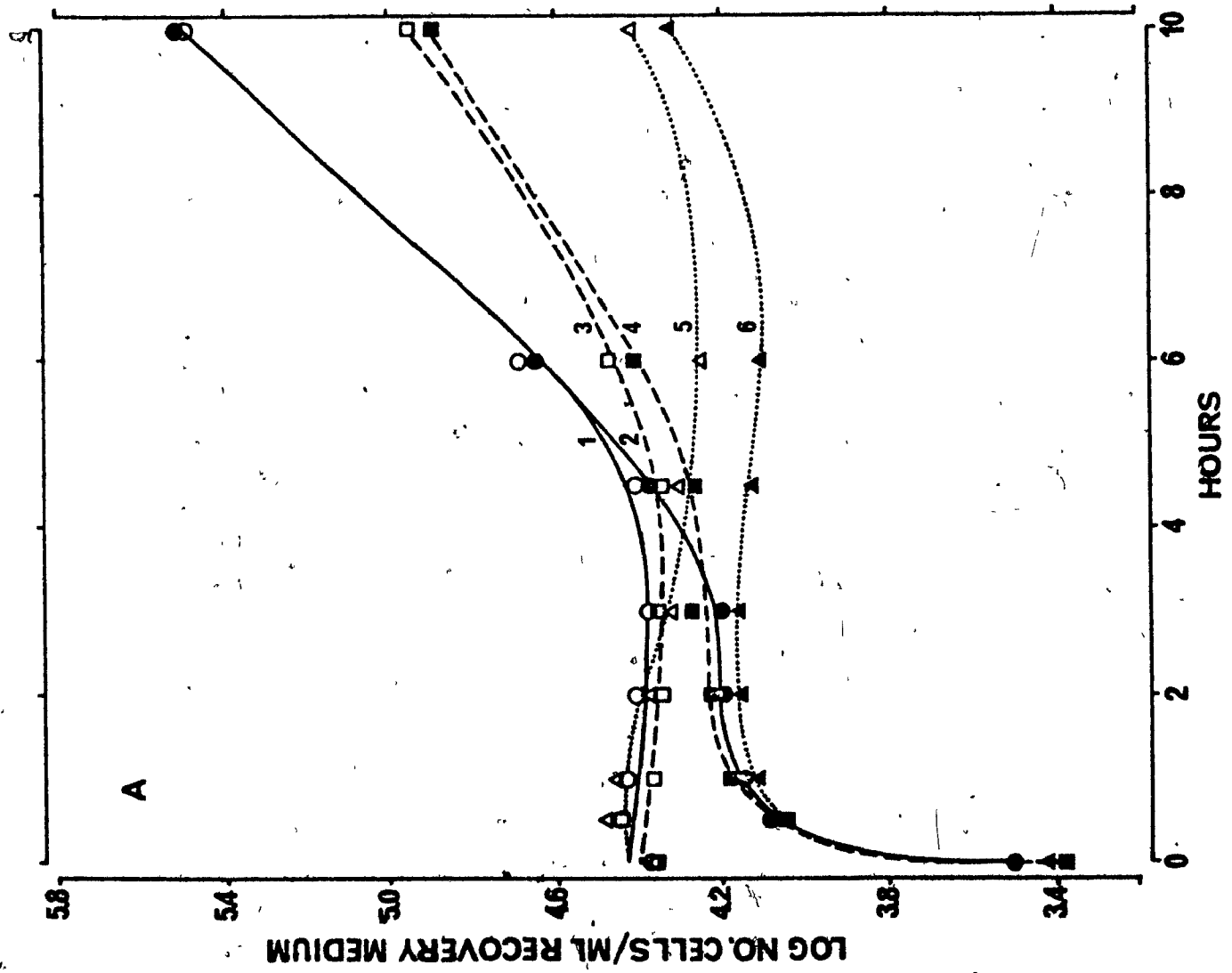
Figure 6 (A and B)

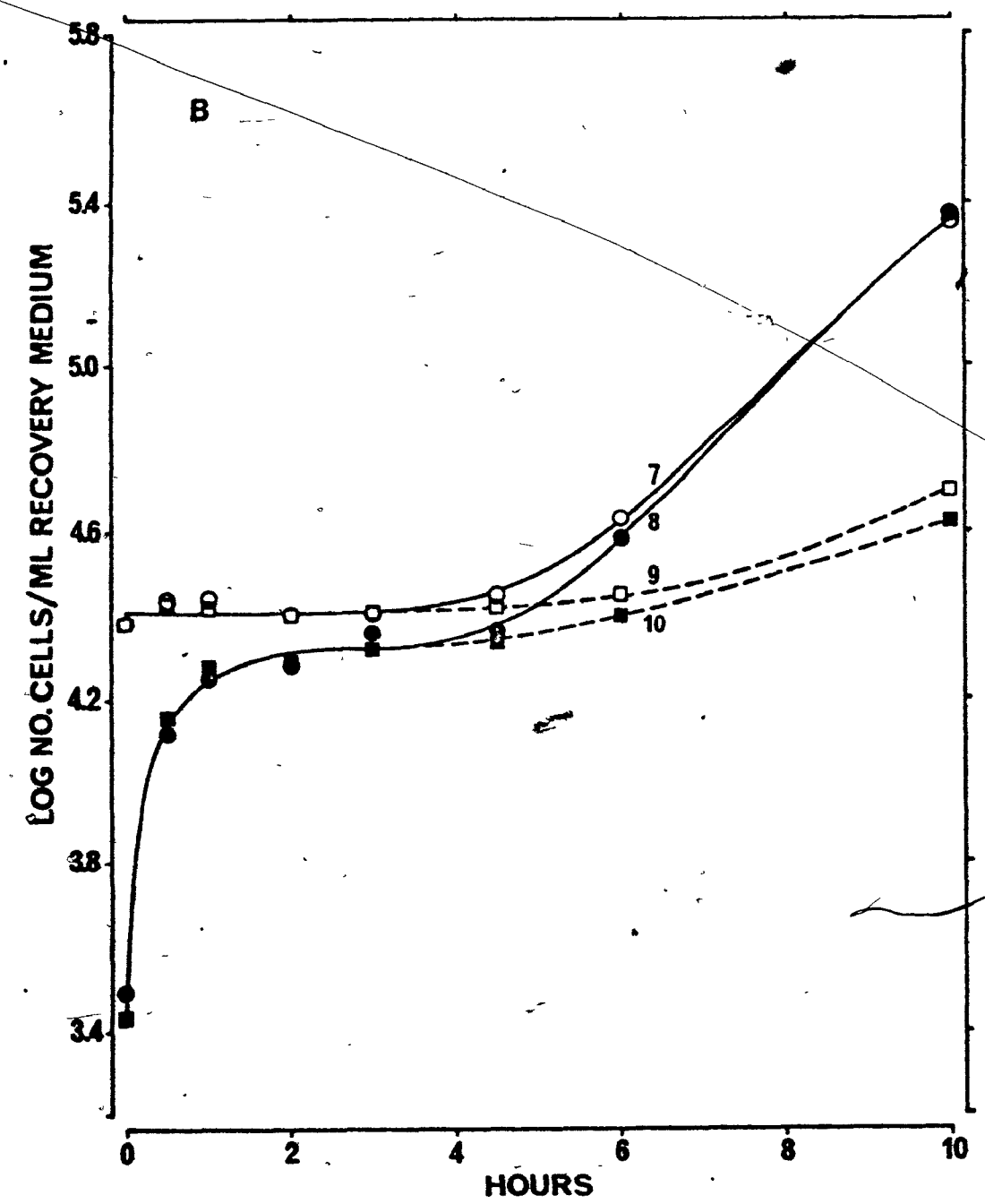
Effect on frozen and thawed cells of substitution of aspartate in the AKM recovery medium by other amino acids.

Concentrations of K^+ and Mg^{++} in all recovery media were the same as in AKM.

Curve	Plating Medium	Recovery Medium
1	TSA	0.005 aspartate + 0.01 M K^+ + 0.001 M Mg^{++}
2	TSAS	
3	TSA	0.005 M alanine + K^+ + Mg^{++}
4	TSAS	
5	TSA	0.005 M serine + K^+ + Mg^{++}
6	TSAS	
7	TSA	0.005 M proline + K^+ + Mg^{++}
8	TSAS	
9	TSA	0.005 M phenylalanine + K^+ + Mg^{++}
10	TSAS	

The results in Figures 6A and 6B are directly comparable. (see legend to Figure 5).


A
1
2
3
4
5
6
LOG NO. CELLS/ML RECOVERY MEDIUM
5.8
5.4
5.0
4.6
4.2
3.8
3.4
HOURS
0
2
4
6
8
10


B
LOG NO.CELLS/ML RECOVERY MEDIUM
5.8
5.4
5.0
4.6
4.2
3.8
3.4
0
2
4
6
8
10
HOURS
7
8
9
10

increase in salt tolerance. It was of interest, therefore, to compare AKM recovery medium with the minimal medium ± aspartate, prepared without agar and used as recovery media. The results are seen in Figure 7.

Incubation in all three media led to a recovery of salt tolerance of the cells. When AKM or minimal medium + aspartate was used as the recovery medium, the cells began to increase in numbers after recovery of salt tolerance. This was not the case with cells incubated in minimal medium without aspartate.

Since recovery took place in the absence of aspartate, this suggested that some component other than aspartate was responsible for the return of salt tolerance of cells incubated in the minimal medium.

Role of Basal Salts in Recovery of Salt Tolerance

Further to the previous experiment, a frozen and thawed suspension was incubated in basal salts alone and the results were compared with incubation in the AKM medium. The results are seen in Figure 8.

In the absence of aspartate, no end was seen to the growth lag in the basal salts medium (curve 3). However, recovery (curve 4) comparable to that in the AKM medium (curve 2) did take place and suggested that some or all of the components in the basal salts medium

FIGURE 7

Figure 7

Comparison of AKM and minimal medium ± aspartate as recovery media for frozen and thawed cells.

Curve	Plating Medium	Recovery Medium
1	TSA	AKM
2	TSAS	
3	TSA	minimal medium without agar
4	TSAS	
5	TSA	minimal medium + 0.005 M aspartate without agar
6	TSAS	

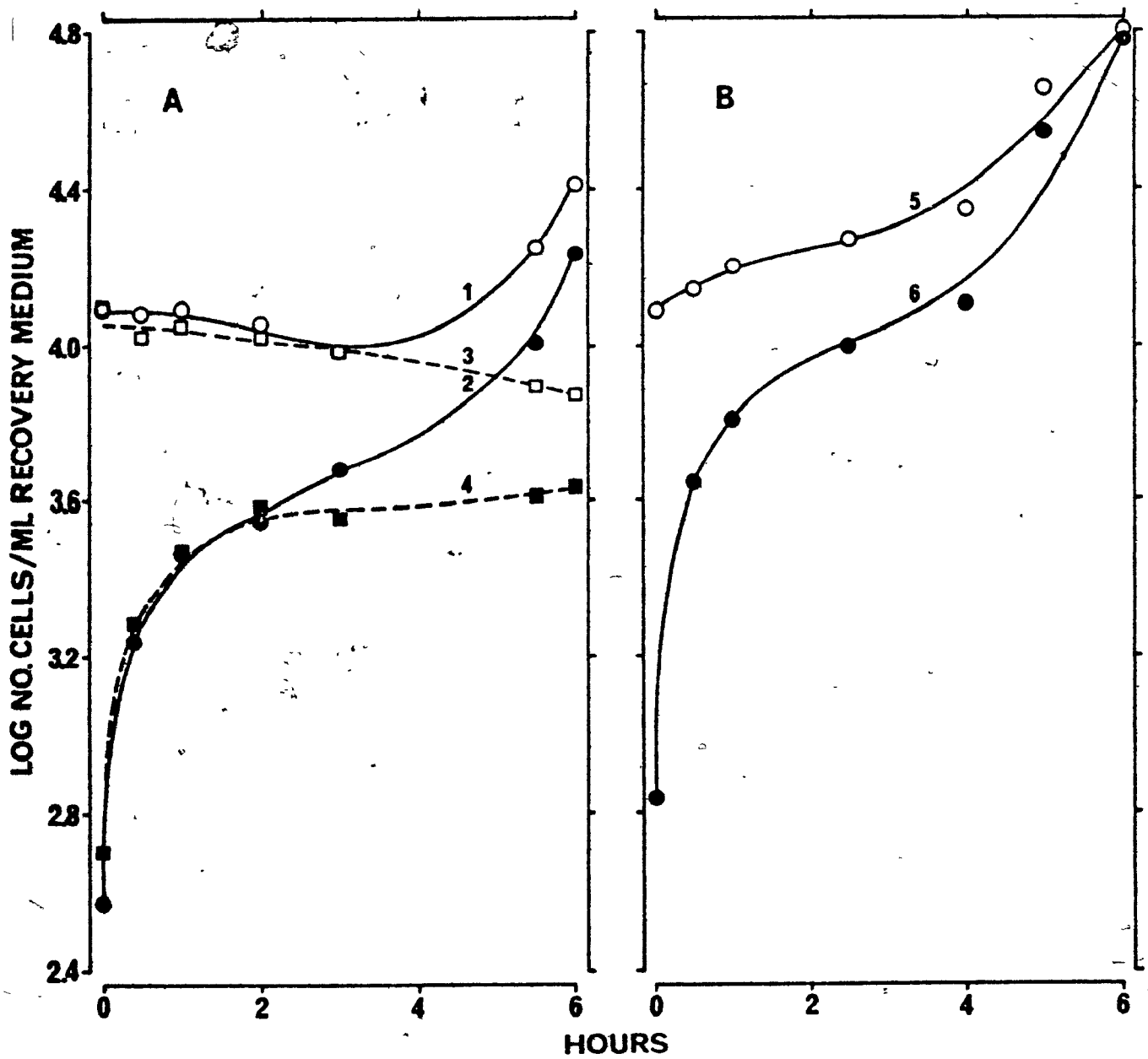
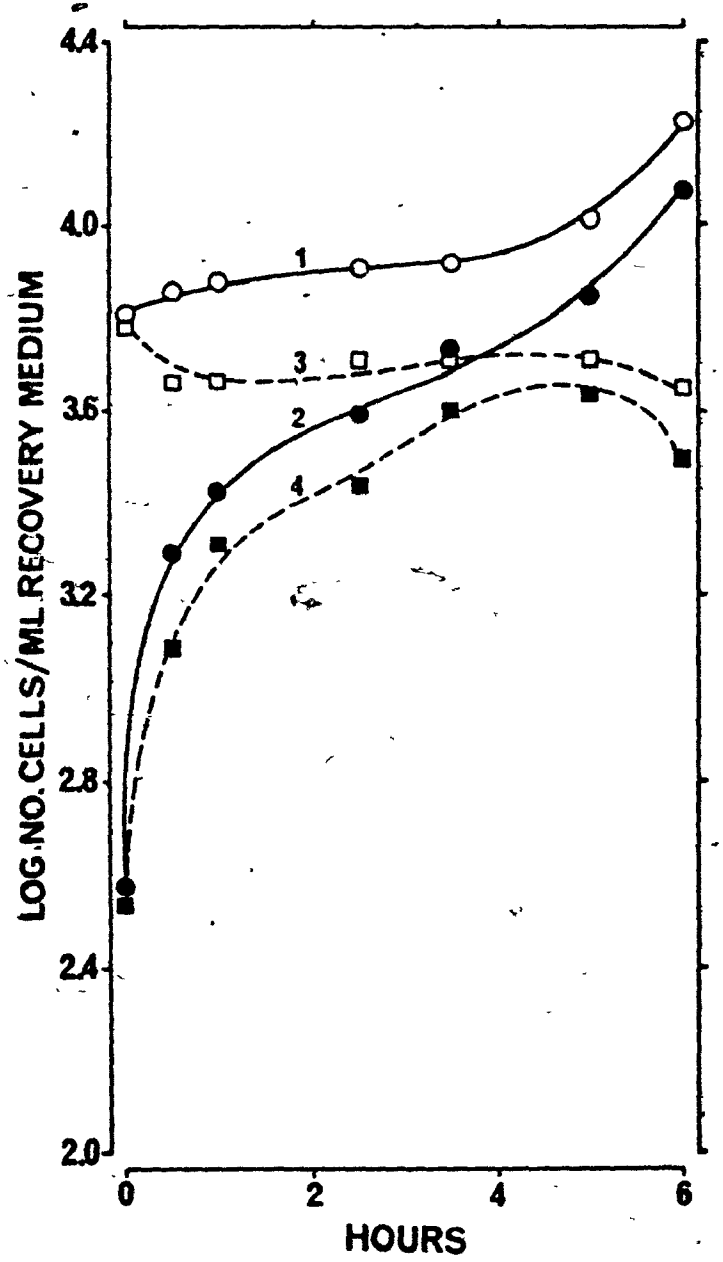

A
B
1
2
3
4
5
6
4.8
4.4
4.0
3.6
3.2
2.8
2.4
LOG NO. CELLS/ML RECOVERY MEDIUM
0
2
4
6
HOURS

FIGURE 8

Figure 8

Comparison of incubation of frozen and thawed cells in AKM and basal salts solution.

Curve	Plating Medium	Recovery Medium
1	TSA	AKM
2	TSAS	
3	TSA	basal salts solution
4	TSAS	


LOG.NO.CELLS/ML.RECOVERY MEDIUM
4.4
4.0
3.6
3.2
2.8
2.4
2.0
0
2
4
6
HOURS
1
2
3
4

could effect a return of salt tolerance in a frozen and thawed suspension of *Escherichia coli*.

Development of PKM as a Recovery Medium

The basal salts solution used in the preparation of minimal medium consisted of: 0.04 M K_2HPO_4, 0.022 M KH_2PO_4, 0.007 M $(NH_4)_2SO_4$, 0.0003 M sodium citrate, and 0.0004 M $MgSO_4 \cdot 7H_2O$). Various concentrations of each component of this formulation were added to solutions of one or more other components held at a constant concentration. These solutions were employed as recovery media and when inoculated, were compared to the effectiveness of a known recovery medium in altering the response of a frozen and thawed suspension of cells to NaCl in the plating medium. Extensive tests with various combinations and concentrations of these components resulted in a solution of NaH_2PO_4, K_2SO_4 and $MgSO_4 \cdot 7H_2O$, adjusted to pH 7.0 with NaOH and containing 0.01 M PO_4^{-3}, 0.01 M K^+ and 0.001 M Mg^{++} (PKM). These concentrations of components were based on the minimum concentration of each component which, when present together with the other components, would allow recovery of salt tolerance by a frozen and thawed suspension of cells comparable to that obtained in basal salts solution, without cell multiplication or loss of viability. Sodium citrate and ammonium sulphate were found not to be required. Figure 9 demonstrates the rate of recovery of salt tolerance when frozen and thawed cells were suspended in recovery media having constant concentrations of PO_4^{-3} and K^+, and various concentrations of Mg^{++}. No difference was seen in the rate of recovery where the concentration of Mg^{++} was 0.01 M (curve 2) or 0.001 M (curve 4) in the recovery medium. A somewhat slower rate of recovery was observed when

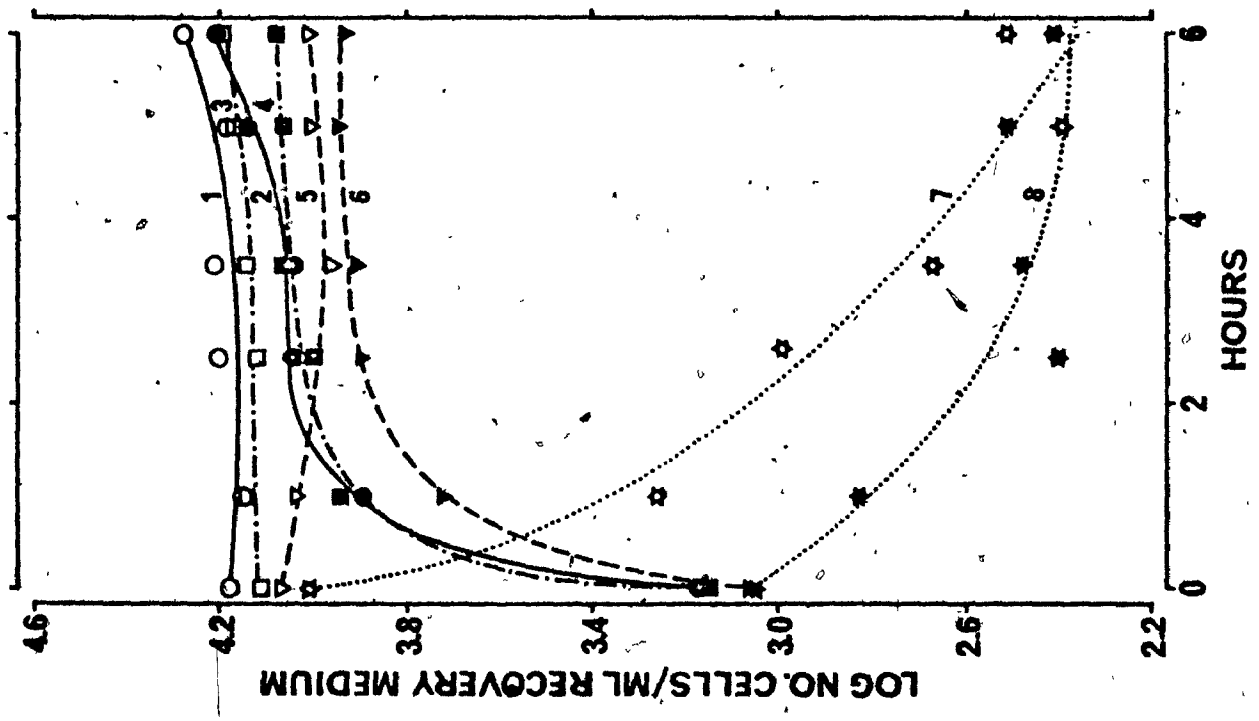
FIGURE 9

Figure 9

Comparison of incubation of frozen and thawed cells in recovery media with various concentrations of Mg^{++}.

All recovery media contained 0.03 M NaH_2PO_4 and 0.03 M K_2SO_4 at pH 7.0.

Curve	Plating Medium	Recovery Medium
1	TSA	0.01 M Mg^{++}
2	TSAS	
3	TSA	0.001 M Mg^{++}
4	TSAS	
5	TSA	0.0001 M Mg^{++}
6	TSAS	
7	TSA	no Mg^{++}
8	TSAS	


LOG NO. CELLS/ML RECOVERY MEDIUM
4.6
4.2
3.8
3.4
3.0
2.6
2.2
HOURS
0
2
4
6
1
2
3
4
5
6
7
8

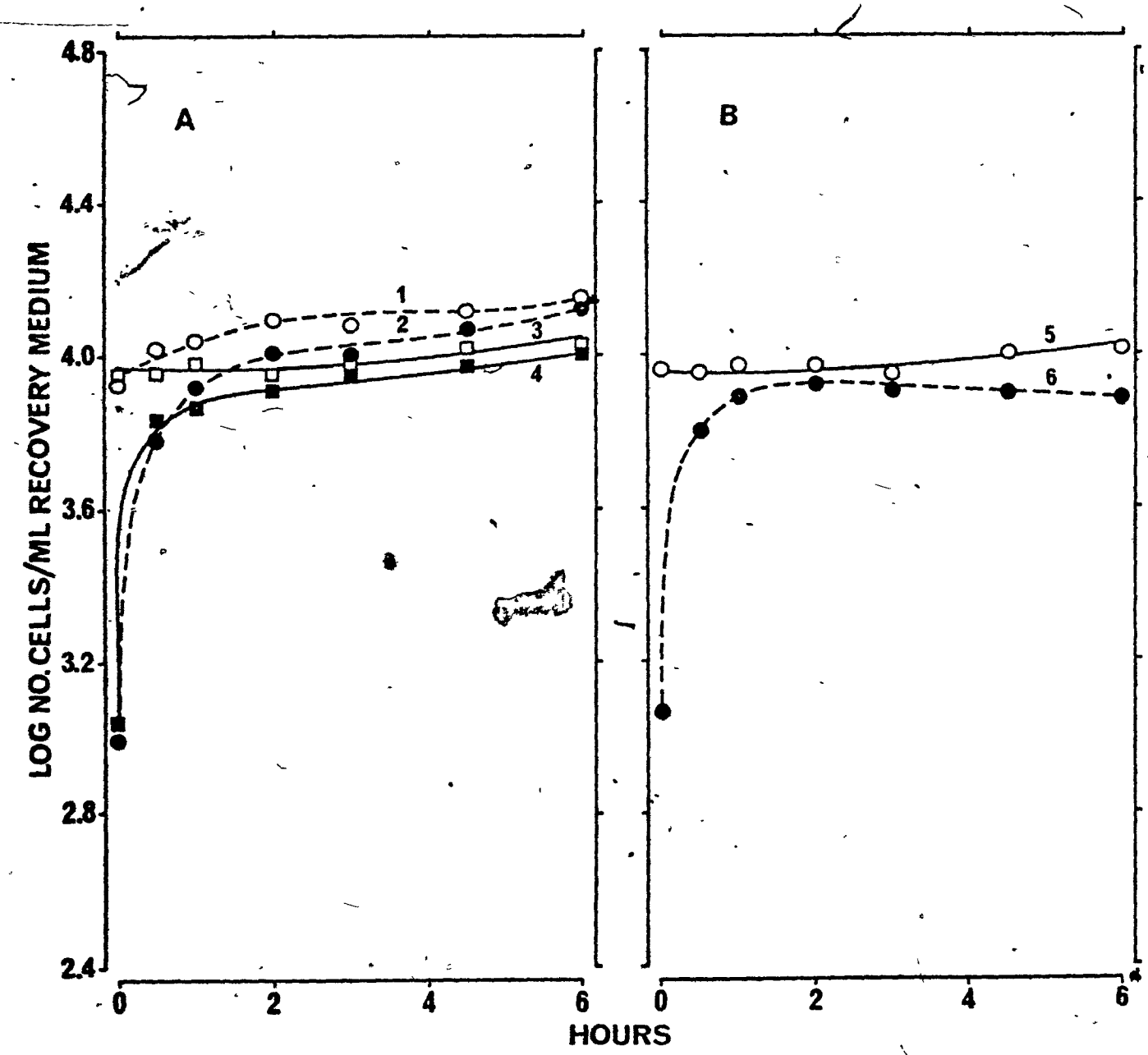
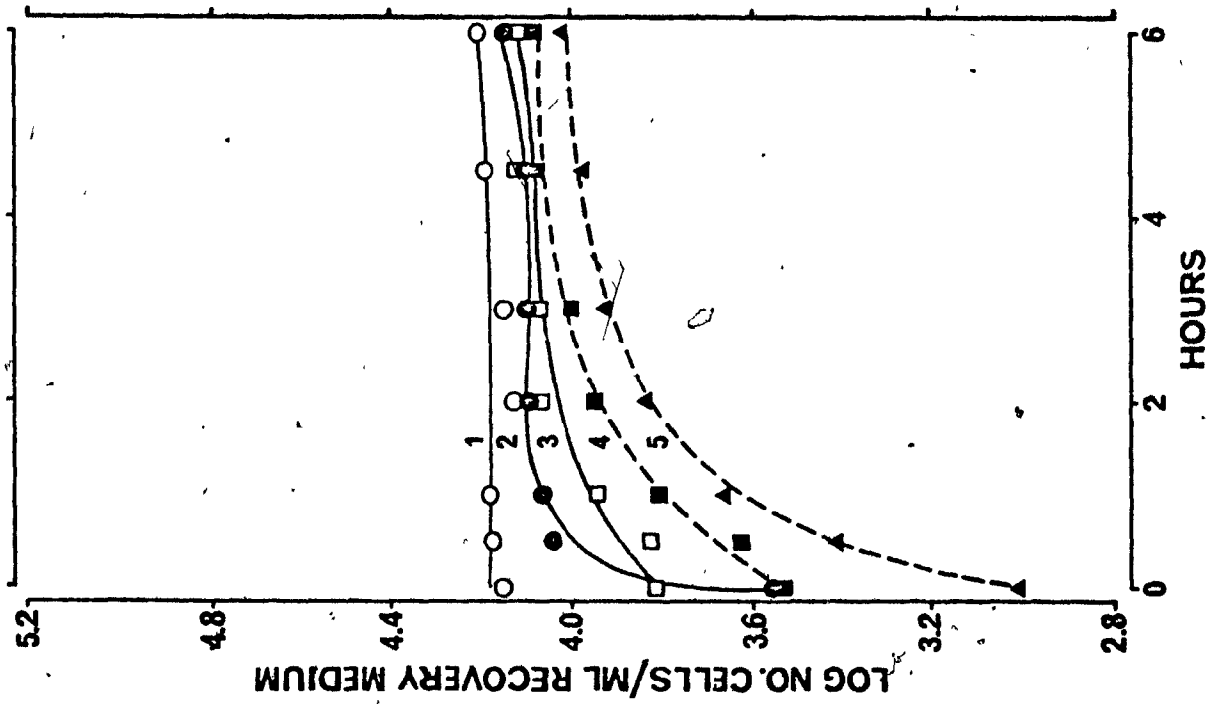

A
B
LOG NO.CELLS/ML RECOVERY MEDIUM
4.8
4.4
4.0
3.6
3.2
2.8
2.4
1
2
3
4
5
6
0
2
4
6
HOURS

Figure 14

Effectiveness of incubation in PKM recovery medium in increasing the counts of frozen and thawed cells on various plating media.

Curve	Plating Medium
1	TSA
2	TSAS
3	minimal agar + 0.005 M aspartate
4	minimal agar
5	minimal agar + 0.005 M aspartate + 0.3 M NaCl

Numerical values for the graph are given in Appendix A.


LOG NO. CELLS/ML RECOVERY MEDIUM
5.2
4.8
4.4
4.0
3.6
3.2
2.8
HOURS
0
2
4
6
1
2
3
4
5

the AKM medium could be involved in the capacity of this medium to enhance salt tolerance.

Sodium phosphate, magnesium sulphate and potassium sulphate were added separately to aspartate and the resulting solutions were compared, as recovery media, to the AKM medium, and to aspartate alone as a recovery medium. The results are seen in Figure 15. With aspartate alone in the recovery medium, there was a decrease in viability of the cells in suspension as measured using the TSA plating medium (curve 3). After reaching a steady state level and remaining there for about two hours, the cell population began to increase. Those cells which survived recovered their salt tolerance as indicated by the increase in numbers of cells in suspension able to form colonies on TSAS medium (curve 4). The addition of PO_4^{-3}, or K^+ to the aspartate solution did not change this pattern significantly. The addition of Mg^{++}, on the other hand, prevented the decrease in viability observed initially and increased the rate of recovery of salt tolerance considerably. Mg^{++} appeared to be the major ionic component of the AKM medium, and its deletion resulted in a considerable loss of viability in the recovery medium.

Effect of Deletion of Components from PKM Recovery Medium - Addition of Aspartate to the PKM Medium

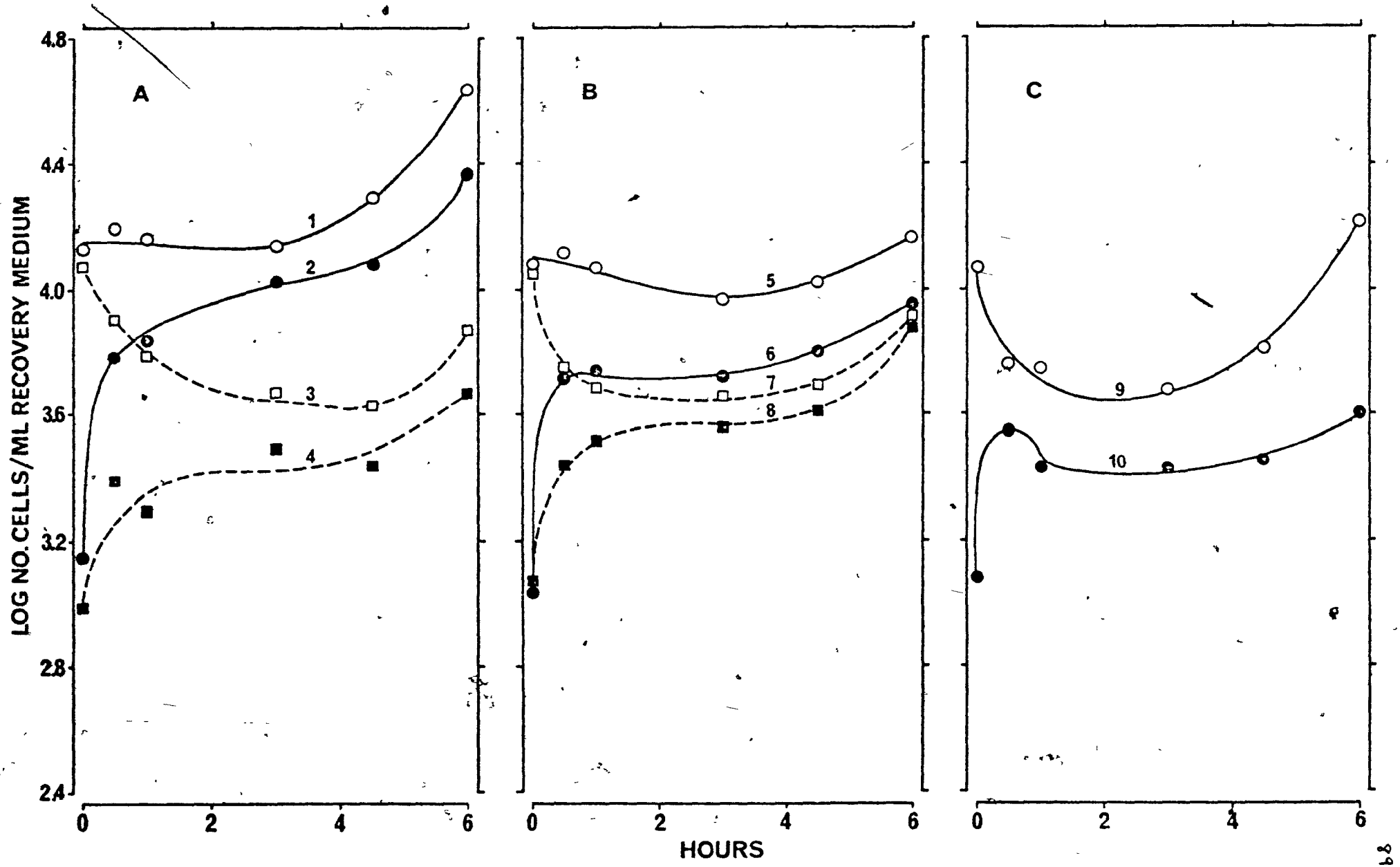
To confirm the effectiveness of PKM as a recovery medium and Mg^{++} as its major component, PKM medium was prepared with and without

Figure 15

Involvement of ions in the capacity of AKM recovery medium to enhance salt tolerance of frozen and thawed cells.

All recovery media were adjusted to pH 7.0 with 1.0 M NaOH.

Curve	Plating Medium	Recovery Medium
1	TSA	AKM (0.005 M aspartate + 0.01 M K^+ + 0.001 M Mg^{++})
2	TSAS	
3	TSA	0.005 M aspartate
4	TSAS	
5	TSA	aspartate + 0.001 M Mg^{++}
6	TSAS	
7	TSA	aspartate + 0.01 M K^+
8	TSAS	
9	TSA	aspartate + 0.01 M PO_4^{-3}
10	TSAS	


A
B
C
LOG NO. CELLS/ML RECOVERY MEDIUM
4.8
4.4
4.0
3.6
3.2
2.8
2.4
0
2
4
6
HOURS
1
2
3
4
5
6
7
8
9
10

0.005 M aspartate. As well, recovery media were prepared in the concentrations of the PKM medium with PO_4^{-3} deleted; with PO_4^{-3} and K^+ deleted; and finally with PO_4^{-3} and Mg^{++} deleted. A medium consisting of glass-distilled water alone served as a control. A frozen and thawed suspension of cells was inoculated into each of these media and aliquots were subsequently plated into TSA and TSAS. The results are seen in Figure 16. The addition of aspartate to the PKM medium did not result in an appreciably greater rate of recovery of salt tolerance. Its addition, however, resulted in the rapid multiplication of cells in the suspension after incubation for three hours.

The deletion of PO_4^{-3}, or PO_4^{-3} + K^+ from PKM medium resulted in a gradual loss of viability of the cells in the incubating suspension but not before a return of salt tolerance which was comparable to that seen with PKM medium. In the absence of PO_4^{-3} and Mg^{++}, that is, with K^+ alone as the recovery medium, there was a rapid loss of viability of the incubating suspension without a return of salt tolerance. This result was comparable to that seen with water alone as the suspending medium.

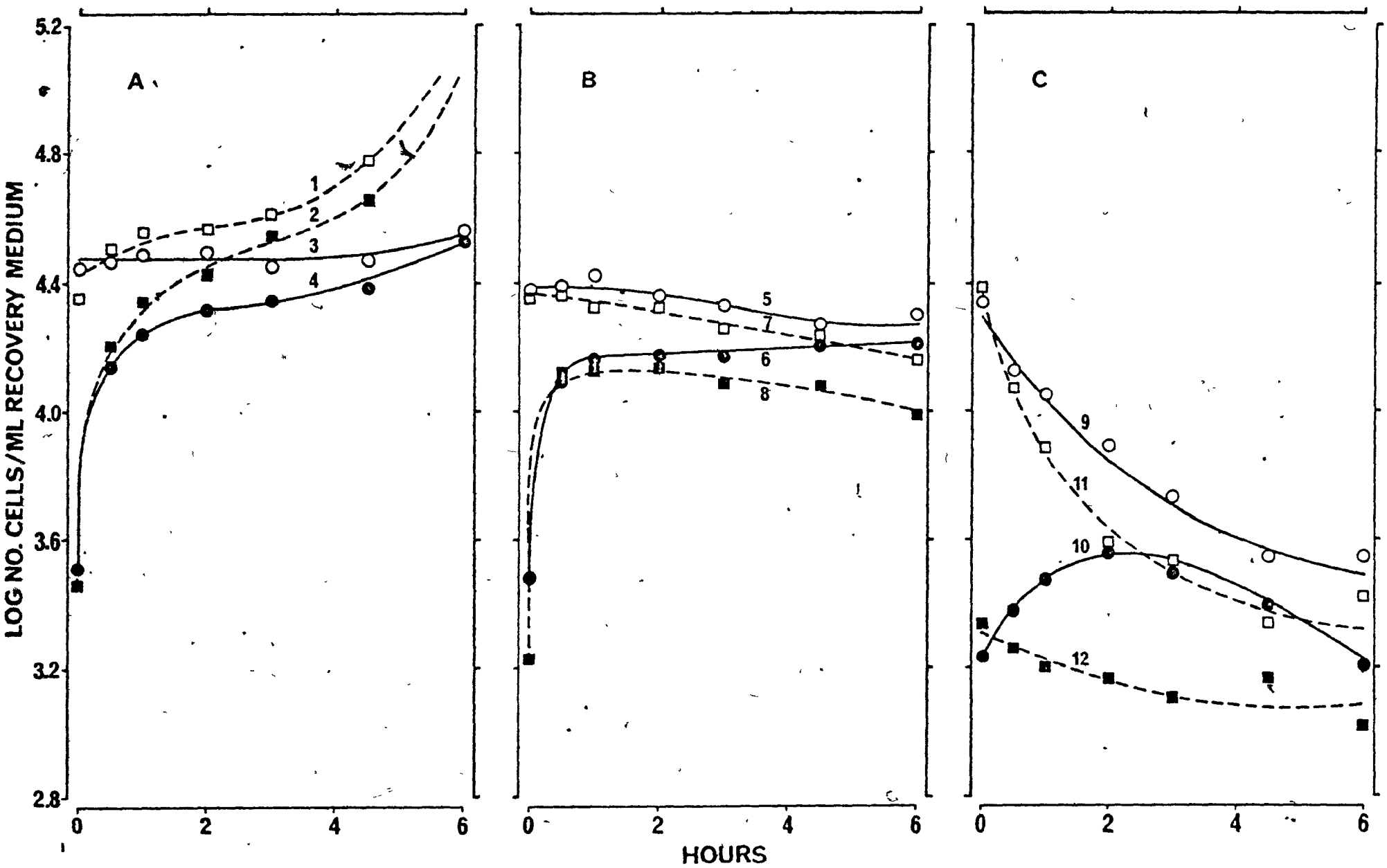
The results in this and the previous experiments suggested that Mg^{+2} was critical to the recovery of salt tolerance by a frozen and thawed suspension. Aspartate appeared to function as a source of carbon and energy for cell multiplication — a function which could be assumed by other amino acids and TCA intermediates (Figures 5 and 6).

Figure 16

Effect of deletion of components from PKM recovery medium. Addition of aspartate to the PKM medium.

All recovery media were adjusted to pH 7.0 with 1.0 M NaOH.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM + 0.005 M aspartate
2	TSAS	
3	TSA	PKM
4	TSAS	
5	TSA	0.01 M K^+ + 0.001 M Mg^{++}
6	TSAS	
7	TSA	0.001 M Mg^{++}
8	TSAS	
9	TSA	0.01 M K^+
10	TSAS	
11	TSA	H_2O alone
12	TSAS	


A
B
C
LOG NO. CELLS/ML RECOVERY MEDIUM
5.2
4.8
4.4
4.0
3.6
3.2
2.8
0
2
4
6
HOURS
1
2
3
4
5
6
8
9
10
11
12

Effect of Magnesium Alone as a Recovery Medium

It had been found in previous experiments that the use of Mg^{++} alone as a recovery medium resulted in varying degrees of recovery of salt tolerance of frozen and thawed suspensions of cells (see Figures 4 and 16). Greater recovery in Mg^{++} alone appeared to be coincident with some degree of growth in the PKM medium, and occurred when initial plate counts were higher. It seemed possible, therefore, that recovery in Mg^{++} alone might be related to increased amounts of intracellular solutes being present as leakage products after larger populations of frozen cells had been thawed. Previous work had demonstrated intracellular leakage into the freezing menstruum (Kuo, 1969) and the inoculation of 0.5 ml of this menstruum into the recovery medium could introduce sufficient alteration of the defined PKM medium to produce an enhanced recovery in Mg^{++} alone, and a slight degree of growth in PKM.

To explore this possibility, a frozen and thawed suspension of cells was prepared in the usual fashion and 1.0 and 4.0 ml aliquots were inoculated into PKM and a recovery medium consisting of 0.001 M $MgSO_4 \cdot 7\ H_2O$. Aliquots of the various recovery media were subsequently plated in TSA and TSAS and the results are seen in Figure 17. Some degree of growth was evident in the PKM medium, where 4.0 ml of the freezing menstruum were employed as the inoculum (curves 5, 6), while little was seen where 1.0 ml was employed (curves 1, 2). Little

Figure 17

Comparison of the effect of two sizes of inocula on the recovery of salt tolerance by frozen and thawed cells.

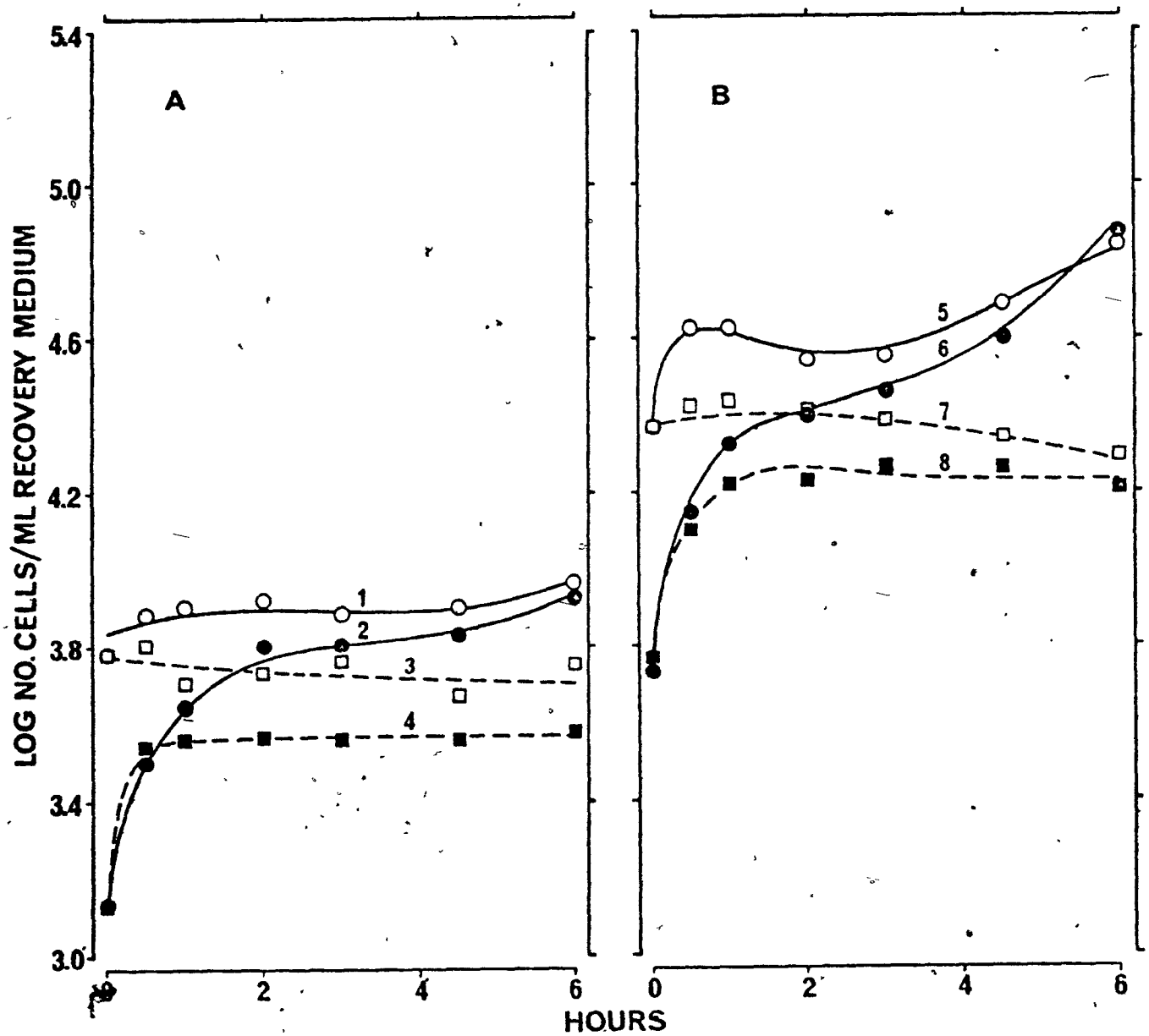
Volumes of 1.0 and 4.0 ml of a frozen and thawed suspension of cells were added to PKM and 0.001 M Mg^{++} and aliquots from these recovery media were subsequently plated on TSA and TSAS.

A. 1.0 ml inoculum

Curve	Plating Medium	Recovery Medium
1	TSA	PKM
2	TSAS	
3	TSA	0.001 M Mg^{++}
4	TSAS	

B. 4.0 ml inoculum

Curve	Plating Medium	Recovery Medium
5	TSA	PKM
6	TSAS	
7	TSA	0.001 M Mg^{++}
8	TSAS	


A
B
LOG NO. CELLS/ML RECOVERY MEDIUM
5.4
5.0
4.6
4.2
3.8
3.4
3.0
0
2
4
6
HOURS
1
2
3
4
5
6
7
8

difference, however, was seen with the two sizes of inocula in their response to Mg^{++} alone. Both demonstrated decreasing viability with some recovery of salt tolerance and were comparable to the result obtained in Figure 16.

Utilization of Oxygen by a Frozen and Thawed Population in the Presence of Aspartate or Glucose

For respirometry, it was necessary to use a higher cell concentration than that used for plating experiments. Cells were prepared in the usual manner, but the suspension to be frozen was increased to a density of $4x10^8$ cells/ml. After freezing for twenty-four hours and thawing, 0.5 ml portions of this suspension were added to the triplicate sets of respirometry flasks chilled with ice and containing various media. These included PKM, PKM + aspartate, PKM + glucose, and water alone. Distilled water was added to one triplicate set of PKM flasks to serve as a control.

After allowing the flasks to equilibrate at 37°C in the water bath, the valves were closed and changes in pressure were recorded up to 17.5 hours. The results are seen in Figure 18B. Respiratory values of inoculum in distilled water were subtracted in each case in order to measure the effect of the PKM medium on endogenous respiration.

Respiration in PKM recovery medium was seen to remain low throughout the course of the experiment (curve 3) and very little increase was seen when glucose was added to this medium (curve 2).

Figure 18

A. Uptake of oxygen by a suspension of fresh (unfrozen) cells incubated in various media at 37°C.

Curve 1. incubation in PKM + 0.005 M aspartate

Curve 2. incubation in PKM + 0.005 M glucose

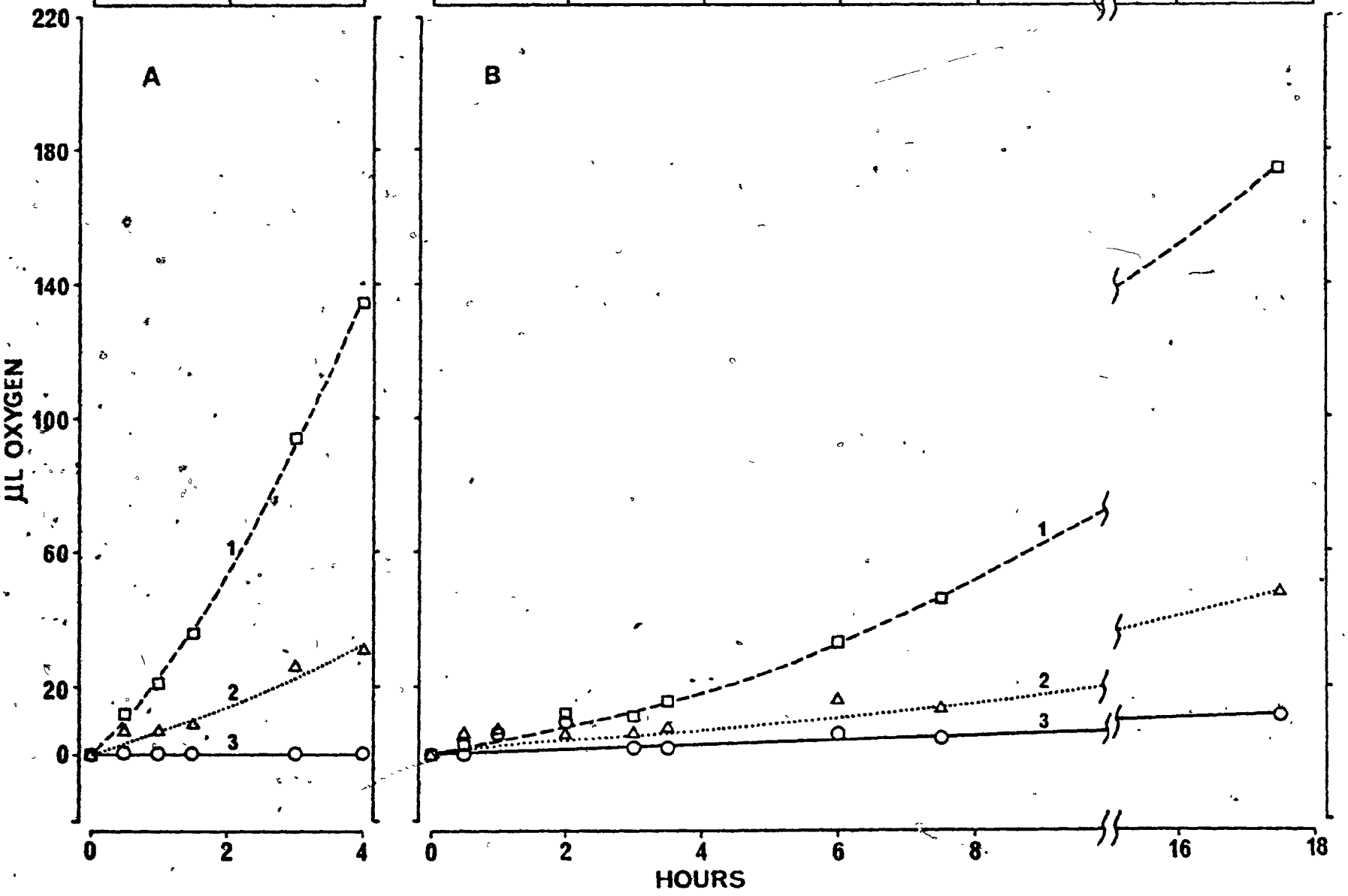
Curve 3. incubation in PKM alone.

B. Uptake of oxygen by a suspension of frozen and thawed cells incubated in various media at 37°C.

Curve 1. incubation in PKM + 0.005 M aspartate

Curve 2. incubation in PKM + 0.005 M glucose

Curve 3. incubation in PKM alone.


A
B
220
180
140
100
60
20
0
µL OXYGEN
1
2
3
0
2
4
6
8
16
18
HOURS

However, an increasing rate of respiration was noted where aspartate was added to the PKM medium (curve 1) — a rate which became particularly apparent after 3-4 hours.

The same experiment was repeated with a fresh cell inoculum diluted ten fold in 0.85% NaCl to approximate the viable count of the frozen and thawed inoculum and the results are seen in Figure 18A. The results were similar to those seen with the frozen and thawed suspensions but rates of respiration were much higher. In both experiments, however, it was evident that glucose did not stimulate respiration to any large extent.

A plating experiment was performed using the same media and the usual frozen and thawed inoculum. Aliquots of the incubating suspensions were taken up to twelve hours. Results are seen in Figure 19. Recovery of salt tolerance was evident in all three media and proceeded at about the same rate. However, no multiplication of cells was seen with PKM or PKM + glucose up to 10 hours, although growth was initiated in the PKM + aspartate medium after a three hour lag. These data corresponded closely to that seen with manometric measurements and support the conclusion that neither aspartate nor glucose enhanced recovery of salt tolerance but that aspartate was a very suitable substrate for growth and division after a period of recovery.

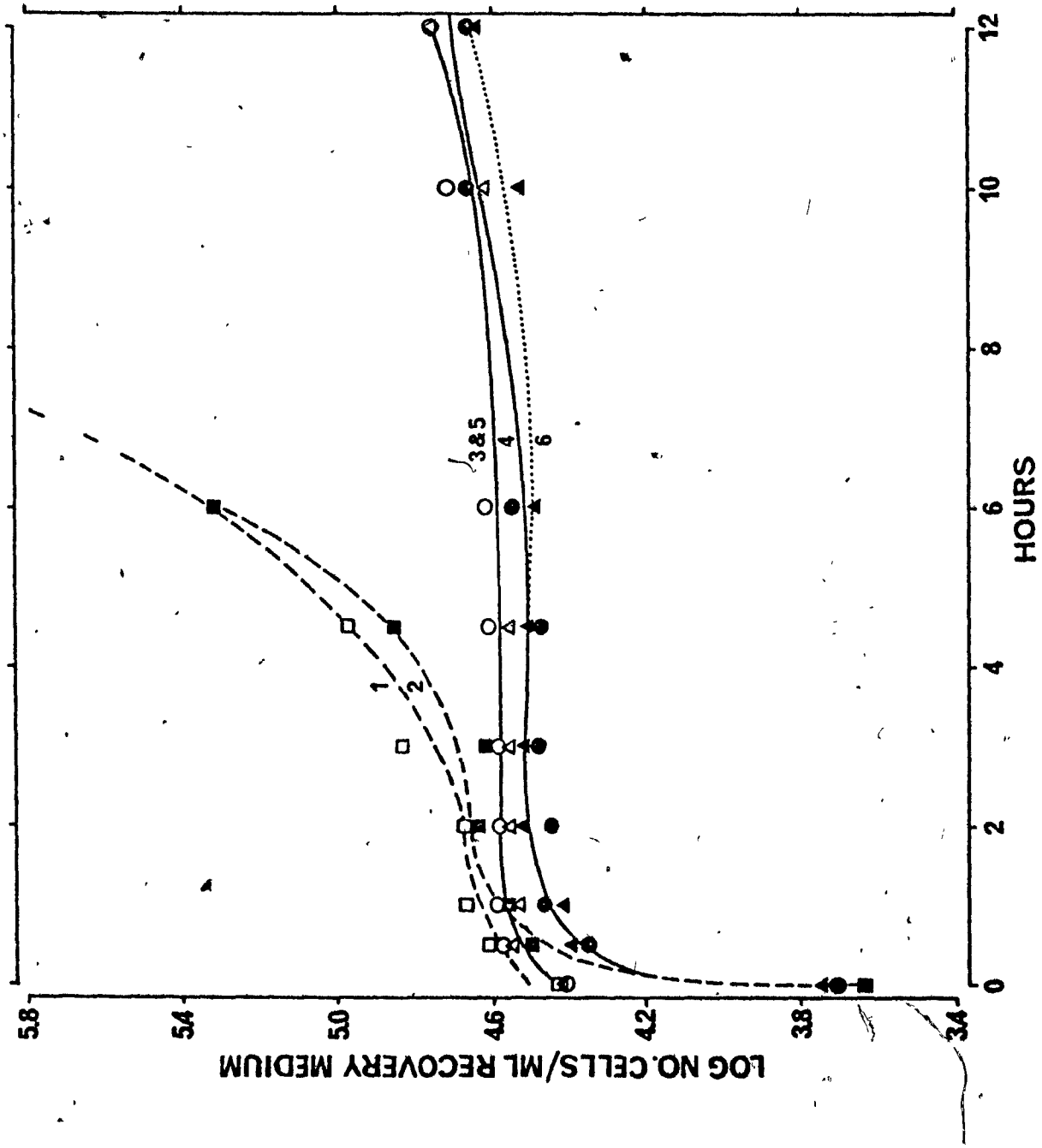
Figure 19

Incubation of a suspension of frozen and thawed cells in various recovery media for twelve hours.

Aliquots of the incubating suspensions of cells were subsequently plated in TSA and TSAS.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM + 0.005 M aspartate
2	TSAS	
3	TSA	PKM alone
4	TSAS	
5	TSA	PKM + 0.005 M glucose
6	TSAS	

Counts of aliquots of the incubating suspensions of cells obtained on the plating media were plotted against the interval of incubation in the recovery media.


LOG NO. CELLS/ML RECOVERY MEDIUM
5.8
5.4
5.0
4.6
4.2
3.8
3.4
HOURS
0
2
4
6
8
10
12
3.85
4
6
1
2

Substitution of Other Divalent Cations for Magnesium in the PKM Medium

Since Mg^{++} appeared to have a major role in the recovery of salt tolerance of a frozen and thawed population, it was of interest to determine whether other divalent cations could substitute for Mg^{++} in the PKM medium.

Equimolar amounts of $CaCl_2$, $MnCl_2$, $SrCl_2$, and $MgCl_2 \cdot 2H_2O$ were added to different recovery media containing NaH_2PO_4 and K_2SO_4. The pH in each case was adjusted to 6.5 to prevent any precipitate from forming. Media containing PO_4^{-3} and K^+ with or without Mg^{++}, at pH 7.0, served as controls. A frozen and thawed suspension was inoculated into these media and aliquots were subsequently plated into TSA and TSAS. The results are seen in Figure 20.

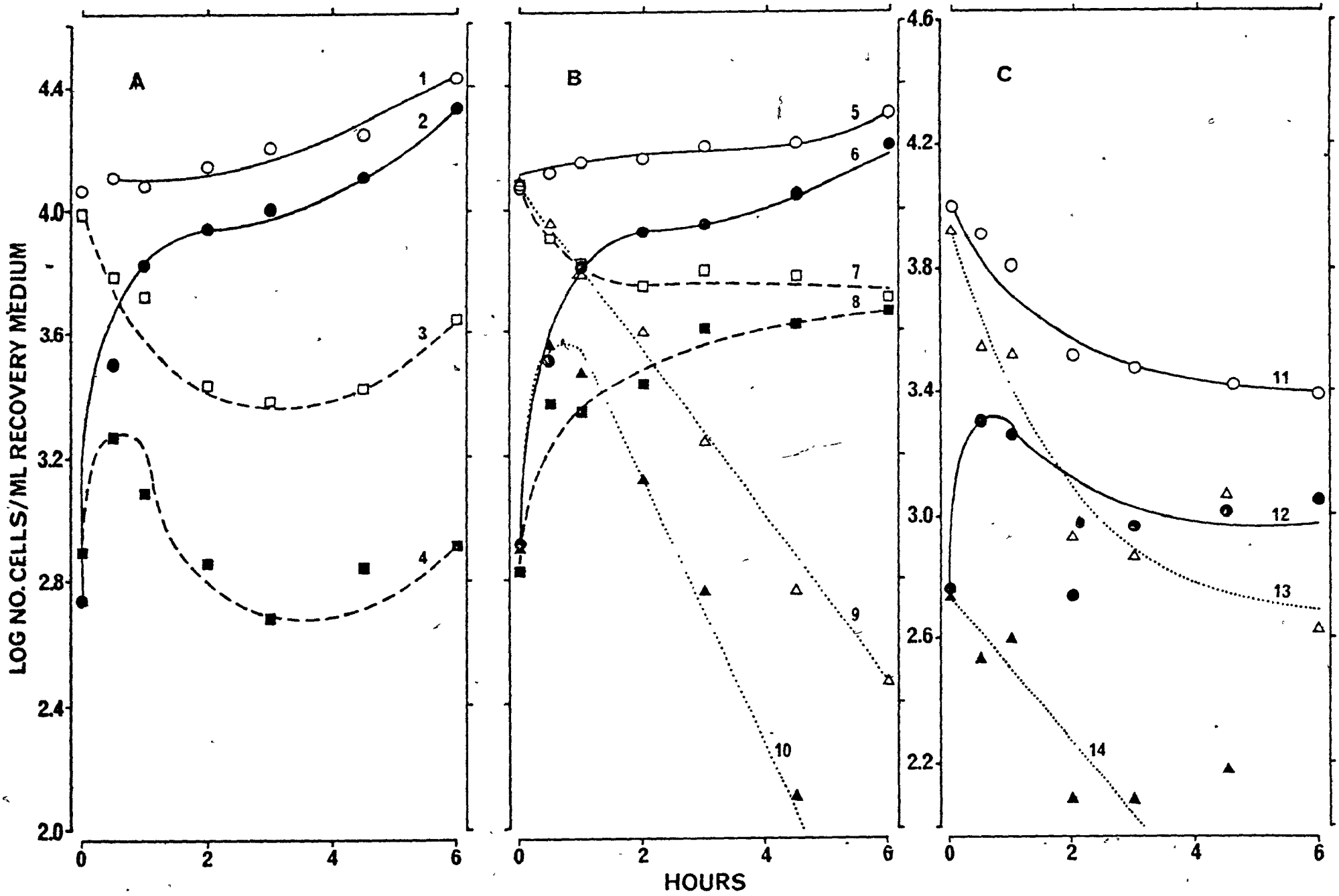
Incubation of a frozen and thawed suspension in PKM containing $MgSO_4 \cdot 7H_2O$ at pH 7.0 (curves 1, 2) or $MgCl_2 \cdot 2H_2O$ at pH 6.5 (curves 5, 6) resulted in a similar recovery of salt tolerance. In the absence of Mg^{++}, there was a rapid loss of viability. This loss was not overcome by the addition of Mn^{++} or Sr^{++} but where Ca^{++} was added, loss of viability was not so rapid and some degree of recovery of salt tolerance was seen. Recovery, however, was limited and did not compare favourably with incubation in PKM.

Figure 20

Substitution of other divalent cations for Mg^{++} in the PKM recovery medium and the effect on recovery of salt tolerance by frozen and thawed cells.

In all cases, concentrations of PO_4^{-3} and K^+ were 0.01 M. The pH was adjusted with 1.0 M NaOH.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM (pH 7.0)
2	TSAS	
3	TSA	PO_4^{-3} + K^+(pH 7.0)
4	TSAS	
5	TSA	PKM (pH 6.5)
6	TSAS	
7	TSA	0.001 M Ca^{++} + PO_4^{-3} + K^+ (pH 6.5)
8	TSAS	
9	TSA	0.001 M Mn^{++} + PO_4^{-3} + K^+ (pH 6.5)
10	TSAS	
11	TSA	0.001 M Sr^{++} + PO_4^{-3} + K^+ (pH 6.5)
12	TSAS	
13	TSA	H_2O alone
14	TSAS	


A
B
C
1
2
3
4
5
6
7
8
9
10
11
12
13
14
LOG NO. CELLS/ML RECOVERY MEDIUM
HOURS
4.4
4.0
3.6
3.2
2.8
2.4
2.0
4.6
4.2
3.8
3.4
3.0
2.6
2.2
0
2
4
6

Effect of Sodium on the Recovery of Salt Tolerance

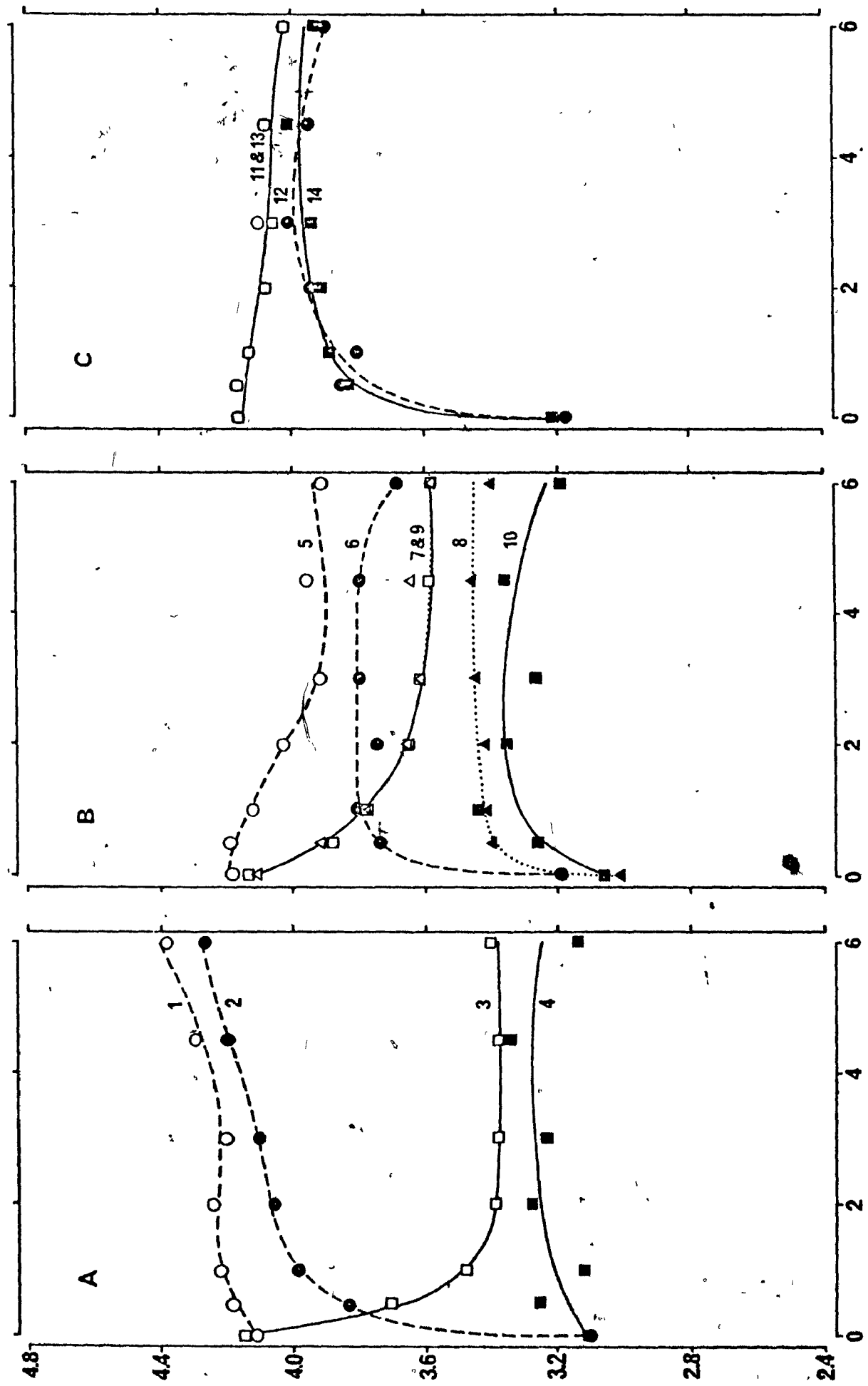
In previous experiments, recovery media had been prepared by adjusting the pH with 0.1 M NaOH, and using NaH_2PO_4 as the source of phosphate. To ascertain whether or not Na^+ enhanced recovery of salt tolerance in the presence of Mg^{++}, solutions of either 0.005 M Na_2SO_4 or 0.005 M K_2SO_4 were prepared with and without 0.001 M $MgSO_4 \cdot 7H_2O$. In the case of the solution of the Na^+ salt, the pH of the solution was adjusted with NaOH. Where the K^+ salt was employed, KOH was used to adjust pH. Frozen and thawed cells were added to each solution as well as to PKM medium and glass-distilled water to serve as controls. The results are seen in Figure 21.

Incubation of a suspension in the absence of Mg^{++} resulted in a rapid loss of viability as evidenced by the counts of the TSA plates which were similar whether incubation occurred in Na^+ or K^+. The addition of Mg^{++} to these solutions resulted in the viability of the suspensions decreasing at a much slower rate – a rate comparable to incubation in Mg^{++} alone. Similarly, although Mg^{++} alone permitted almost complete recovery, some loss in viability of the cells in suspension was evident. Both Na^+ and K^+, when added with Mg^{++}, reduced the loss of viability in the presence of Mg^{++} alone and to an almost identical extent.

Figure 21

Effect of Na^+ on the recovery of salt tolerance.

Curve	Plating Medium	
1	TSA	PKM
2	TSAS	
3	TSA	H_2O
4	TSAS	
5	TSA	0.001 M Mg^{++}
6	TSAS	
7	TSA	0.01 M Na^+
8	TSAS	
9	TSA	0.01 M K^+
10	TSAS	
11	TSA	0.01 M K^+ + 0.001 M Mg^{++}
12	TSAS	
13	TSA	0.01 M Na^+ + 0.001 M Mg^{++}
14	TSAS	


A
B
C
1
2
3
4
5
6
7&9
8
10
11&13
12
14
LOG NO. CELLS/ML RECOVERY MEDIUM
4.8
4.4
4.0
3.6
3.2
2.8
2.4
0
2
4
6
HOURS

Effect of pH on the Recovery of Salt Tolerance in PKM

To examine the effect of pH on recovery of salt tolerance, three volumes of PKM medium were prepared in the usual way but sufficient NaOH was added to the three volumes to achieve a final pH of 5.5, 7.0 and 8.5. A frozen and thawed suspension of cells was added to the three media in the usual manner with subsequent plating of aliquots into TSA and TSAS. The results are seen in Figure 22.

Viability and recovery of salt tolerance were similar at pH 7.0 and 5.5 Even brief exposure of the cells to pH 8.5, however, caused approximately half to lose their viability as indicated by the count in TSA at zero time. The viability of the remaining cells decreased rapidly as the suspension was plated on TSA over a period of six hours (curve 5). The initial counts on TSAS from this suspension (curve 6) were very low, but recovery of salt tolerance of the viable cells was seen up to six hours.

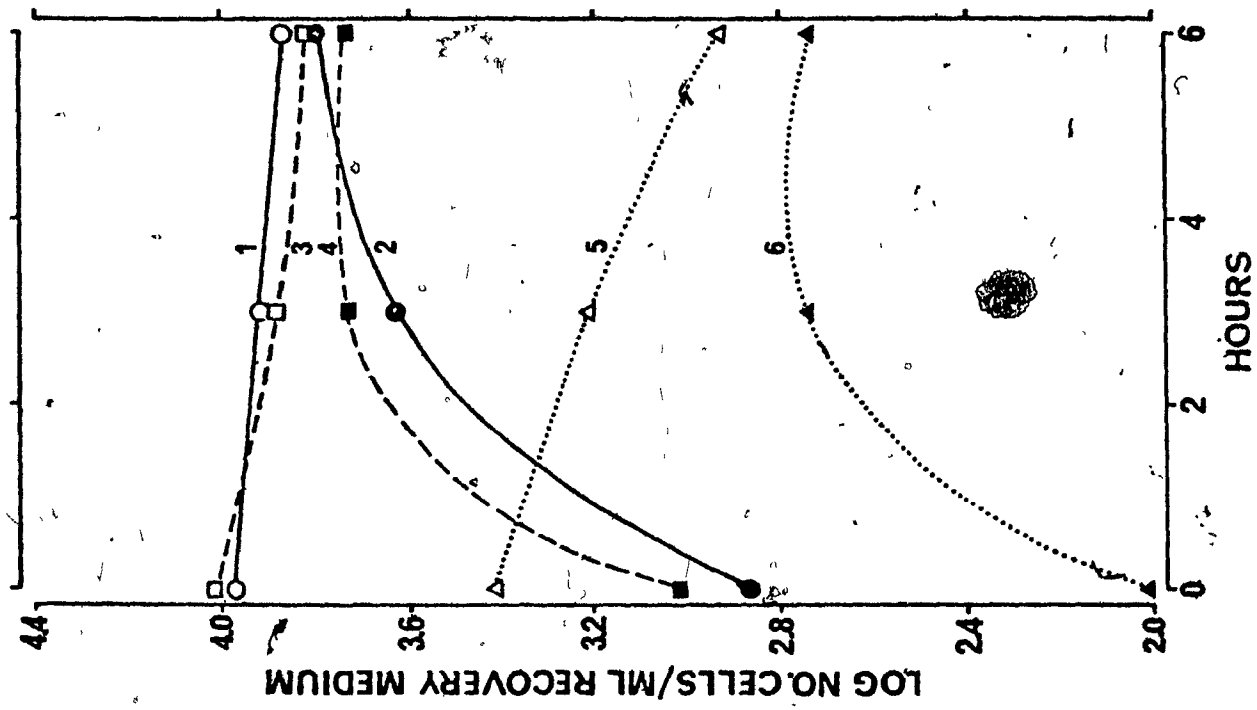
Effect of Temperature on Recovery of Salt Tolerance in PKM Medium

Since the recovery of salt tolerance of a frozen and thawed population occurred in the PKM medium without an added carbon source, it was of interest to note the effect of the incubating temperature upon this recovery. Accordingly, a frozen and thawed suspension was added to PKM media and incubated simultaneously at different temperatures in

Figure 22

Effect of pH on the recovery of salt tolerance in PKM recovery medium.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM at pH 7.0
2	TSAS	
3	TSA	PKM at pH 5.5
4	TSAS	
5	TSA	PKM at pH 8.5
6	TSAS	


LOG NO. CELLS/ML RECOVERY MEDIUM
4.4
4.0
3.6
3.2
2.8
2.4
2.0
HOURS
0
2
4
6
1
2
3
4
5
6

four waterbath shakers. The temperatures selected were 15°, 20°, 30° and 37°C. Aliquots from these incubating suspensions were plated into TSA and TSAS in the usual fashion and the results are seen in Figure 23.

In all cases, the counts obtained on TSAS were similar to those on TSA after six hours. The rate of recovery of salt tolerance at 30°C was found to be similar to the rate at 37°C as evidenced by the TSAS counts of the incubating suspensions at those temperatures.

At the lower temperatures, however, a slight decrease in the viability of the incubating populations was shown by the TSA counts, and the rate of recovery of salt tolerance was somewhat slower. After one hour, the counts on TSAS had increased by approximately log 0.3 at 15°C as compared to log 0.44 at 37°C.

Although slower, the rate of recovery at 15°C was significant and complete recovery of salt tolerance had occurred after six hours.

Addition of Cyanide to the PKM Recovery Medium

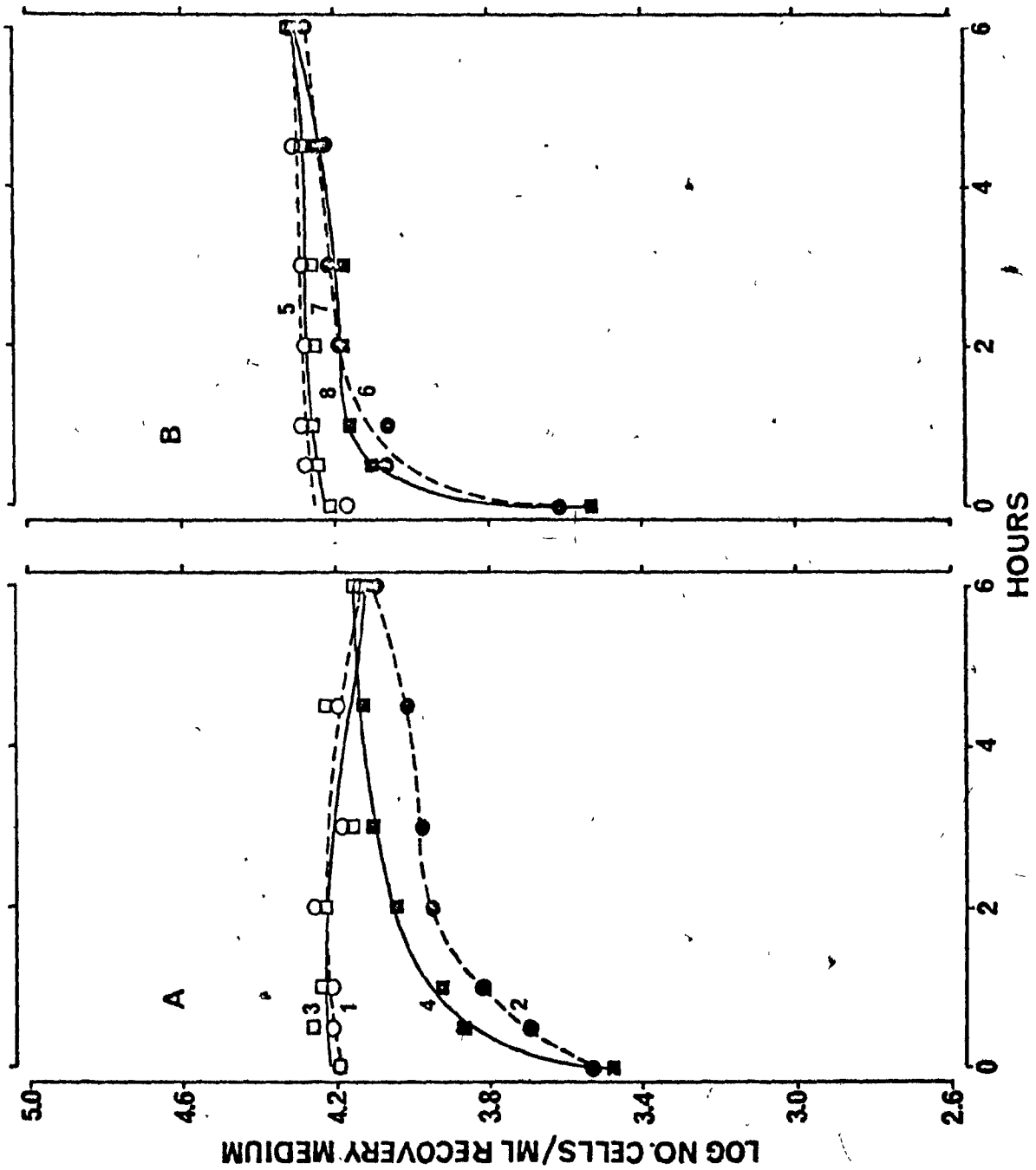
Recovery of salt tolerance in PKM was not inhibited by incubation of a frozen and thawed suspension of cells at 15°C and it was of interest to ascertain whether or not this recovery mechanism was affected by the addition of CN^- to the recovery medium.

To establish the optimum concentration of CN^-, a potent

Figure 23

Effect of temperature on recovery of salt tolerance in PKM recovery medium.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM at 15°C
2	TSAS	
3	TSA	PKM at 20°C
4	TSAS	
5	TSA	PKM at 30°C
6	TSAS	
7	TSA	PKM at 37°C
8	TSAS	


A
B
1
2
3
4
5
6
7
8
5.0
4.6
4.2
3.8
3.4
3.0
2.6
LOG NO. CELLS/ML RECOVERY MEDIUM
0
2
4
6
HOURS

inhibitor of the electron transport chain (Harold, 1972), a fresh, unfrozen suspension of cells was incubated in various recovery media with and without CN^-. The optimum concentration of CN^- for these experiments was considered to be one which would inhibit the multiplication of fresh, unfrozen cells without decreasing the viability of the cells up to six hours.

Fresh, unfrozen cells were incubated in PKM medium with and without aspartate in the presence and absence of 0.5 mM sodium cyanide. The results are seen in Figure 24. The addition of aspartate to the PKM medium resulted in the multiplication of cells after two hours as seen in the counts on both TSA and TSAS. The addition of CN^- to this medium prevented this multiplication without decreasing viability. Where Mg^{++} alone was employed as the suspending medium, the addition of CN^- resulted in a loss of viability of the cells.

The same experiment was performed with a frozen and thawed suspension and the results are seen in Figure 25. In the presence of PKM or PKM + aspartate, recovery of salt tolerance was rapid as evidenced by the counts on TSAS plates. When CN^- was added to these media, recovery of salt tolerance was retarded but not inhibited and the counts on TSAS approximated those on TSA after six hours. The multiplication of cells in PKM + aspartate was completely inhibited. The addition of CN^- to the Mg^{++} recovery medium resulted in a rapid loss of viability without any recovery of salt tolerance.

Figure 24

Addition of cyanide to various recovery media and its effect on a suspension of fresh (unfrozen) cells.

Cyanide was added as 0.5 mM NaCN and pH was adjusted to pH 6.5 with 1.0 M NaOH.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM (pH 6.5)
2	TSAS	
3	TSA	PKM + CN^-
4	TSAS	
5	TSA	PKM + aspartate (pH 6.5)
6	TSAS	
7	TSA	PKM + aspartate + CN^-
8	TSAS	
9	TSA	0.001 M Mg^{++}
10	TSAS	
11	TSA	0.001 M Mg^{++} + CN^-
12	TSAS	

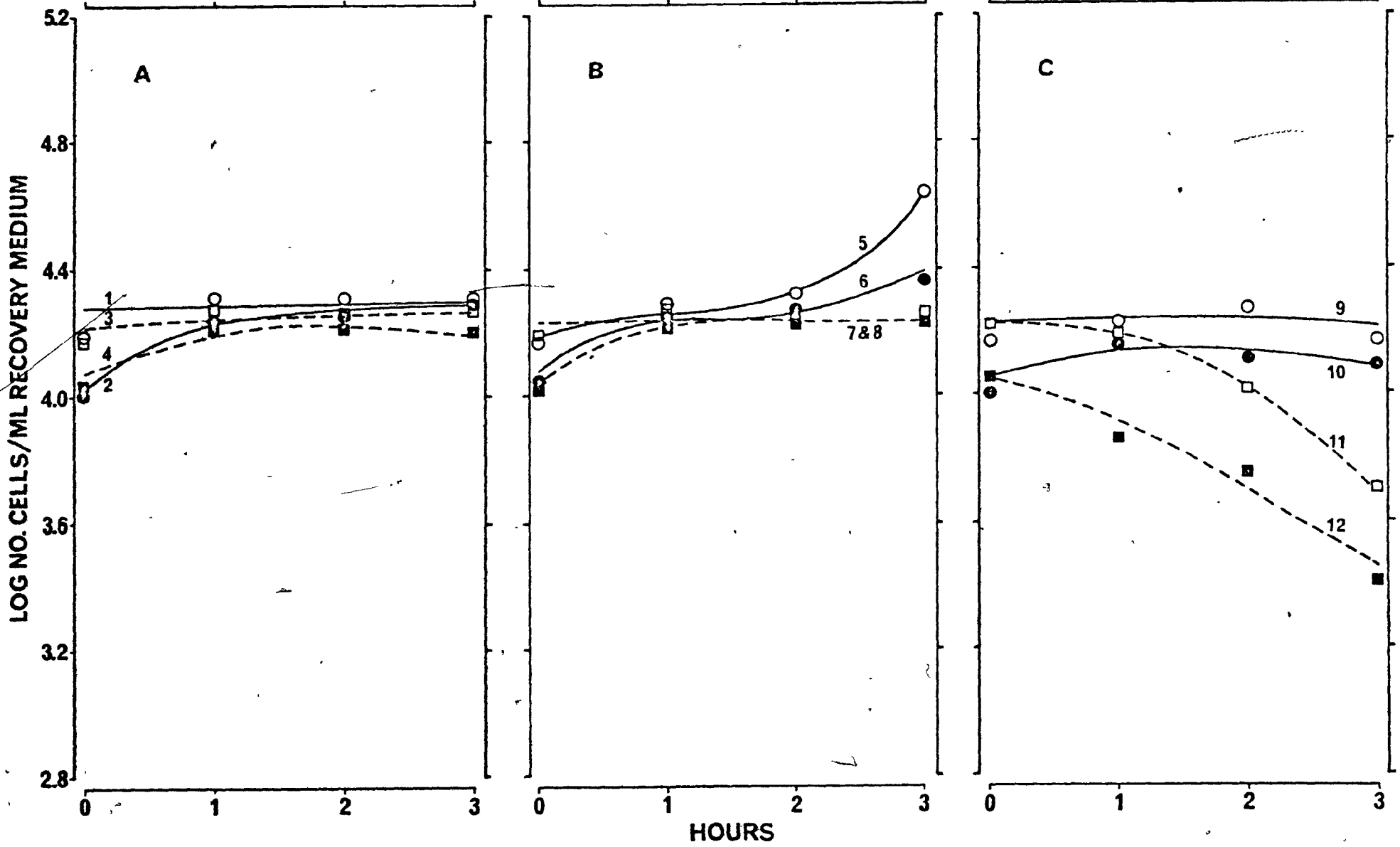
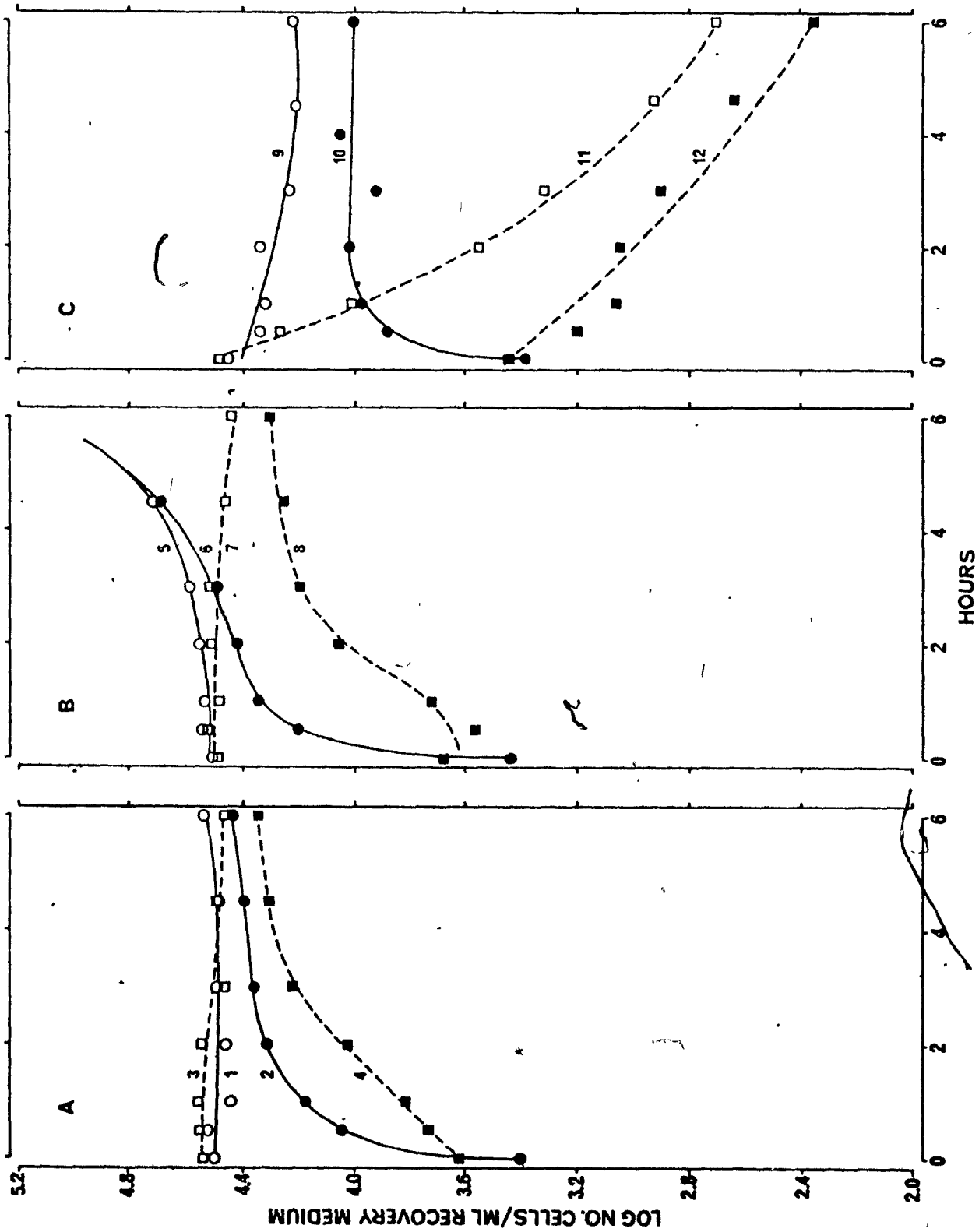

A
B
C
LOG NO. CELLS/ML RECOVERY MEDIUM
5.2
4.8
4.4
4.0
3.6
3.2
2.8
0
1
2
3
HOURS
1
2
3
4
5
6
7&8
9
10
11
12

Figure 25

Addition of cyanide to various recovery media and its effect on a suspension of frozen and thawed cells.

Cyanide was added as 0.5 mM NaCN and pH was adjusted to pH 6.5 with 1.0 M NaOH.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM (pH 6.5)
2	TSAS	
3	TSA	PKM + CN^-
4	TSAS	
5	TSA	PKM + aspartate (pH 6.5)
6	TSAS	
7	TSA	PKM + aspartate + CN^-
8	TSAS	
9	TSA	0.001 M Mg^{++}
10	TSAS	
11	TSA	0.001 M Mg^{++} + CN^-
12	TSAS	


A
B
C
1
2
3
4
5
6
7
8
9
10
11
12
LOG NO. CELLS/ML RECOVERY MEDIUM
5.2
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
0
2
4
6
HOURS

The results demonstrated that recovery of salt tolerance occurred in a concentration of CN^- sufficient to inhibit multiplication of cells.

Addition of CN^- + Iodoacetate to the PKM Recovery Medium

Although recovery had been demonstrated to occur in the presence of cyanide, there remained a possibility that recovery might be a function of energy derived from endogenous substrate-linked phosphorylation in the frozen and thawed cells.

To clarify this possibility, iodoacetic acid, an inhibitor of substrate-linked phosphorylation (Winkler and Wilson, 1966), was added to recovery media in the presence and absence of cyanide. Several concentrations of iodoacetate were tested on a suspension of frozen and thawed cells and 0.25 mM was found to maintain viability up to six hours in PKM.

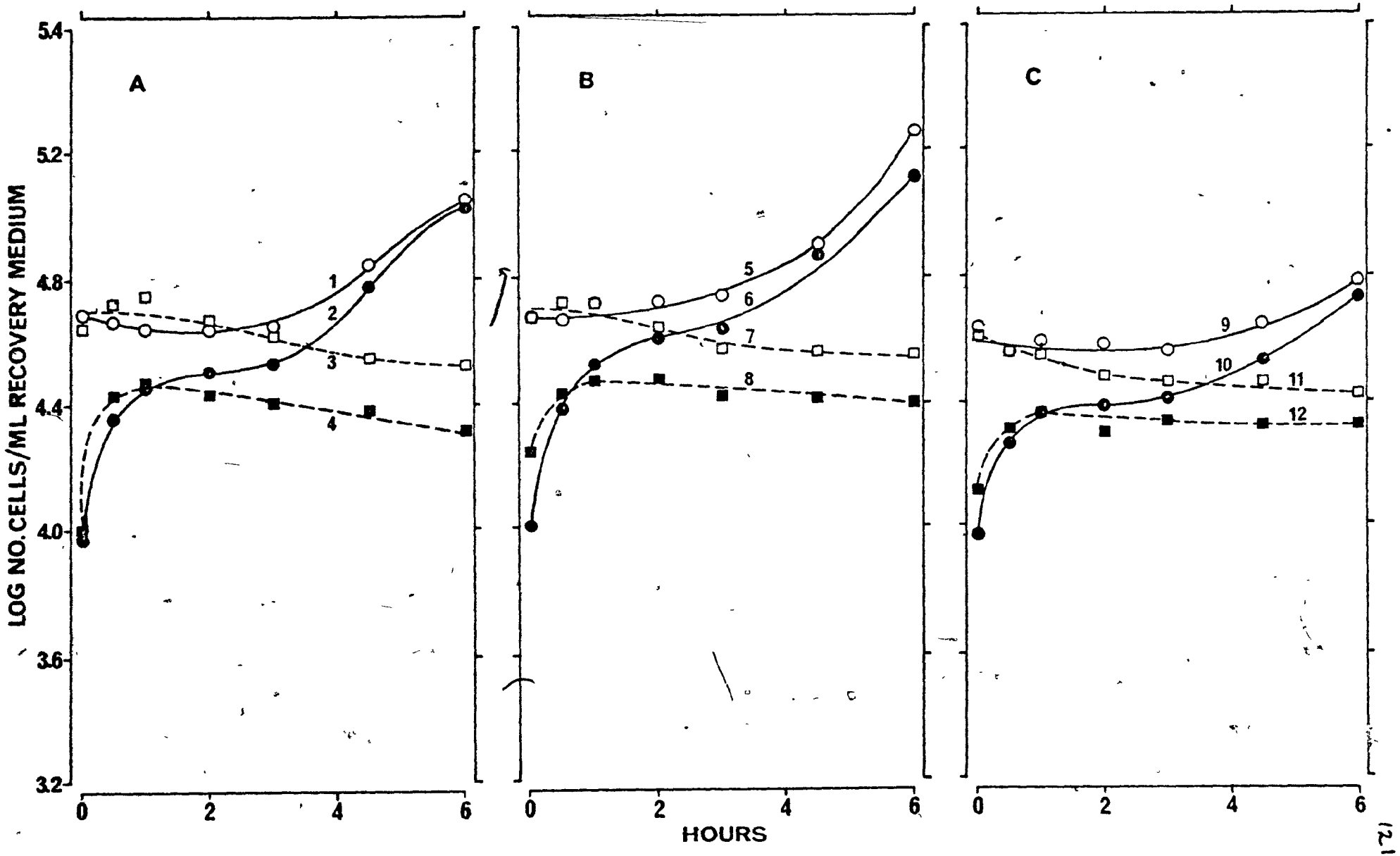
A frozen and thawed suspension of cells was added to PKM + CN^- and PKM + aspartate + CN^- recovery media with and without iodoacetate. PKM, PKM + aspartate, and PKM + aspartate + 0.5 mM CN^- served as controls. The results are seen in Figure 26. Recovery of salt tolerance was evident when PKM or PKM + aspartate were supplemented by CN^- and iodoacetate, although the viability of the population slowly decreased during the six-hour incubation period. Iodoacetate alone at the

Figure 26

Addition of iodoacetate to various recovery media supplemented with cyanide and its effect on a suspension of frozen and thawed cells.

Iodoacetate was added as 0.25 mM iodoacetic acid and cyanide as 0.5 mM NaCN. The pH was adjusted to 6.5 in all cases with 1.0 M NaOH.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM (pH 6.5)
2	TSAS	
3	TSA	PKM + CN^- + iodoacetate
4	TSAS	
5	TSA	PKM + 0.005 M aspartate
6	TSAS	
7	TSA	PKM + aspartate + CN^- + iodoacetate
8	TSAS	
9	TSA	PKM + aspartate + iodoacetate
10	TSAS	
11	TSA	PKM + aspartate + CN^-
12	TSAS	


A
B
C
LOG NO.CELLS/ML RECOVERY MEDIUM
5.4
5.2
4.8
4.4
4.0
3.6
3.2
0
2
4
6
HOURS
1
2
3
4
5
6
7
8
9
10
11
12

concentration used retarded but did not inhibit cell multiplication in PKM + aspartate recovery medium which suggested that the assimilation of aspartate was not inhibited by iodoacetate.

Addition of Chloramphenicol to the PKM Recovery Medium

Recovery of salt tolerance in PKM did not appear to be greatly affected by the presence of CN^- or CN^- + iodoacetate in the medium and it was of interest to note the effect of chloramphenicol on the recovery mechanism.

To establish the optimum concentration of chloramphenicol, an inhibitor of protein synthesis (Iandolo and Ordal, 1966), a suspension of fresh, unfrozen cells was incubated in various recovery media with and without chloramphenicol. As in previous experiments, the optimum concentration of chloramphenicol was considered to be one which would inhibit the multiplication of fresh, unfrozen cells without decreasing the viability of the cells in the suspension after incubating up to six hours.

Fresh, unfrozen cells were incubated in PKM medium with and without aspartate in the presence and absence of 100 µg/ml of chloramphenicol. The results are seen in Figure 27.

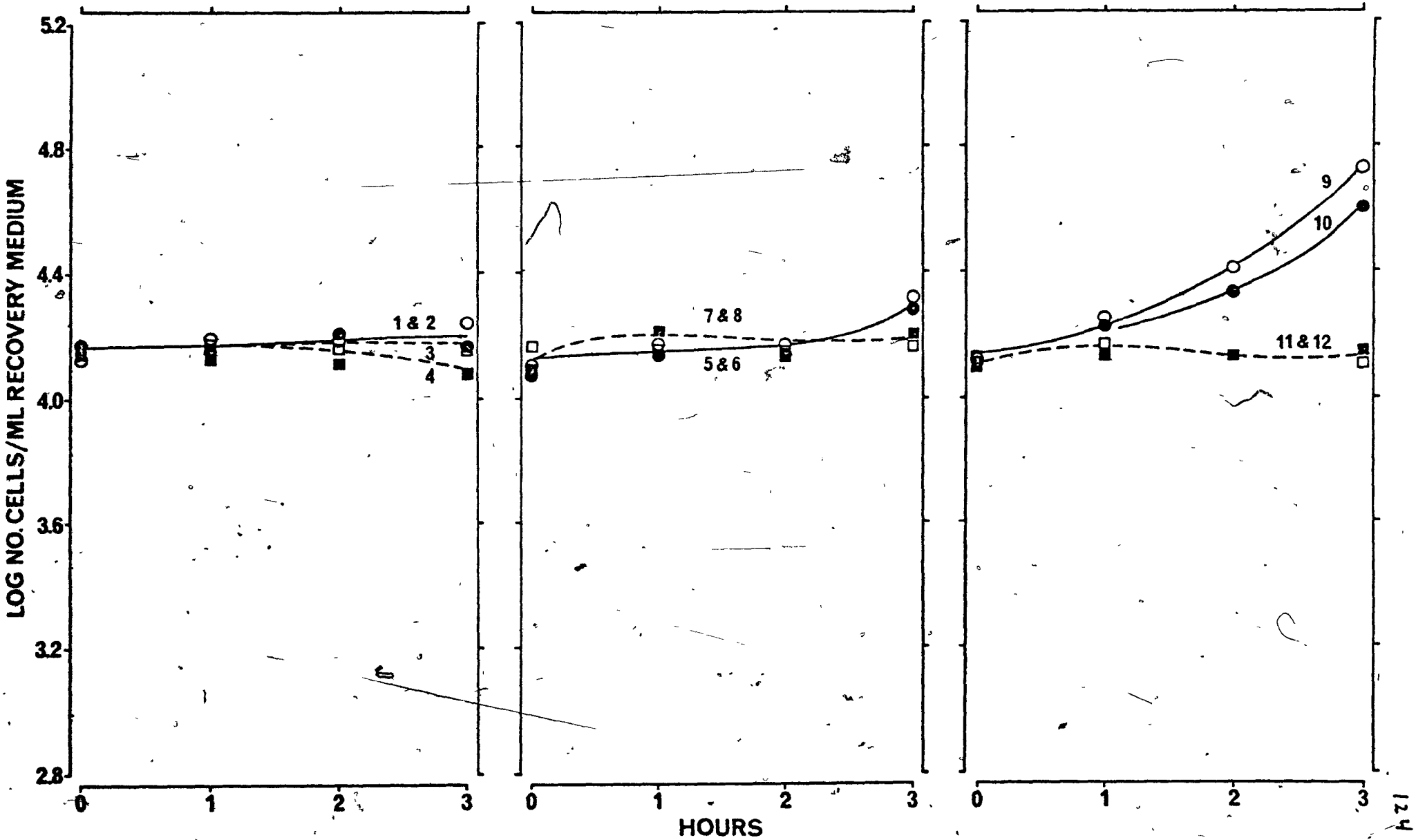
The addition of aspartate to the PKM medium resulted in the multiplication of cells after one hour as seen in the counts of both

Figure 27

Addition of chloramphenicol to various recovery media and its effect on a suspension of fresh (unfrozen) cells.

A sterile stock solution of chloramphenicol was added to prepared recovery media to yield a final concentration of 100 µg/ml.

Curve	Plating Medium	Recovery Medium
1	TSA	0.001 M Mg^{++}
2	TSAS	
3	TSA	0.001 M Mg^{++} + chloramphenicol
4	TSAS	
5	TSA	PKM
6	TSAS	
7	TSA	PKM + chloramphenicol
8	TSAS	
9	TSA	PKM + 0.005 M aspartate
10	TSAS	
11	TSA	PKM + 0.005 M aspartate + chloramphenicol
12	TSAS	


LOG NO. CELLS/ML RECOVERY MEDIUM
5.2
4.8
4.4
4.0
3.6
3.2
2.8
0
1
2
3
1 & 2
3
4
7 & 8
5 & 6
9
10
11 & 12
HOURS

TSA and TSAS. The addition of chloramphenicol to this medium prevented the multiplication of cells without decreasing the viability. Where Mg^{++} alone was employed as the suspending medium, the addition of chloramphenicol did not alter viability.

The same experiment was performed with a suspension of frozen and thawed cells and the results are seen in Figure 28.

The addition of chloramphenicol to the various media did not inhibit recovery of salt tolerance to any degree, although the multiplication of cells was completely inhibited in the PKM + aspartate medium as seen in the TSA counts from this suspension.

From the results, it could be concluded that the recovery process was not affected by the inhibition of protein synthesis. At the same time, the same concentration of chloramphenicol completely inhibited the multiplication of cells.

Addition of Actinomycin D
to the PKM Recovery Medium and to TSA Plates

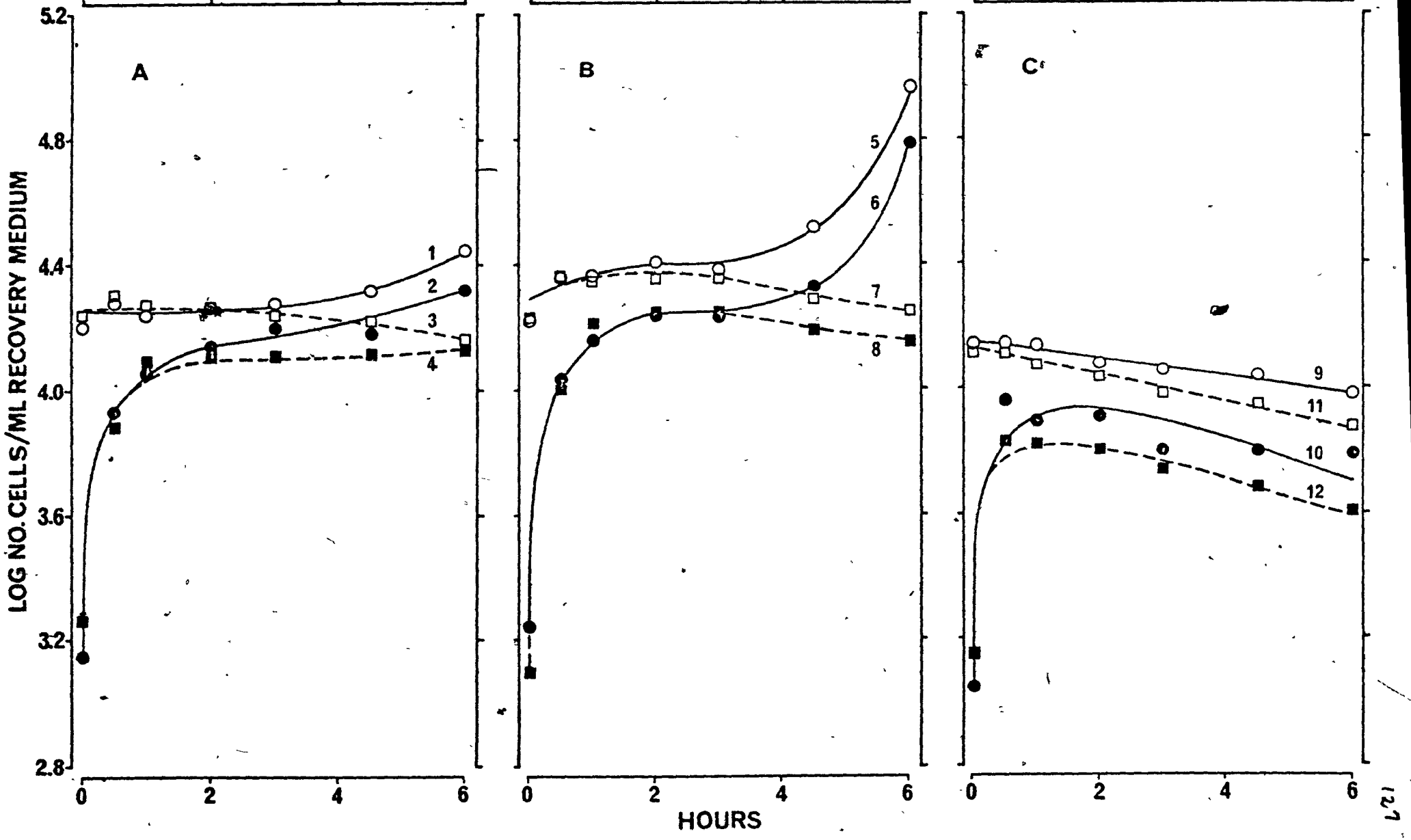
Actinomycin D has been found to inhibit the synthesis of messenger RNA in Gram-positive cells. Its ineffectiveness against Gram-negative cells has been attributed to the impermeability of the Gram-negative membrane and wall (Singh *et al.*, 1972). The purpose of these experiments was to demonstrate the increased permeability of the membrane of *Escherichia coli* 451R due to freezing and thawing.

Figure 28

Addition of chloramphenicol to various recovery media and its effect on a suspension of frozen and thawed cells.

A sterile stock solution of chloramphenicol was added to prepared recovery media to yield a final concentration of 100 µg/ml.

Curve	Plating Medium	Recovery Medium
1	TSA	PKM
2	TSAS	
3	TSA	PKM + chloramphenicol
4	TSAS	
5	TSA	PKM + 0.005 M aspartate
6	TSAS	
7	TSA	PKM + 0.005 M aspartate + chloramphenicol
8	TSAS	
9	TSA	0.001 M Mg^{++}
10	TSAS	
11	TSA	0.001 M Mg^{++} + chloramphenicol
12	TSAS	


A
B
C
LOG NO. CELLS/ML RECOVERY MEDIUM
5.2
4.8
4.4
4.0
3.6
3.2
2.8
0
2
4
6
HOURS
1
2
3
4
5
6
7
8
9
10
11
12

A fresh unfrozen suspension of cells was incubated in PKM medium with subsequent plating of aliquots of the incubating suspension into plates containing TSA, TSAS, and TSA + 50 µg/ml of actinomycin D (Merck, Sharp & Dohme). To prepare the last medium, sterile actinomycin D was aseptically added with a disposable syringe to tubes of TSA medium which had been sterilized and cooled to 45°C. The results are seen in Figure 29A. The counts obtained on the three media were similar in all cases. The addition of actinomycin D to TSA had no effect on colony formation.

In a similar experiment with a frozen and thawed suspension of cells (Figure 29B), the cells in the initial aliquot taken from PKM medium showed a sensitivity to the presence of actinomycin D in TSA plating medium (curve 6) although not to the same degree as sensitivity to NaCl (curve 5). Incubation of the frozen and thawed suspension in PKM medium resulted in a recovery of tolerance to actinomycin D. At six hours the counts on all three plating media were similar.

The addition of a frozen and thawed suspension to PKM medium containing 50 µg/ml of actinomycin D resulted in a rapid loss of viability (curve 7) without any demonstrable recovery of salt tolerance (curve 8).

Figure 29

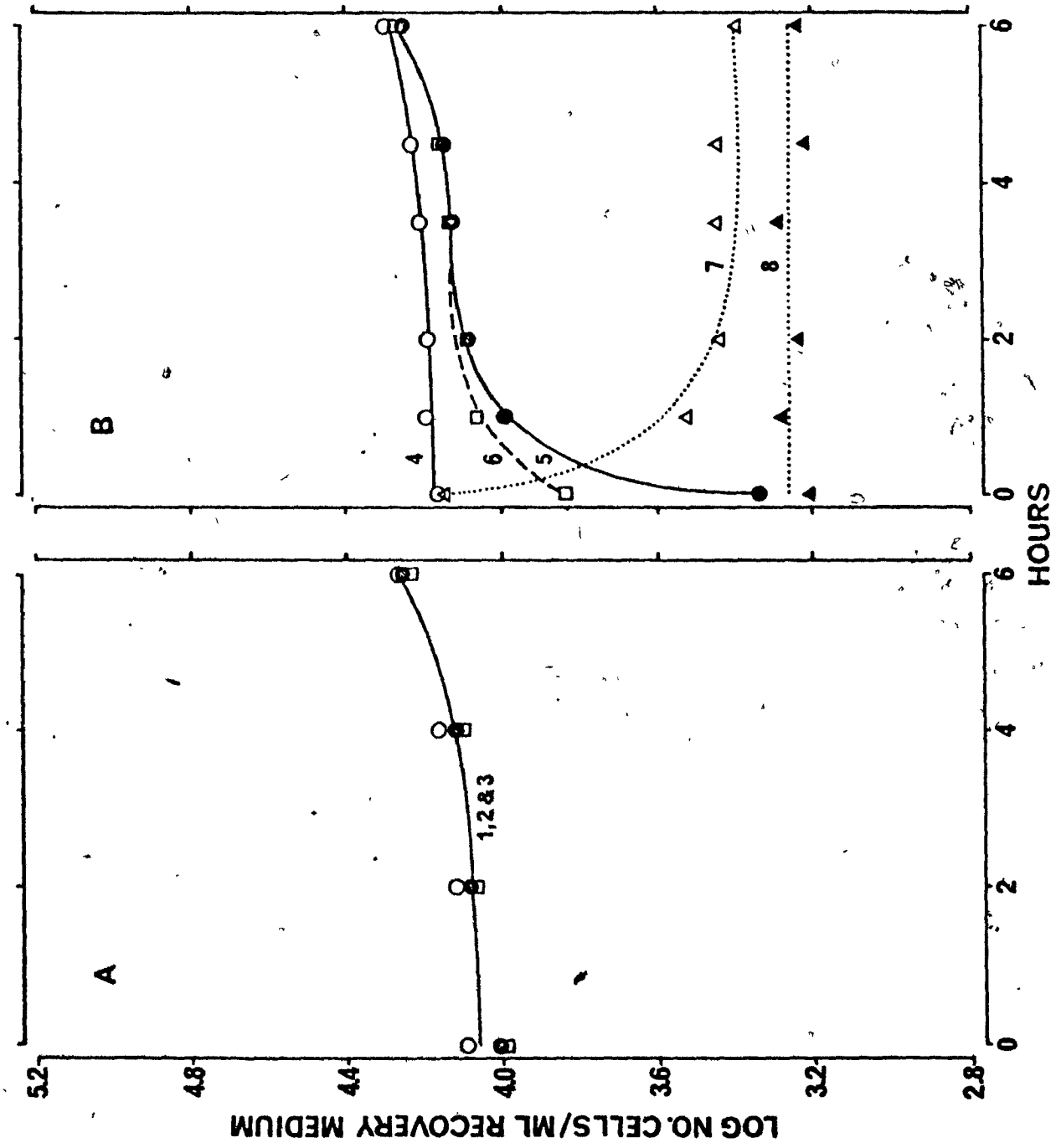
A. Addition of actinomycin D to the TSA plating medium and its effect on fresh (unfrozen) cells.

Curve	Plating Medium	Recovery Medium
1	TSA	
2	TSAS	PKM
3	TSA + actinomycin D	

B. Addition of actinomycin D to the PKM recovery medium and to the TSA plating medium and its effect on frozen and thawed cells.

Curve	Plating Medium	Recovery Medium
4	TSA	
5	TSAS	PKM
6	TSA + actinomycin D	
7	TSA	PKM + actinomycin D
8	TSAS	

A sterile stock solution of actinomycin D was added to prepared media to yield a final concentration of 100 µg/ml.


LOG NO. CELLS/ML RECOVERY MEDIUM
5.2
4.8
4.4
4.0
3.6
3.2
2.8
A
1,2&3
B
4
6
5
7
8
0
2
4
6
HOURS

Tolerance of a Frozen and Thawed Population to Deoxycholate in the Plating Medium

Several investigators have employed nutritionally-adequate plating media with and without deoxycholate as a criterion of freezing damage. Sodium deoxycholate, a surface-active agent (Sinskey and Silverman, 1970), has been found to inhibit the growth of damaged cells in plating media, at a concentration which allows colony formation of undamaged cells.

To investigate the effect of this agent on *Escherichia coli* 451B, TSA plates containing 0.2% sodium deoxycholate (TSAC) were prepared at pH 7.0. Aliquots of a fresh, unfrozen suspension of cells were found to be capable of growth on this medium. A frozen and thawed population of cells was prepared in the usual fashion and inoculated into PKM and the same medium with Mg^{++} deleted. Aliquots of both suspensions were subsequently plated into TSA, TSAS and TSAC and the results are seen in Figure 30.

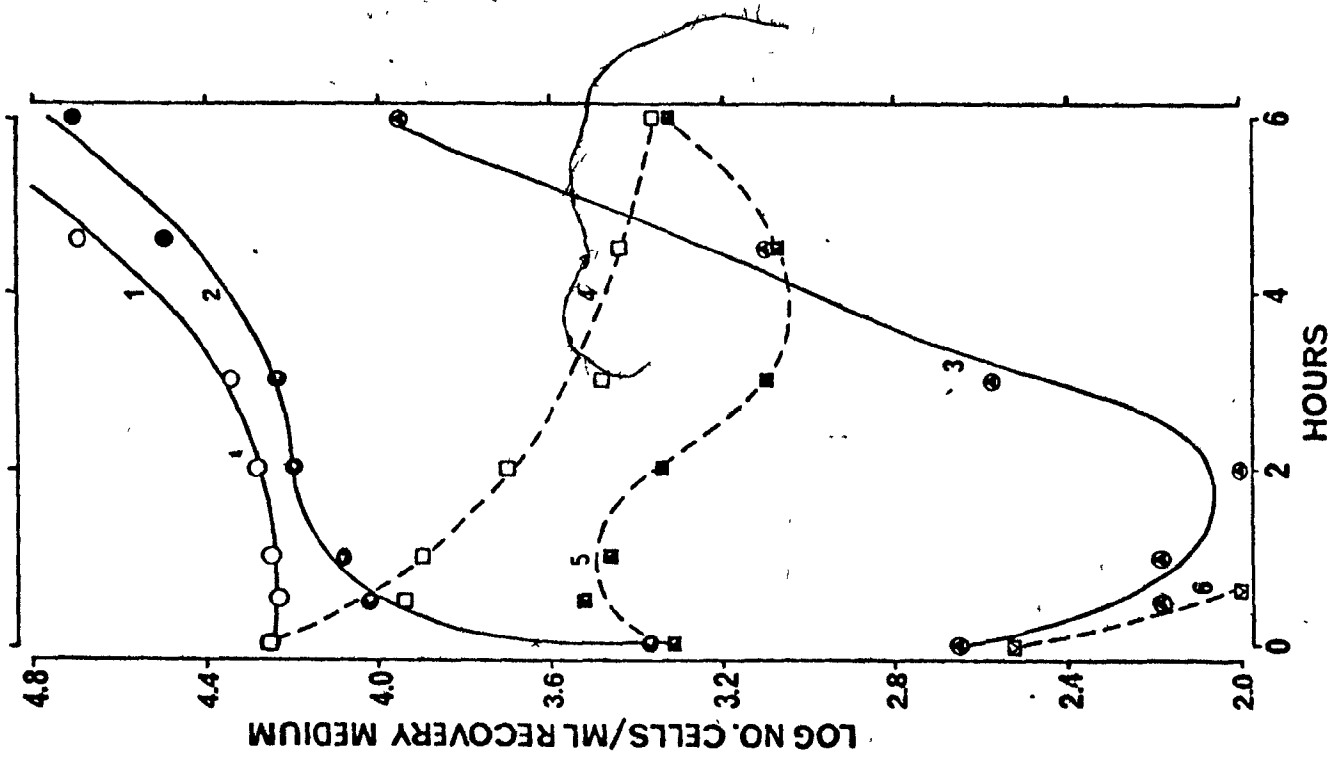
Recovery of salt tolerance was seen after incubation in PKM (curve 2), but the count on TSAC (curve 3) declined rapidly in the first two hours of incubation. After two hours, the increasing count on TSAC paralleled the increase seen on TSA plates (curve 1). In the absence of Mg^{++} from the recovery medium, no recovery of salt tolerance was observed (curve 5) and the viability of the suspended cells decreased rapidly (curve 4). After 0.5 hours incubation in this recovery medium,

Figure 30

Tolerance of a suspension of frozen and thawed cells to deoxycholate in the plating medium.

Deoxycholate was added to the plating medium as 0.2% sodium deoxycholate and the pH was adjusted to pH 7.0 with 1.0 M NaOH.

Curve	Plating Medium	Recovery Medium
1	TSA	
2	TSAS	PKM
3	TSA + deoxycholate	
4	TSA	
5	TSAS	PKM without Mg^{++}
6	TSA + deoxycholate	


LOG NO. CELLS/ML RECOVERY MEDIUM
4.8
4.4
4.0
3.6
3.2
2.8
2.4
2.0
HOURS
0
2
4
6
1
2
3
4
5
6

subsequent plating of the suspension on TSAC did not produce any colonies (curve 6). Although the PKM medium supported the recovery of salt tolerance by damaged cells, similar suspensions of cells failed to grow when plated on a deoxycholate medium.

DISCUSSION

DISCUSSION

MacLeod, Kuo and Gélinas (1967) found a difference in count when a suspension of frozen and thawed cells of *Aerobacter aerogenes* was plated on minimal medium with and without Trypticase. A higher count was obtained in the presence of Trypticase. The difference was demonstrated to result from the presence of Cu^{++} in the plating diluent. The inhibitory effect of Cu^{++} was overcome by the addition either of Trypticase or cysteine, a chelating agent, to the minimal medium or by employing glass-distilled water in the preparation of the plating diluent. A similar difference in count was observed with frozen and thawed cells of *Escherichia coli* 451B, but was not overcome by the addition of cysteine to the minimal plating medium and was improved but not eliminated by removing Cu^{++} from the plating diluent. Acid-hydrolysed Trypticase yielded the same counts as Trypticase when employed as a supplement to the plating medium. Aspartic acid, glutamic acid and alanine were as effective as Trypticase as supplements.

Kuo and MacLeod (1969) proposed two possibilities to account for the increased count on minimal medium supplemented with aspartic acid:

1. Aspartate was required to replenish amino acid pools leaked in

the freezing and thawing process.

2. Aspartate was specifically required in a mechanism to repair damage resulting from freezing and thawing.

In the present work, initial experiments confirmed the findings of Kuo and MacLeod that aspartate added to a minimal plating medium increased the plate count of a suspension of *Escherichia coli* 451B after freezing, although approximately 90% of the initial population remained incapable of forming colonies. It was also shown, however, that preliminary incubation of frozen and thawed cells in the minimal medium containing aspartate but without agar increased the number of cells able to grow upon subsequent plating in the medium two fold and eliminated the response to aspartate in this plating medium. Similarly, incubation in a simpler preincubation medium of aspartate and potassium and magnesium salts eliminated the subsequent response to aspartate in the plating medium.

When salt tolerance was employed as a criterion of metabolic damage, rather than response to aspartate in the plating medium, the number of injured cells detectable in the population after freezing was increased several fold. This was in part due to the fact that when salt tolerance was tested, the plating medium used was Trypticase soy agar, a complex medium permitting many more of the injured cells to grow than the minimal medium plus aspartate. The addition of 0.55 M NaCl to this plating medium reduced the plate count on a suspension of *Escherichia coli*

after freezing. If the cells in the suspension were preincubated in minimal medium with or without aspartate or in a solution containing aspartate together with potassium and magnesium salts, the plate count on the suspension obtained using the NaCl-containing plating medium was restored to that obtained using the plating medium without added NaCl. In other words, preincubation had restored the salt tolerance of the frozen and thawed cells.

When attempts were made to determine which components of the recovery medium were required to obtain recovery of salt tolerance, it was discovered that the presence of aspartate or any other carbon source was not required. A formulation consisting of phosphate, potassium and magnesium salts (PKM recovery medium) was derived from the basal salts solution and its composition was based on the minimum concentration of each component which, when present together with the other components, would allow recovery of salt tolerance without cell multiplication or loss of viability. Recovery of cells in the PKM recovery medium was not affected by the addition of aspartate.

When a frozen and thawed suspension of cells was plated on several media after various periods of preincubation in PKM recovery medium, the counts on all media ultimately arrived at values similar to those obtained on Trypticase soy agar. A large proportion of the suspension was unable initially to form colonies on Trypticase soy agar containing 0.55 M NaCl, but this proportion was rapidly reduced by

incubation in PKM recovery medium. A proportion of the total viable cells in the suspension was also unable to form colonies on minimal plating medium initially but gained this ability during incubation in the PKM recovery medium. It cannot be ascertained whether the cells in suspension which failed to grow initially on both plating media were, in fact, composed of cells with similar or the same kinds of cellular damage. PKM recovery medium restored salt tolerance. It also restored the ability of some frozen and thawed cells to initiate multiplication and colony formation in the minimal plating medium.

Mg^{++} is the most important component of the PKM recovery medium and its addition to a recovery medium composed of aspartate and potassium salts enhanced recovery of salt tolerance. In the absence of Mg^{++}, recovery in all suspending media tested was minimal and viability of the cells in suspension after freezing was maintained only where Mg^{++} was present.

In some experiments, a solution of a Mg^{++} salt alone as the suspending medium was found to effect a degree of recovery of salt tolerance, but not in all experiments. Recovery in this case appeared to occur where a larger population of cells was present in the recovery media. It seemed possible that in a cell suspension of greater cell density, a higher concentration of leakage products from the cells would be present in the suspending medium. This leakage could contribute factors responsible for repair of metabolic damage. An experiment was

designed to test this possibility, but the results were inconclusive.

In the recovery of salt tolerance during incubation in PKM recovery medium, the initial lesion to be repaired is probably membrane permeability. This is indicated by the rapid recovery of salt tolerance. Neither aspartate nor glucose affected the rate of this recovery. That a carbon source is required for subsequent multiplication and growth but not for an early return of salt tolerance was shown by manometric measurements accompanied by a concurrent plating experiment. Utilization of oxygen by frozen and thawed cells in PKM recovery medium supplemented with aspartate occurred after four hours, as multiplication of cells began, whereas recovery of salt tolerance occurred within one-half hour. When glucose was added to the PKM recovery medium, recovery of salt tolerance was again rapid but the rate of respiration was very slow and cell multiplication had not begun after ten hours of incubation.

Differences in rate of respiration were similarly noted with fresh, unfrozen cells incubated in PKM recovery medium supplemented with glucose or aspartate. Utilization of oxygen by fresh, unfrozen cells was much slower in PKM recovery medium supplemented with glucose as compared to incubation in the same medium supplemented with aspartate.

Ray and Speck (1972a) found that glucose alone was ineffective in repairing metabolic injury. This was attributed to freeze-injury of some component(s) of the glucose transport system. Sanwal (1970) has noted that the enteric bacteria live largely by anaerobic glycolysis

even when growing on glucose aerobically. A number of crucial enzymes of the citric acid cycle such as succinic dehydrogenase, succinic thiokinase, α-ketoglutarate dehydrogenase and cytochromes are repressed in the presence of glucose. Specifically, the reduced NADH produced by glycolysis inhibits citrate synthetase such that the citric acid cycle serves only a biosynthetic function and ATP is produced primarily through substrate phosphorylation in the glycolytic pathway.

Both fresh and frozen and thawed cells of *Escherichia coli* 451B presumably utilize glucose via the glycolytic pathway. This situation would be reflected in a glucose plating medium, such as minimal plating medium, where a proportion of the cells plated from a frozen and thawed suspension would utilize a carbon source which gives rise to only two moles of ATP per mole of glucose. Consequently, they might lose their viability during the long growth lag following freezing and thawing. When supplemented with aspartate, however, the minimal plating medium could provide a more suitable energy and carbon substrate since much more ATP is synthesized per mole of aspartate metabolized. This could explain why approximately twice as many injured cells are able to form colonies when aspartate is present.

The effect on frozen and thawed cells of supplementing recovery media or minimal plating medium with aspartate, therefore, appears to have a nutritional basis. The same would be true of other organic substrates that are not metabolized by the glycolytic pathway. Aspartate

probably participates in the replenishment of essential metabolites during the lag before growth and division but does not appear to have a specific role in the early repair of frozen and thawed cells.

A requirement for Mg^{++} in the recovery of salt tolerance was demonstrated by substituting other ions for Mg^{++} in the PKM recovery medium. Only Ca^{++} appeared to effect some degree of recovery and complete viability of the suspension was not maintained. No other divalent or monovalent cation tested had any pronounced effect on a frozen and thawed suspension of cells except Mn^{++}, which caused a rapid decrease in viability. These results are in contrast to those obtained by Ray, Janssen and Busta (1972) working with *Salmonella anatum*, and Ray and Speck (1972a, b) working with *Escherichia coli*. In both cases phosphate proved to be the major ionic component responsible for repair of freeze-injury. In both cases, Mg^{++} increased the degree of recovery of tolerance to deoxycholate but was not essential to the process.

In the above cited work, optimum phosphate repair occurred at pH 8.0 for *Salmonella anatum* and pH 8.5 for *Escherichia coli*. In the case of *Escherichia coli* 451B, repair was effected at pH 7.0 or lower, and viability decreased rapidly at pH 8.5. The phosphate + magnesium solution of Ray and Speck contained the same ionic species as the PKM recovery medium, although at different concentrations. The results would suggest that different factors are involved in the recovery

of tolerance to deoxycholate.

At 15°C, recovery of *Escherichia coli* 451B in PKM was reduced to about half the rate at 30 or 37°C. That recovery did occur would indicate that Mg^{++} had been effective at that temperature. In contrast, Ray and Speck (1972a) found no repair with phosphate at 15°C and optimal repair occurred between 25 and 35°C — observations which suggested a definite energy requirement to the authors. In the case of *Escherichia coli* 451B, the rate of recovery of salt tolerance in PKM recovery medium appeared to be dependent on temperature. Dependence of rate on temperature might suggest the involvement in the repair mechanism of a chemical reaction such as chelation.

To ascertain the role of metabolic energy in the recovery process, cyanide and iodoacetate were employed as inhibitors of energy metabolism. Cyanide has been shown to be an effective inhibitor of oxidative phosphorylation and iodoacetate has been employed to inhibit substrate-linked phosphorylation. In the present study, a concentration of cyanide was chosen which would inhibit growth of unfrozen cells without decreasing the viability of the population. Since the level of iodoacetate could not be correlated with inhibition of growth, the maximum concentration which would not decrease the viability of a frozen and thawed suspension was chosen. This concentration was within the range of concentrations employed by other investigators to inhibit glycolysis (Green, Needham and Dewan, 1937).

Recovery was apparent in the PKM recovery medium with or without added aspartate and was retarded but not inhibited by the addition of CN^-. When the PKM recovery medium was supplemented by aspartate and CN^-, however, the multiplication of cells following recovery was completely inhibited. In other words, CN^--sensitive processes required for cell multiplication were not required for recovery, and probably include respiration. Interestingly, when a recovery medium consisting of a solution of Mg^{++} alone was supplemented with CN^-, there was a rapid decline in viability whether the cells were fresh or frozen and thawed. The reason for this remains unknown.

When CN^- and iodoacetate were added to the PKM recovery medium, recovery was apparent in the presence and absence of aspartate, but cell multiplication was inhibited. That iodoacetate and CN^- had different effects on the cell suspension was apparent from the multiplication of cells which occurred in the PKM recovery medium supplemented with aspartate and iodoacetate. The utilization of aspartate was not inhibited by iodoacetate presumably because substrate-linked phosphorylation is not important to this process. Since no criterion was established for the effectiveness of iodoacetate as an inhibitor of substrate-linked phosphorylation, it can only be assumed that the concentration employed was sufficient to inhibit this process.

In the study of Ray, Janssen and Busta (1972), repair of freeze-injured *Salmonella anatum* was almost completely inhibited by CN^-.

Repair of a frozen and thawed suspension of *Escherichia coli* was reduced 75% in the investigation of Ray and Speck (1972b). In both cases, the repair was induced by phosphate buffer and these authors concluded that energy metabolism was a requirement in the phosphate repair process. In the present study, energy metabolism was not considered to be a factor in Mg^{++}-mediated recovery of salt tolerance.

The addition of chloramphenicol to PKM recovery medium did not inhibit recovery although multiplication of both fresh and frozen and thawed cells was inhibited. Similar results were obtained by Ray, Janssen and Busta (1972) and Ray and Speck (1972b). In a study of sublethally heat-treated *Staphylococcus aureus* (Iandolo and Ordal, 1966), repair of heat injury was not affected by chloramphenicol. However, chloramphenicol did inhibit the recovery of heat-treated *Salmonella typhimurium* (Tomlins and Ordal, 1971). In this instance, protein synthesis appeared to be required for the synthesis of ribonucleotide protein. In the present study of freeze-damage, protein synthesis did not appear to be a prerequisite to recovery of salt tolerance.

An altered permeability of the cell membrane of *Escherichia coli* 451B after freezing, was demonstrated in several ways. Knowles (1971) determined that *Escherichia coli* was impermeable to 0.2 M NaCl. Fresh, unfrozen cells of *Escherichia coli* 451B were capable of forming the same number of colonies in Trypticase soy agar plates with or without 0.55 M NaCl. After freezing and thawing, however, the same

suspension of cells showed a sensitivity to the presence of NaCl in the plating medium since colony formation was greatly reduced compared to counts obtained on Trypticase soy agar without NaCl. This suggested an alteration of permeability to NaCl.

Frozen and thawed cells of *Escherichia coli* 451B demonstrated a sensitivity to actinomycin D. When a suspension of fresh, unfrozen cells of *Escherichia coli* 451B was plated on Trypticase soy agar with and without actinomycin D, the counts obtained were similar in both cases. Like other gram negative bacteria (Singh *et al.*, 1972), the growth of *Escherichia coli* 451B was unaffected by actinomycin D a potent inhibitor of ribonucleic acid (RNA) synthesis. When a suspension of frozen and thawed cells was plated on similar media, however, an initial sensitivity was indicated by lower counts in the presence of actinomycin D than those obtained on Trypticase soy agar alone.

Singh *et al.* (1972) demonstrated that *Escherichia coli* mutants produced by irradiation had altered cytoplasmic membranes which resulted in varying responses to actinomycin D and varying degrees of β-galactosidase leakage (an intracellular or membrane-bound enzyme). A wild strain of *Escherichia coli* was found to be insensitive to actinomycin D as were spheroplasts of this strain produced by sucrose-lysozyme treatment. Spheroplasts of the wild strain produced by ethylenediaminotetraacetate (EDTA)-lysozyme treatment, however,

demonstrated a sensitivity to actinomycin D similar to that seen with the mutant strains. Since both preparations of spheroplasts had an altered cell envelope but only EDTA-treated spheroplasts were sensitive to actinomycin D, the authors concluded that the cytoplasmic membrane, not the outer lipopolysaccharide (LPS) component of the envelope, was the ultimate barrier to actinomycin D penetration into gram negative cells. Cells exposed to EDTA underwent an alteration of the cytoplasmic membrane as well as the outer lipopolysaccharide allowing entry of actinomycin D.

The evidence presented by Singh *et al.* would suggest that sensitivity of frozen and thawed cells to actinomycin D results from an altered cytoplasmic membrane. A frozen and thawed suspension of *Escherichia coli* 451B sensitive to actinomycin D demonstrated an even greater sensitivity to NaCl. This was indicated by the counts obtained when a frozen and thawed suspension was plated on Trypticase soy agar supplemented with actinomycin D or NaCl. If membrane damage is assumed, then the degree of damage in a proportion of cells was sufficient to allow entry of NaCl but not actinomycin D. Those cells sensitive to actinomycin D were presumably sensitive to NaCl as well. It is reasonable to conclude that colonies on plates containing actinomycin D possessed intact membranes or membranes with sufficient integrity to be impermeable to actinomycin D. Since the count on plates containing actinomycin D increased after incubation of a frozen and thawed suspension in PKM recovery medium, it was concluded that this recovery medium

rendered the cells impermeable to actinomycin D by repairing lesions in the membrane. The addition of actinomycin D to the recovery medium itself resulted in a rapid inactivation of the cell suspension with a resultant loss of colony-forming ability on Trypticase soy agar. Recovery of salt tolerance in this instance was not observed.

Leive (1965a, 1965b) treated *Escherichia coli* with EDTA and obtained release of lipopolysaccharide accompanied by the appearance of sensitivity to actinomycin D. This surface action of EDTA suggested that *Escherichia coli*, normally impermeable to actinomycin D, was rendered permeable by the loss of 30-50% of the lipopolysaccharide component of the cell wall. Citing this literature, Bretz and Kocka (1967) concluded that sensitivity of *Escherichia coli* to actinomycin D after freezing and thawing resulted from damage to the wall when bacteria were held at freezing temperatures. These authors further stated that if the environmental conditions were appropriate and the cell membrane remained intact, although "injured", then the cell could recover to produce viable progeny. If the disruption of the wall was sufficiently great and the post-thawing environment was not appropriate for recovery of the wall, then presumably the membrane also lost its integrity, cytoplasmic constituents were lost, and death resulted. In a subsequent paper, Leive (1968) suggested that EDTA acted by chelating a metal ion on the cell surface — presumably Mg^{++}. Once the cell surface had been exposed to EDTA, adding back metal ions would not restore the permeability barrier of the wall unless the cell

was allowed to metabolize. Since synthesis of protein, ribonucleic acid (RNA) or mucopeptide was not required, the results suggested to Leive that the initial action of EDTA was followed by a steric or chemical change in the cell surface which required energy metabolism for its reversal.

Ray and Speck (1972a, 1972b) demonstrated what they considered to be an energy-dependent repair process in frozen and thawed cells of *Escherichia coli* when incubated in a recovery medium consisting of phosphate buffer. Cells were initially sensitive to lysozyme, sodium lauryl sulphate and deoxycholate, but increasing tolerance was observed after incubation in phosphate buffer. Citing Bretz and Kocka (1967) and Leive (1968), the authors suggested that cells demonstrating such sensitivities had undergone alterations of the lipopolysaccharide component of the outer envelope and would necessarily be permeable to actinomycin D. Recovery of tolerance to deoxycholate occurred after incubation in phosphate buffer supplemented with actinomycin D, and since permeability to actinomycin D was assumed, the authors concluded that RNA synthesis was not involved in the energy-dependent repair process. They suggested that the repair of metabolic injury involving the synthesis of protein and RNA as seen in sublethal heat treatment (Tomlins and Ordal, 1971) and freeze-drying (Sinskey and Silverman, 1970) was different from what they concluded was ATP-dependent repair of freeze-injury in phosphate buffer. Similar results and conclusions have been reported by Ray, Janssen and Busta (1971). In subsequent

work, Ray, Speck and Dobrogosz (1973) further characterized freeze-damage to the lipopolysaccharide with bacteriophage studies. T-series phages, specific for lipopolysaccharide, failed initially to adsorb on frozen and thawed cells, but partial adsorption was accomplished after one hour of incubation in phosphate buffer. Electron micrographs of frozen and thawed cells did not demonstrate any difference between uninjured, injured and non-viable cells. Freezing damage was suspected, therefore, to be a minor change in the molecular configuration of the lipopolysaccharide component of the cell wall. It was proposed that this change was reversible and repaired in K_2PO_4, probably through the synthesis and utilization of ATP.

In view of the results of Singh *et al.* (1970), previous investigators of freeze-damage, in citing Leive (1965a, 1965b, 1968), may have erroneously suggested that the well-documented damage to the lipopolysaccharide component of the cell wall after freezing and thawing would allow penetration of actinomycin D. Damage to the cytoplasmic membrane suggested by sensitivity to actinomycin D, was not indicated in their conclusions.

Ray, Janssen and Busta (1971) and Ray and Speck (1972b) noted but did not elaborate on the observation that a proportion of their frozen and thawed population lost its ability to form colonies on an enriched control plating medium when actinomycin D was added to the recovery medium before subsequent plating. It is possible that this

proportion was rendered permeable to actinomycin D because of an altered cytoplasmic membrane and became nonviable in its presence. The increase in count on deoxycholate plating medium after incubation in phosphate recovery medium supplemented with actinomycin D could, therefore, have been accomplished by repair of the outer envelope of the remaining proportion of cells which possessed intact membranes. The authors should not have concluded that RNA synthesis was not involved in this energy-dependent surface repair of lipopolysaccharide since actinomycin D probably did not penetrate the membrane of this proportion of injured cells to inhibit RNA synthesis.

In the previously cited studies of phosphate repair, the addition of Mg^{++} to a phosphate recovery medium resulted in an enhanced count on deoxycholate plating medium. The authors suggested that Mg^{++} might be necessary for the activity of enzyme involved in the repair process or for maintaining stability of the cell wall, or both. It is also possible that Mg^{++} contributed to the repair of membrane lesions in that proportion of cells which were sensitive to actinomycin D. In phosphate recovery medium alone, this proportion of cells, having an altered membrane permeability, could become nonviable in the absence of Mg^{++}. In phosphate recovery medium supplemented with Mg^{++}, membrane lesions could be repaired by Mg^{++}, and this, coupled with energy-dependent phosphate repair, might result in the increased count on deoxycholate plating medium which was reported.

In the determination of freezing damage, Ray, Janssen and Busta (1971) and Ray and Speck (1972a) employed plating media with and without deoxycholate. After freezing and thawing, only cells unaffected by freezing were capable of colony formation on plates supplemented with deoxycholate. In the absence of deoxycholate, both injured cells and cells unaffected by the freezing process formed colonies. The inability of cells to form colonies on deoxycholate plating medium was interpreted to be a result of cell wall damage. By this criterion, these authors demonstrated a repair of cell wall damage, that is, a recovery of tolerance to deoxycholate, by incubating frozen and thawed cells in a phosphate recovery medium lacking magnesium. In contrast, when frozen and thawed cells of *Escherichia coli* 451B were incubated in a similar phosphate recovery medium lacking Mg^{++}, and subsequently plated into Trypticase soy agar with or without NaCl or deoxycholate, the viability of the suspension was observed to decline, and there was no recovery of tolerance to NaCl or deoxycholate. In view of previous experiments, this result would be expected. While other investigators obtained recovery by this procedure, it would appear that the degree of damage in *Escherichia coli* 451B after freezing necessitates the presence of Mg^{++}. In the absence of Mg^{++} from the phosphate recovery medium, alterations of membrane permeability were not repaired and the observed loss of viability occurred. The addition of Mg^{++} to the phosphate recovery medium, which yielded the formulation of PKM medium, sustained the viability of the suspension of cells and

allowed recovery of salt tolerance. At the same time, there was no recovery of tolerance to deoxycholate. The results would suggest that after freezing and thawing, the cells of *Escherichia coli* 451B could not be repaired sufficiently to permit colony formation in the presence of deoxycholate. Although membrane integrity had been restored, unrepaired lesions in the outer envelope could result in a lack of colony formation on deoxycholate plating medium.

In the studies of Ray, Janssen and Busta (1971) and Ray and Speck (1972a), it would appear that a lesser degree of freezing damage to the cell suspension occurred. In these instances, Mg^{++} was not required for repair or stability of the membrane. This may possibly be explained by the freezing procedure employed by these authors. In their investigations, cells were suspended in distilled water and subjected to rapid freezing followed by slow thawing. Kuo (1969) demonstrated that this was the procedure least injurious to the cell suspension. Washing and suspending the cells in 0.85% NaCl before slow freezing greatly increased the degree of death and damage to the suspended cells, and for this reason, was employed in the present investigation. Previous authors (Strange and Shon, 1964; Forsberg, Costerton and MacLeod, 1970; Sato, Izaki and Takahashi, 1972) have demonstrated that Mg^{++} is washed from the cell envelope by a saline solution. After washing *Escherichia coli* 451B in 0.85% saline, some loss of Mg^{++} may occur. Such a loss may make the cell more susceptible to the changes produced by the freeze-thaw process which result

in injury and death.

It would appear that freezing and thawing of *Escherichia coli* 451B in the presence of salts results in an altered permeability of the cytoplasmic membrane, and this alteration is reversed by incubation in PKM recovery medium. Mg^{++} appears to be the essential component of this medium and effects a repair of the membrane without protein synthesis or an energy requirement. The rapid recovery of salt tolerance of a frozen and thawed population would suggest that Mg^{++} participates in a chelating effect in the membrane to bind lesions which may result from loss of Mg^{++} from the membrane during the freeze-thaw process.

APPENDIX A

APPENDIX A

Plate counts obtained after incubation of a suspension of frozen and thawed cells in PKM recovery medium and subsequent plating of aliquots of this suspension on various plating media at several time intervals.

Mean values of quadruplicate plates are given together with the standard deviations and common logarithms of these means. The logarithmic values were employed to construct the graph in Figure 14. The values given in this appendix are typical of the values obtained in all plating experiments.

Plating Medium	Time (hrs.)	Mean	Standard Deviation	Log_{10}
1. Trypticase soy agar	0	138.3	8.34	4.1407
	½	149.3	3.10	4.1739
	1	149.0	20.22	4.1732
	2	133.5	11.15	4.1255
	3	138.3	9.71	4.1407
	4½	153.5	2.38	4.1861
	6	160.0	9.42	4.2041
2. Trypticase soy agar + 0.55 M NaCl	0	36.3	2.36	3.5593
	½	109.8	10.59	4.0404
	1	116.5	6.14	4.0663
	2	130.0	7.75	4.1139
	3	129.3	8.22	4.1114
	4½	126.0	15.49	4.1004
	6	137.8	9.00	4.1391
3. minimal agar + 0.005 M aspartate	0	64.8	12.61	3.8112
	½	67.0	11.75	3.8261
	1	87.8	12.92	3.9432
	2	118.0	9.90	4.0719
	3	122.0	8.04	4.0864
	4½	126.0	13.59	4.1004
	6	124.5	13.92	4.0952
4. minimal agar	0	35.5	7.85	3.5502
	½	42.5	9.68	3.6284
	1	64.3	6.29	3.8079
	2	88.3	8.26	3.9457
	3	98.5	7.59	3.9934
	4½	121.3	6.65	4.0837
	6	125.3	10.56	4.0978

Plating Medium	Time (hrs.)	Mean	Standard Deviation	Log_{10}
5. minimal agar	0	10.0	2.00	3.0000
+ 0.005 M	½	25.8	2.87	3.4108
aspartate	1	46.0	7.35	3.6628
+ 0.3 M NaCl	2	67.3	6.75	3.8277
	3	83.8	5.97	3.9230
	4½	94.8	13.28	3.9766
	6	106.0	12.19	4.0253

BIBLIOGRAPHY

BIBLIOGRAPHY

Allwood, M. C. and A. D. Russell. 1968.
Thermally induced RNA degradation and leakage of substances from the metabolic pool in *Staphylococcus aureus*.
J. Bacteriol. *95*: 345-349.

Arpai, J. 1962.
Nonlethal freezing injury to metabolism and motility of *Pseudomonas fluorescens* and *Escherichia coli*.
Appl. Microbiol. *10*: 297-301.

Arpai, J. and Y. Tomishava. 1966.
Effect of freezing on the induction of streptomycin resistance by chemical mutagens.
Mikrobiol. Z. Akad. Nauk. Ukr. *28*: 34-41.

Bayer, M. E. and C. C. Remsen. 1970.
Structure of *Escherichia coli* after freeze-etching.
J. Bacteriol. *101*: 304-313.

Bluhm, L. and Z. J. Ordal. 1969.
Effect of sublethal heat on the metabolic activity of *Staphylococcus aureus*.
J. Bacteriol. *97*: 140-150.

Bretz, H. W. and F. E. Kocka. 1967.
Resistance to actinomycin D of *Escherichia coli* after frozen storage.
Can. J. Microbiol. *13*: 914-917.

Brock, T. D. 1962.
Effects of magnesium ion deficiency on *Escherichia coli* and possible relation to the mode of action of novobiocin.
J. Bacteriol. *84*: 679-682.

Eagon, R. G. and K. J. Carson. 1965a.
Lysis of cell walls and intact cells of *Pseudomonas aeruginosa* by ethyl-enediamine tetracetic acid and by lysozyme.
Can. J. Microbiol. *11*: 193-201.

Eagon, R. G., G. P. Simmons and K. J. Carson. 1965b.
Evidence for the presence of ash and divalent metals in the cell wall of *Pseudomonas aeruginosa*.
Can. J. Microbiol. *11*: 1041-1042.

Farrell, J. and A. H. Rose. 1968.
Cold shock in a mesophilic and a psychrophilic pseudomonad.
J. Gen. Microbiol. *50*: 429-439.

Fill, A. and D. Branton. 1969.
Changes in the plasma membrane of *Escherichia coli* during magnesium starvation.
J. Bacteriol. *98*: 1320-1327.

Forsberg, C. W., J. W. Costerton and R. A. MacLeod. 1970.
Separation and localization of cell wall layers of a gram-negative bacterium.
J. Bacteriol. *104*: 1338-1353.

Gordon, R. C. and R. A. MacLeod. 1966.
Mg^{++} phospholipids in cell envelopes of a marine and a terrestrial pseudomonad.
Biochem. Biophys. Res. Commun. *24*: 684-690.

Green, D. E., D. M. Needham and J. G. Dewan. 1937.
Dismutations and oxidoreductions.
Biochem. J. *31*: 2327-2352.

Hansen, I. A. and P. M. Nossal. 1955.
Morphological and biochemical effect of freezing on yeast cells.
Biochem. Biophys. Acta *16*: 502-512.

Harold, F. M. 1972.
Conservation and transformation of energy by bacterial membranes.
Bacteriol. Rev. *36*: 172-230.

Iandolo, J. J. and Z. J. Ordal. 1966.
Repair of thermal injury of *Staphylococcus aureus*.
J. Bacteriol. *91*: 134-142.

Kahane, I., Z. Ne'eman and S. Razin. 1973.
Divalent cations in native and reaggregated Mycoplasma membranes.
J. Bacteriol. *113*: 666-671

Knowles, Ch. J. 1971.
Salt induces changes of turbidity and volume of *Escherichia coli*.
Nature *228*: 154-155.

Kocka, F. E. and H. W. Bretz. 1969.
Survival of *Escherichia coli* after storage in various frozen menstrua.
Antonie van Leeuwenhoek *35*: 65-76.

Kohn, A. and W. Szybalski. 1959.
Lysozyme spheroplasts from thawed *Escherichia coli* cells.
Bacteriol. Proc. 126-127.

Kuo, S. C. 1966.
Metabolic injury to bacteria on freezing and storage.
M. Sc. Thesis, McGill University, Montreal, Canada.

Kuo, S. C. 1969.
Studies on the mechanism of loss of viability of bacterial cells on freezing.
Ph. D. Thesis, McGill University, Montreal, Canada.

Kuo, S. C. and R. A. MacLeod. 1969.
Capacity of aspartic acid to increase the bacterial count on suspensions of *Escherichia coli* after freezing.
J. Bacteriol. *98*: 651-658.

Leder, I. G. 1972.
Interrelated effects of cold shock and osmotic pressure on the permeability of the *Escherichia coli* membrane to permease accumulated substrates.
J. Bacteriol. *111*: 211-219.

Leive, L. 1965a.
Actinomycin sensitivity in *Escherichia coli* produced by EDTA.
Biochem. Biophys. Res. Commun. *18*: 13-17.

Leive, L. 1965b.
Release of lipopolysaccharide by EDTA treatment of *Escherichia coli*.
Biochem. Biophys. Res. Commun. *21*: 290-296.

Leive, L. 1968.
Studies on the permeability change produced in coliform bacteria by EDTA.
J. Biol. Chem. *243*: 2373-2380.

Lindeberg, G. and A. Lode. 1963.
Release of ultraviolet-absorbing material from *Escherichia coli* at subzero temperatures.
Can. J. Microbiol. *9*: 523-530.

Lovelock, J. F. 1957.
The denaturation of lipid-protein complexes as a cause of damage by freezing.
Proc. Roy. Soc. Brit. *147*: 427-433.

MacLeod, R. A., S. C. Kuo and R. Gélinas. 1967.
Metabolic injury to bacteria. II. Metabolic injury induced by distilled water or Cu^{++} in the plating diluent.
J. Bacteriol. *93*: 961-969.

MacLeod, R. A., L. D. H. Smith and R. Gélinas. 1966.
Metabolic injury to bacteria. I. Effect of freezing and storage on the requirements of *Aerobacter aerogenes* and *Escherichia coli* for growth.
Can. J. Microbiol. *12*: 61-71.

Mazur, P. 1963.
In "Culture Collections: Perspectives and Problems. Proceedings of the specialists' conference on culture collections".
(S. M. Martin, ed.), pp. 59-70.
University of Toronto Press, Canada.

Mazur, P. 1970.
Cryobiology: The freezing of biological systems.
Science *168*: 939-949.

McClure, C. W. F. 1967.
Bonding between proteins and lipids in the envelopes of *Halobacterium halobium*.
Nature *216*: 766-771.

Moss, C. W. and M. L. Speck. 1963.
Injury and death of *Streptococcus lactis* due to freezing and frozen storage.
Appl. Microbiol. *11*: 326-329.

Moss, C. W. and M. L. Speck. 1966a.
Identification of nutritional components in trypticase responsible for recovery of *Escherichia coli* injured by freezing.
J. Bacteriol. *91*: 1098-1104.

Moss, C. W. and M. L. Speck. 1966b.
Release of biologically active peptides from *Escherichia coli* at subzero temperatures.
J. Bacteriol. *91*: 1105-1111.

Pierson, M. D., R. I. Tomlins and Z. J. Ordal. 1971.
Biosynthesis during recovery of heat-injured *Salmonella typhimurium*.
J. Bacteriol. *105*: 1234-1236.

Postgate, J. R. and J. R. Hunter. 1963.
Metabolic injury in frozen bacteria.
J. Appl. Bacteriol. *26*: 405-414.

Ray, B., D. W. Janssen and F. F. Busta. 1972.
Characterization of the repair of injury induced by freezing *Salmonella anatum*.
Appl. Microbiol. *23*: 803-809.

Ray, B., J. J. Jezeski and F. F. Busta. 1971.
Repair of injury in freeze-dried *Salmonella anatum*.
Appl. Microbiol. *22*: 401-407.

Ray, B. and M. L. Speck. 1972a.
Repair of injury induced by freezing *Escherichia coli* as influenced by recovery medium.
Appl. Microbiol. *24*: 258-263.

Ray, B. and M. L. Speck. 1972b.
Metabolic process during the repair of freeze-injury in *Escherichia coli*.
Appl. Microbiol. *24*: 585-590.

Ray, B., M. L. Speck and W. J. Dobrogosz. 1973.
Cell wall lipopolysaccharide damage in frozen and thawed *Escherichia coli*.
Abst. Ann. Meet. ASM. E 106. p. 18.

Rosenthal, L. J. and J. J. Iandolo. 1970.
Thermally induced intracellular alteration of ribosomal ribonucleic acid.
J. Bacteriol. *103*: 833-835.

Sanwal, B. D. 1970.
Allosteric controls of amphibolic pathways in bacteria.
Bacteriol. Rev. *34*: 20-39.

Sato, M. and H. Takahashi. 1969.
Cold shock of bacteria. II. Magnesium-mediated recovery from cold shock and existence of two critical temperature zones in various bacteria.
J. Gen. Appl. Microbiol. *15*: 217-229.

Sato, M. and H. Takahashi. 1970.
Cold shock of bacteria. IV. Involvement of DNA ligase reaction in recovery of *Escherichia coli* from cold shock.
J. Gen. Appl. Microbiol. *16*: 279-290.

Sato, T., Y. Suzuki, K. Izaki and H. Takahashi. 1971.
Some features of saline-sensitive phenomenon in *Escherichia coli* — Protection and recovery by magnesium ion.
J. Gen. Appl. Microbiol. *17*: 371-382.

Sato, T., K. Izaki and H. Takahashi. 1972.
Recovery of cells of *Escherichia coli* from injury induced by NaCl.
J. Gen. Appl. Microbiol. *18*: 307-317.

Scheirer, R. and P. Gerhardt.. 1973.
Influence of Mg^{++} ions on porosity of the *Bacillus megaterium* cell wall and membrane.
J. Bacteriol. *114*: 888-890.

Shikama, K. 1965.
Effect of freezing and thawing on the stability of double helix of DNA.
Nature *207*: 529-530.

Siminovitch, D. and D. Chapman. 1971.
Liposome bilayer model systems of freezing living cells.
FEBS Letters *16*: 207-212.

Singh, A. P., K.-J. Cheng, J. W. Costerton, E. S. Idziak and J. M. Ingram. 1972.
Sensitivity of normal and mutant strains of *Escherichia coli* to actinomycin D.
Can. J. Microbiol. *18*: 909-915.

Sinskey, T. J. and G. J. Silverman. 1970.
Characterization of injury incurred by *Escherichia coli* upon freeze-drying.
J. Bacteriol. *101*: 429-437.

Sogin, S. J. and Z. J. Ordal. 1967.
Regeneration of ribosomes and ribosomal ribonucleic acid during repair of thermal injury to *Staphylococcus aureus*.
J. Bacteriol. *94*: 1082-1087.

Sorrells, K. M., M. L. Speck and J. A. Warren. 1970.
Pathogenicity of *Salmonella gallinarum* after metabolic injury by freezing.
Appl. Microbiol. *19*: 39-43.

Squires, R. W. and S. E. Hartsell. 1955.
Survival and growth initiation of defrosted *Escherichia coli* as affected by frozen storage menstrua.
Appl. Microbiol. *3*: 40-45.

Straka, R. P. and J. L. Stokes. 1959.
Metabolic injury to bacteria at low temperatures.
J. Bacteriol. *78*: 181-185.

Strange, R. E. and M. Shon. 1964.
Effect of thermal stress on viability and RNA of *Aerobacter aerogenes* in aqueous suspension.
J. Gen. Microbiol. *34*: 99-114.

Tempest, D. W. and R. E. Strange. 1966.
Variation in content and distribution of magnesium, and its influence on survival, in *Aerobacter aerogenes*, grown in a chemostat.
J. Gen. Microbiol. *44*: 273-279.

Tomlins, R. I. and Z. J. Ordal. 1971a.
Requirements of *Salmonella typhimurium* for recovery from thermal injury.
J. Bacteriol. *105*: 512-518.

Tomlins, R. I. and Z. J. Ordal. 1971b.
Precursor ribosomal ribonucleic acid and ribosome accumulation in vivo during the recovery of *Salmonella typhimurium* from thermal injury.
J. Bacteriol. *107*: 134-142.

Tomlins, R. I., M. D. Pierson and Z. J. Ordal. 1971.
Effect of thermal injury on the TCA cycle enzymes of *Staphylococcus aureus* MF 31 and *Salmonella typhimurium* 7136.
Can. J. Microbiol. *17*: 759-765.

Tomlins, R. I., G. L. Vaaler and Z. J. Ordal. 1972.
Lipid biosynthesis during the recovery of *Salmonella typhimurium* from thermal injury.
Can. J. Microbiol. *18*: 1015-1021.

Weibull, C. 1956.
Bacterial protoplasts; their formation and characteristics.
Symp. Soc. Gen. Microbiol. *6*: 111-126.

Winkler, H. H. and T. H. Wilson. 1966.
The role of energy coupling in the transport of β-galactosides by *Escherichia coli*.
J. Biol. Chem. *241*: 2200-2211.

Wisdom, C. and N. E. Welker. 1973.
Membranes of *Bacillus stearothermophilus*: Factors affecting protoplast stability and thermostability of alkaline phosphatase and reduced nicotinamide adenine dinucleotide oxidase.
J. Bacteriol. *114*: 1336-1345.

Wolin, M. J. 1966.
Lysis of *Vibrio succinogenes* by EDTA or lysozyme.
J. Bacteriol. *91*: 1781-1786.

Zimmerman, L. 1964.
Freezing and freeze-drying of *Serratia marcescens* suspended in sodium chloride.
U.S. Army Biol. Lab. Tech. Manuscript 181: 1-11.