EFFECT OF TRANSPORT METHODS ON RECOVERY

OF BACTERIA FROM GROUND BEEF SAMPLES

by

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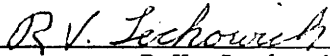
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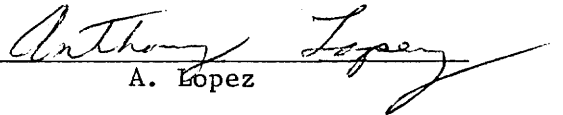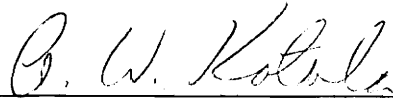
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INTRODUCTION

Freezing is a convenient and commonly used method for transporting or subsequently storing food samples intended for microbiological analysis. Freezing accomplishes a twofold purpose: during transport the total bacterial population in a sample does not increase in number; and overgrowth of a particular microorganism by others present is avoided. All bacterial cells are not equally resistant to sub-zero exposure. Clostridium perfringens, a common food-borne pathogen, dies quite readily upon freezing (Barnes et al., 1963; Strong and Canada, 1964). Index microorganisms (e.g., Escherichia coli) can also lose viability and may undergo sublethal stresses which injure cells and hamper the ability of current assay procedures to detect their presence.

In recent years considerable effort has been devoted to studying the response of microbial cells to freezing stress. Straka and Stokes (1959) determined that freezing might result in metabolically injured cells; cells which were more exacting in their nutritional requirements. More recently, Ray et al. (1973) described a state of injury in which cells were rendered highly sensitive to many surface-active compounds. These cells were termed "structurally injured." Researchers have suggested modifications to existing assays for the recovery of injured cells to include various nonselective, pre-enrichment steps which allow for a period of repair. Pre-enrichment presents no problems when qualitative information alone (e.g., determining the presence or absence of Salmonella organisms) is required. Quantitative

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recovery of selected microorganisms from a mixed population, however, requires the use of selective media and/or restrictive growth conditions.

A second approach to the freeze-injury problem has been to limit the extent of cell injury by controlling freezing and thawing rates, storage temperature, or through the use of cryoprotective compounds. In most instances, the influence of these variables on cell recovery rates has only been tested on pure bacterial suspensions in vitro. Only one study reports the direct application of these variables to the transport of food substances. Hauschild and Hilsheimer (1974) determined that viable recoveries of C. perfringens could be improved by proper selection of transport temperature and by mixing food samples 1:1 (wt/vol) with 20% glycerol prior to transport. This suggested that similar conditions might improve the survival of other microorganisms and allow their quantitative recovery from foods with existing, selective methodologies. The purpose of this investigation was to assess and compare present transport methodologies and to test modifications which would minimize injury and loss of viability of microorganisms during transport.

REVIEW OF LITERATURE

A. Transport Methods

Guidelines for the transport of perishable food samples suspected of containing pathogenic microorganisms have been suggested (APHA, 1966; FDA, 1976; USDA, 1974): generally, samples should be delivered as rapidly as possible; freezing of refrigerated samples should be avoided, if possible, since destruction of certain microorganisms will occur; frozen samples should be kept frozen and shipped in prechilled containers; thawed samples of frozen products should be discarded as results from these will have doubtful meaning.

Methodologies have also been suggested for cases where frozen transport, due to immediate lack of laboratory facilities, becomes necessary. The Animal and Plant Health Inspection Service (APHIS), U.S. Department of Agriculture (Johnson, Director of Microbiology, personal communication), makes use of Trans Temp Heat Exchange Units to ship frozen samples. APHIS recommends that all perishable food samples for chemical, antibiotic, or microbiological analysis be shipped frozen. Trans Temp units are available which will maintain temperatures of -33°C, -21°C, -11°C, -3°C, and -2°C for varying periods of time, though a temperature of -3°C is specifically used by APHIS.

The Food and Drug Administration (1976), the American Public Health Association (1966), and the International Commission on Microbiological Specifications for Foods (1974), recommend that frozen samples be collected in prechilled containers and placed in a freezer immediately after collection for sufficient time

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to become solidly frozen. Samples should be transported in an appropriate insulated container with dry ice as the refrigerant.

Frozen transport accomplishes a twofold purpose: the total bacterial population in a sample is held more or less stationary; and overgrowth of a particular microorganism by others present is avoided. An important disadvantage, especially if quantitative relationships are being sought, is that certain microorganisms (e.g., Clostridium perfringens) are intolerant to sub-zero exposure. To avoid lethal consequences of freezing, clinical microbiologists prefer to transport samples at ambient temperature and have devised a number of transport media for preserving incriminated samples during shipment or storage. A buffered glycerol solution (BGS) described by Teague and Clurman (1916) and modified by Sachs(1939) has been used with some success for transporting stool specimens containing Shigella, Salmonella and cholera vibrios. Hajna (1955) devised a specimen preservative (SP) solution and compared shigellae and salmonellae recoveries from 565 fecal specimens emulsified in SP and BGS. Twice the number of shigellae and salmonellae were isolated from the SP solution.

Shipe et al. (1960a, 1960b) investigated the use of EDTA in glycerol saline solutions as a preservative. Artifically contaminated stools were used (Shipe et al., 1960a) to compare recoveries after 1 and 4 days storage at 25°C in EDTA solution, BGS, and Hajna's SP mixture. The EDTA preservative proved to be superior in recovering shigellae and other enteric pathogens. These and

several additional methods for transport of stool specimens have been reviewed by Ewing (1971). Due to a lack of comparative data no one method could be recommended.

Hauschild and Hilsheimer (1974) investigated methods for transporting a variety of beef, pork and poultry products which were artificially inoculated with Clostridium perfringens. Storage temperature was found to play an important role in the recovery of this very cold-intolerant microorganism. After one week of storage at 4°C, log reductions (base 10) ranged from <0.2 to >3. Comparable losses occurred at -18°C. In dry ice losses were considerably lower. These experiments indicated that losses could be reduced further by mixing samples 1:1 (wt/vol) with 20% glycerol prior to storage in dry ice. Recently, the "Bacteriological Analytical Manual for Foods" (1976) has also recommended the addition of a 20% buffered glycerin solution to samples prior to frozen transport when C. perfringens contamination is suspected.

B. Freezing as a Lethal Stress

Freezing is a convenient and commonly used method of preparation for transporting or subsequently storing food samples intended for microbiological analysis. All microorganisms, however, are not equally resistant to sub-zero exposure. In recent years considerable effort has been devoted to studying the lethal effects of freezing on common food-borne pathogens and index microorganisms.

Gunderson and Rose (1948) investigated the survival of six salmonellae strains in frozen chicken chow mein. After two weeks of storage

at -25°C, recoveries decreased 33 to 92% depending on the strain tested. Georgala and Hurst (1963) inoculated ground beef with Salmonella typhimurium and compared recoveries from samples stored at -2°C and -20°C. Recoveries after three months storage were much greater at -20°C (50%) than at -2°C (1%).

The survival of Clostridium perfringens in frozen beef (Barnes et al., 1963) and chicken gravy (Strong and Canada, 1964) has been reported. Blast freezing immediately reduced the numbers of vegetative cells in beef by tenfold. In chicken gravy less than 20% of the cells survived storage at -17.7°C for 24 hours. Trakulchang and Kraft (1977) inoculated hamburger and frozen precooked food products with C. perfringens. Samples were frozen and stored at -29°C. After 24 hours losses ranged from 38% in chicken pot pie to 75% in hamburger. Unlike vegetative cells, the spores of C. perfringens appear to be highly resistant to freezing (Barnes et al., 1963; Canada et al., 1964).

Staphylococcus aureus is generally considered the most freeze-resistant of the common food-borne pathogens (Georgala and Hurst, 1963). Woodburn and Strong (1960) described the survival of S. aureus inoculated and subsequently frozen in four suspending media. Waxy rice flour, low-dextrin corn sirup, egg white and sodium alginate were tested. After 24 hours storage at -21°C, recoveries ranged from 60% for cells frozen in corn sirup to no apparent survival in sodium alginate.

Early reports on the persistence of index microorganisms in frozen foods were primarily concerned with the comparative ability of coliforms and enterococci to survive low temperature storage.

Coliforms were generally found less able to withstand frozen storage
(Burton, 1949; Brown and Gibbons, 1950; Larkin et al., 1955). Kereluk
and Gunderson (1959) suspended a mixed inoculum of Escherichia coli -
Aerobacter aerogenes cells in sterile chicken gravy. A 5 log reduction
in counts was observed after an extended frozen storage period of 481
days at -6°F. In a more recent study, Ray and Speck (1973a) suspended
E. coli cells in four foods: 10% reconstituted nonfat dry milk, meat
broth, crab meat, and whole egg liquid. These suspensions were frozen
in dry ice - acetone (-78°C) for 10 min., then thawed at 8°C for 30
minutes. Cell death was 40% or higher in foods other than milk (15%).
The mechanism of death in frozen-thawed cells remains unclear.
Keith (1913) and Weiser and Osterud (1945) believed death during freez-
ing resulted from the crushing action of extracellular ice. Support
for this theory was based on quantitative studies of bacteria suspended
in a number of different media. Lethality was greatest when cells were
frozen in water. The addition of certain solutes to the freezing medium,
such as colloids, sugars, etc., was seen to exert a protective effect,
presumably by preventing the complete and crushing crystallization of
the medium.

Harrison (1956) opposed the "mechanical destruction" explanation
and suggested that the primary cause of death in frozen suspensions
appeared to be solute which was concentrated concommitantly with the
solidification of the suspension. Haines (1938) believed that concen-
tration of extracellular solutes could lead to denaturation and subse-
quent flocculation of cellular protein components. The protection af-
forded bacteria in frozen suspensions by glycerol and other substances

was due to the ability of these compounds to counteract the lethal effect of concentrated solutes (Haines, 1938; Squires and Hartsell, 1955; Harrison, 1956).

Other workers have suggested that the principal cause of freezing death may be intracellular ice formation (Nei et al., 1969). Nei et al. (1967) studied freezing patterns in E. coli cells using electron microscopy techniques. The effect of cooling rate on cell viability was also examined. Cells which were frozen slowly at rates from 0.1^o to 10^oC/min. appeared shrunken in electromicrographs, but showed a high rate of post-thawing survival. In contrast, rapidly frozen cells retained their original shape but numerous small intracellular cavities were in evidence. The post-thawing survival rate for these cells was very low. Mazur (1970) offered this explanation for the phenomenon of intracellular freezing: As cells are subjected to subzero temperatures. the external medium first becomes supercooled, and then begins to freeze. The cells, however, can remain unfrozen, and therefore super-cooled, to -10° or -15^oC, even when ice is present in the external medium (Mazur, 1963). Since supercooled water has a higher vapor pressure than ice an equilibrium must be re-established. The manner in which cells regain equilibrium depends on the rate at which they are cooled and on the permeability to water. If they are cooled slowly or if their permeability to water is high, cells will equilibrate by losing water to the external environment; if, however, cells are cooled rapidly or if their permeability to water is low they will equilibrate, at least in part, by intracellular freezing. Other investigators (Luyet, 1961), using electron microscopic techniques,

have noted the shrunken appearance of bacterial cells when slowly cooled, and the contrasting normal size of rapidly cooled cells. Rapidly cooled cells had numerous intracellular cavities, presumably occupied by ice crystals.

Mazur (1960, 1966, 1970) proposed a two-factor hypothesis to account for the death of frozen bacteria; one factor being responsible at low, and the other at high cooling rates. At low cooling rates, solution effects were responsible for bacterial death; with rapid cooling, intracellular freezing was responsible. However, even with the formation of intracellular ice, the extent of death was found to depend on both the size of the crystals formed during cooling and the subsequent rate of warming used to thaw the cells. Rapid cooling results in the formation of small intracellular ice crystals which are, due to their high surface energies, unstable and so tend to reduce their surface energies by fusing with other small crystals. Mazur (1960), using rapidly cooled cells of Pasteurella tularensis, found that subsequent rapid warming gave greater recoveries than slowly warmed suspensions. It was suggested that the lethal event may not be intracellular ice formation per se, but the subsequent tendency for ice grains to recrystallize. Slow warming rates, which gave lower recoveries, encouraged migratory recrystallization.

Litvan (1972) suggested that the two factors involved in bacterial death may be dehydration at low, and membrane damage at high cooling rates. Mazur (1963) argued that when a difference in vapor pressure existed between the unfrozen intracellular water and the

frozen extracellular medium equilibrium was restored by water migrating out of the cell leading to increased salt concentrations and diminished vapor pressure of the remaining cellular fluid. Damage resulted from the increased salt concentration and the eventual formation of intra-cellular ice. Litvan (1972) suggested that equilibrium was restored by the gradual release of bound water (desorption) and subsequent excretion of the free water from the cell. As the amount of water decreases, denaturation occurs because the protein molecules lose their protective aqueous layer (Levitt, 1962) and also, because of this loss, the cell volume decreases. On rapid cooling the intracellular water is released at a rate faster than the permeability of the membrane allows it to flow out resulting in membrane destruction. According to Litvan's (1972) hypothesis, intracellular ice does not form in intact cells and in cases where it is observed it did not precede and cause cell destruction but rather followed it.

C. Injury and Recovery

The complex phenomena of freeze injury and recovery of micro-organisms has recently been reviewed by Ray and Speck (1973b). Straka and Stokes (1959) found that three strains of Pseudomonas and one of Escherichia coli before freezing gave the same colony counts on a minimal agar medium composed of inorganic salts, $(NH_4)_2SO_4$, citrate, and glucose, as on trypticase soy agar. After freezing, the counts on minimal agar were much less than those on trypticase soy agar. Those bacteria capable of growth on TSA, but not on minimal agar, were termed "metabolically injured." Similar observations were

reported with <u>Aerobacter</u> <u>aerogenes</u> (Postgate and Hunter, 1963),
<u>Pseudomonas</u> <u>fluorescens</u> (Arpai, 1962), <u>Shigella</u> <u>sonnei</u> (Nakamura
and Dawson, 1962), <u>Streptococcus</u> <u>lactis</u> (Moss and Speck, 1963),
and <u>Xanthomonas</u> <u>phaseoli</u> (Quadling, 1960).

More recently Ray <u>et</u> <u>al</u>. (1972) and Ray and Speck (1972) de-
scribed a state of freezing injury in certain bacterial species which
renders the injured cells highly sensitive to many surface-active
compounds. The recovery of frozen <u>Salmonella</u> <u>anatum</u> cells suspended
in water was reported Ray <u>et</u> <u>al</u>., 1972). More than 90% of the sur-
viving cells failed to form colonies on the selective medium (xylose-
lysine-peptone-agar) plating medium. Similarly, 90% higher recoveries
of surviving <u>E</u>. <u>coli</u> cells were obtained by using trypticase soy
agar containing 0.3% yeast extract (TSYA) as a recovery medium than
by using TSYA containing 0.1% deoxycholate (Ray and Speck, 1972).
These cells were designated as "structurally injured." Structurally
injured <u>E</u>. <u>coli</u> cells have also been reported sensitive to bile salts
and lauryl sulfate (Ray and Speck, 1973a; Warseck <u>et</u> <u>al</u>., 1973).

Injury in frozen-thawed bacterial cells may be manifested by an
extended lag time (Moss and Speck, 1966a, 1966b; Postgate and Hunter,
1963), increased nutritional requirements (MacLeod <u>et</u> <u>al</u>., 1966;
Morichi, 1969; Morichi and Irie, 1973; Nakamura and Dawson, 1962;
Straka and Stokes, 1959), and an increased sensitivity to surfactants
and other compounds (Bretz and Kocka, 1967; Maxcy, 1970; Morichi and
Irie, 1973; Ray <u>et</u> <u>al</u>., 1972).

Trypticase soy agar (TSA) has been analyzed to identify which
components were responsible for recovery of injured E. coli (Morichi,
1969; Moss and Speck, 1966a; Straka and Stokes, 1959). Of the TSA
components, trypticase was effective in promoting cell recovery while
phytone and acid hydrolyzed casein were inactive. Straka and Stokes
(1959) concluded that the activity of trypticase was due to specific
peptides present in the pancreatic digest and which might be required
for resynthesis of essential enzymes or other proteins destroyed by
freezing. Moss and Speck (1966a) attempted to isolate the active
peptides from trypticase. Most of the biological activity was possessed
by a fraction containing five closely related peptides. Frozen E. coli
cells were found to leak peptides of relatively small molecular weight
(Moss and Speck, 1966b). These compounds protected a dilute cell sus-
pension from death during freezing and also possessed biological activ-
ity for the recovery of injured cells. MacLeod et al. (1966) found
that a mixture of 18 amino acids promoted the recovery of injured A.
aerogenes. Cystine was the most effective amino acid in promoting
recovery, but cysteine was even more effective. It was postulated that
freezing damages the cytoplasmic membrane rendering cells penetrable by
toxic metal ions. Enriched media permit recovery of damaged cells by
providing compounds capable of chelating toxic ions. Morichi and Irie
(1973) also found that cysteine promoted recovery from injury but
attributed this to the reducing ability of the compound.

Frozen-thawed bacterial cells release many micro- and macro-
molecular cellular components into suspending media. Membrane damage
has been indicated by the loss of peptide material (Moss and Speck,

1966b), 260 nm absorbing material (Calcott and MacLeod, 1975; Lindeberg and Lode, 1963; Moss and Speck, 1966b), potassium ions (Calcott and MacLeod, 1975), and amino acids (Gabis, 1970).

Moss and Speck (1966b) found that the nature and quantity of cellular material released by frozen E. coli cells varied with the suspending medium. Cells frozen in phosphate buffer released appreciably more 260 nm absorbing material than cells frozen in distilled water, but no correlation existed between loss of this material and loss of viability. Cells frozen in phosphate buffer or in distilled water both released significant amounts of protein which was peptide in nature. Loss of peptide material paralleled loss of viability. Other investigators (Calcott and MacLeod, 1975; Lindeberg and Lode, 1963), however, found that loss of 260 nm absorbing material did correlate with loss of viability.

The metabolic processes required by cells for repair of freeze-injury have been investigated. Metabolic inhibitors available for the study of repair processes include actinomycin D and rifampin, both inhibitors of RNA synthesis; chloramphenicol and tetracycline, inhibitors of protein synthesis; D-cycloserine, an inhibitor of cell-wall mucopeptide synthesis; 2,4-dinitrophenol, an uncoupler of oxidative phosphorylation; and sodium cyanide, an inhibitor of cytochrome oxidase. Ray et al. (1972) and Janssen and Busta (1973) reported that repair of Salmonella anatum cells was not inhibited by actinomycin D, chloramphenicol, D-cycloserine, rifampin, or by tetracycline. Repair was prevented by addition of either cyanide or 2,4-dinitrophenol to the recovery medium. Injured cells were also highly sensitive to

lysozyme. This suggested that injury involved damage to the lipopoly-saccharide of the cell wall and that adenosine triphosphate synthesis was required for repair. Lysozyme causes degradation of the mucopep-tide layer of the cell wall, but in gram-negative bacteria this layer lies beneath the lipopolysaccharide (LPS) layer of the cell wall and remains inaccessible to lysozyme. However, removal or conformational change of the LPS layer would allow the mucopeptide-wall layer to become accessible to the enzyme. Bretz and Kocka (1967) also concluded that freezing damages the LPS layer after noting that frozen and thawed E. coli cells became sensitive to actinomycin D for a limited time after thawing. E. coli is normally impermeable to actinomycin D. Morichi and Irie (1973) reported that freeze-injured Streptococcus faecalis displayed a sensitivity to NaCl but cells could recover their salt tolerance within 30 min. when incubated in tryptone-yeast extract-glucose broth. Addition of chloramphenicol, puromycin, cycloserine, or penicillin G to the recovery medium did not inhibit recovery. The addition of actinomycin D, however, prevented the recovery of salt tolerance in injured cells.

Factors which have been demonstrated to influence the extent of lethal and nonlethal freezing injury in bacterial cells include the culture age (Ray and Speck, 1973; Toyokawa and Hollander, 1956), aerobiosis during growth (Harrison, 1956; Nei et al., 1969; Nei, 1973), cell concentration (Harrison, 1956; Major et al., 1955; Morichi, 1969; Sato, 1954), the rate of freezing (Calcott and MacLeod, 1975; Mazur, 1966, 1970; Nei et al., 1967, 1969), repeated freezing and thawing (Harrison, 1955, 1956; Hollander and Nell, 1954; Packer et al., 1965), conditions of frozen storage (Gibson et al., 1963; Harrison, 1956;

Johannsen, 1972; Straka and Stokes, 1959; Woodburn and Strong, 1970; Yurchenco et al., 1954), and the suspending medium (Ashwood-Smith and Warby, 1971; Bretz and Kocka, 1967; Calcott and MacLeod, 1975; Hauschild and Hilsheimer, 1974; Mazur, 1970; Nakamura and Dawson, 1962; Postgate and Hunter, 1961; Quadling, 1960; Squires and Hartsell, 1955; Straka and Stokes, 1959).

The sensitivity of bacterial cells to freezing damage may vary with the culture age and the cell concentration tested. E. coli cells were found most susceptible to freezing damage during the period of logarithmic growth; stationary phase cells were more resistant (Ray and Speck, 1973b; Toyokawa and Hollander, 1956). Some reports indicate that the survival of bacterial cells during freezing may be proportional to the cell concentration (Harrison, 1956; Major et al., 1955; Ray and Speck, 1973a). Morichi (1969) explained this observation on the basis of substances released by cells during freezing which might exert a protective effect on the surviving population.

Nei et al. (1969) studied the effect of cooling rate on the survival of aerobically and anaerobically grown E. coli cells. Non-aerated cells showed a much higher rate of intracellular freezing and cell death on rapid cooling than did aerated cells. It was noted that non-aerated cells demonstrated a low permeability to water and freezing. Cells which had previously been dehydrated by exposure to hypertonic solutions became resistant to freezing.

The rate at which bacterial suspensions are frozen and subsequently thawed may have a marked effect upon the extent of survival. Mazur (1966, 1970) compared the effects of cooling velocity on the

survival of yeast cells, human erythrocytes, marrow stem cells, and hamster cells cooled to -196°C, and thawed rapidly. In all cases the relation between cooling velocity and survival was similar in that maximum survival occurred at an optimum rate although the specific value of the optimum rate varied for different cell types. Cooling rates of 7° and 3000°C per minute produced maximum survival of yeast and red blood cells, respectively. It was suggested that an optimum cooling rate arises because of the interaction of two classes of freezing events: solution effects, which are responsible for injury when cooling is slower than optimal, and intracellular freezing, when cooling is faster than optimal. Accordingly, an optimum cooling rate was defined as one just low enough to permit a cell to dehydrate and thus avoid intracellular freezing, yet rapid enough to minimize the length of time cells are exposed to solution effects. Calcott and MacLeod (1975) reported that E. coli cells showed a peak of survival at a cooling rate of 8°C/min with survival decreasing as the cooling rate was increased or decreased from this value. Permeability damage, as measured by release of UV-absorbing material, was dependent on the cooling rate. For cooling rates less than about 8°C/min, there was minimal permeability damage to cells. However, at rates greater than this value, damage and viability were related; the lower the viability the more the damage to the permeability barrier.

The extent of bacterial death and/or injury may increase as suspensions are repeatedly frozen and thawed or with extended frozen storage (Harrison, 1955, 1956; Hollander and Nell, 1954; Johannsen, 1972; Woodburn and Strong, 1960). Generally, bacterial survival will

decrease with extended storage but the highest rate of lethality is noted early in storage (Straka and Stokes, 1959; Gibson et al., 1966; Woodburn and Strong, 1960).

Many substances, when added to a suspending medium prior to freezing, have been reported to reduce the extent of injury and result in higher recovery rates. Such "cryoprotective" agents include a number of small molecular weight organic compounds such as glycerol, sucrose, lactose, and dimethyl sulfoxide (Bretz and Kocka, 1967; Calcott and MacLeod, 1975; Hauschild and Hilsheimer, 1974; Hollander and Nell, 1954; Mead et al., 1960; Nash et al., 1963; Postgate and Hunter, 1961; Smittle et al., 1972; Squires and Hartsell, 1955). Of these, dimethyl sulfoxide (DMSO) and glycerol have been found to most consistently confer protection on a wide variety of microorganisms under varying experimental conditions. Sucrose, for instance, was found just as effective as glycerol in protecting a suspension of A. aerogenes (Postgate and Hunter, 1961), but provided no protection to C. perfringens (Canada et al., 1964), and protected frozen E. coli suspensions only half as well as glycerol (Calcott and MacLeod, 1975). A number of complex media and large molecular weight compounds such as milk, yeast extract, nutrient broth, dextran, and polyvinylpyrrolidone have demonstrated protective capabilities in some cases (Ashwood-Smith and Warby, 1971; Moss and Speck, 1963; Nakamura and Dawson, 1962; Straka and Stokes, 1959). Packer et al. (1967) found that spent growth medium, a stationary culture filtrate, was effective in protecting E. coli cells against death by freezing and thawing.

The protective action of various solutes on bacterial cells are observed facts but the mechanism of cryoprotection remains unclear. Only in the protection of red blood cells by glycerol, DMSO, and similar low molecular weight neutral solutes is there a well-supported hypothesis (Lovelock, 1953, 1954; Lovelock and Bishop, 1959). According to this theory low temperatures are harmful because they produce high solute concentrations. Glycerol and similar neutral solutes protect because their presence reduces the electrolyte concentration in and around the cell. Lovelock (1954) indicated that protection of human red blood cells (RBC's) against haemolysis occured only when the solute was able to permeate the cell. Glycerol, which was completely permeable, protected RBC's completely; glucose, which was partially permeable, had limited ability to protect; while sucrose, which was impermeable, failed to protect RBC's against haemolysis during freezing. Nash (1962, 1966) defined the observable characteristics of known cryoprotectors as compounds of low molecular weight (less than 150), with the ability to permeate cells rapidly and completely, are nontoxic, and have a high affinity for water.

The requirement for complete permeability was questioned when it became clear that some impermeable high molecular weight compounds like polyvinylpyrrolidone and dextran possessed cryoprotective capabilities for erythrocytes and bacteria (Meryman, 1968; Ashwood-Smith and Warby, 1971). Mazur (1970) suggested that the ability of a cell to survive freezing may depend more on protection of the cell surface than on protection of the cell interior. There is no reason, however, to assume that all cryoprotectors protect

different cells under varying freeze-thaw regimes in the same fashion.
Litvan (1972) proposed that permeable cryoprotectors protect by lower-
ing the vapor pressure of the intracellular fluid colligatively and
increasing the vapor pressure of the extracellular solid. Non-penetra-
ting agents protected only by forming glasses upon solidification.
In support of this hypothesis Litvan (1972) cited the experimental
results of Lovelock (1953). Recovery of frozen RBC's dropped from 97
to 63% when permeation by glycerol was blocked by added copper ion,
but recoveries decreased further to 2% in the complete absence of
glycerol. It was suggested that survival increased from 2 to 63%
by virtue of glass formation but could not achieve 97% due to the ab-
sence of penetration.

Much work has been done, as shown by the literature already
cited, on determining experimental conditions which limit the extent
of cell death and injury in pure culture suspensions. Hauschild and
Hilsheimer (1974), whose research dealt with recovery of frozen
vegetative cells of C. perfringens, appear to be the only researchers
to date that have applied this information to minimizing cell losses
in foods during transport.

The purpose of this investigation was to compare and assess
present transport methodologies and to test modifications which would
minimize cell losses during transport.

MATERIALS AND METHODS

A. Ground Beef Source

The ground beef used in this study was fabricated commercially under federal inspection and supplied, unfrozen, in 5-pound chub packs. The product was fabricated from chilled cow beef (rounds and chucks), chilled beef flanks and line trimmings. The meats were passed through a coarse grinder (13 mm plate), then mixed in proportions not to exceed 25% fat content. The product was then passed through a fine grinder (3 mm plate) and finally through a packaging machine which stuffed ground beef into casings to make the 5-pound chub packs. The final product is usually held overnight at $-1.1^{\circ}C$ and shipped to retail stores the following day. Chub packs were obtained from local retail sotres, transported (15 minutes) to the Beltsville Agricultural Research Center, and stored at $5^{\circ}C$ for no longer than three hours prior to use in experiments.

B. Enumeration Methods

Procedures for performing initial 10^{-1} dilutions varied with experimental conditions. Ground beef samples (25 g) used to obtain initial, pre-freezing counts; or samples (control) packaged and frozen without volumes of cryoprotective solutions (Section C) were weighed into a sterile Mason jar containing 225 ml sterile phosphate-buffered diluent and blended two minutes for the initial 10^{-1} dilution. Samples (25 g) packaged with either 25 or 50 ml volumes of cryoprotective media were blended with 200 and 175 ml volumes of diluent respectively. Serial dilutions were prepared by transferring 10 ml

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of the previous dilution to 90 ml of diluent. Butterfield's phosphate-buffered diluent was prepared in accordance with the procedures described in the "Microbiology Laboratory Guidebook" (USDA, 1974).

Aerobic plate counts (APC) were determined by direct pour plating with Plate Count Agar (Difco Laboratories, Detroit, Mich.). Serial dilutions were plated in duplicate. In a preliminary experiment, (Section D) three plate incubation temperatures were compared: $5^{o}C$ (10 days), $20^{o}C$ (5 days), and $35^{o}C$ (48 hr). Based on this study an incubation temperature of $20^{o}C$ was selected for use in further experiments, but plates were then counted after an incubation period of 72 hours. These conditions were felt to give the highest and most rapid estimate of the true spoilage microflora in ground beef samples.

Coliforms and Escherichia coli were assayed by the 3-tube Most Probable Number (MPN) technique as described in "Official Methods of Analysis" (AOAC, 1975). The MPN for coliform organisms was reported on the basis of the number of Brilliant Green Lactose Bile (BGLB) tubes (Baltimore Biological Laboratory, Baltimore, Md.) showing gas production in 48 hours at $35^{o}C$. MPN determinations for E. coli were based on the numbers of gas-positive EC broth (Baltimore Biological Laboratory) tubes confirmed as containing E. coli by production of typical colonies on Levine Eosine Methylene Blue (L-EMB) agar (Difco) and by IMViC procedures. The IMViC series was only completed for experiments described in Section C. Since more than 99% of the typical L-EMB colonies tested were positively confirmed by the IMViC series, production of typical colonies on L-EMB in subsequent experiments (Section H) was considered sufficient confirmation for the

presence of E. coli in gas-positive EC tubes.

Clostridium perfringens was enumerated by direct pour plating with Tryptose-Sulfite-Cycloserine (TSC) agar (Harmon, 1976). Plates were overlaid with an additional 5 to 7 ml of TSC agar and incubated in GasPak 100 anaerobic systems (Baltimore Biological Laboratory) at 35°C for 18-20 hours. Duplicate plates were inoculated for each dilution. Two suspect C. perfringens colonies from each countable plate were confirmed by stab inoculating tubes of lactose-gelatin medium (Harmon, 1976). Tubes were incubated at 35°C for 24 hours then examined for production of acid and gas from lactose. Negative tubes were incubated an additional 24 hours. Tubes were then chilled for 1 hour at 5°C and examined for gelatin liquefaction. Black colonies from TSC agar which subsequently produced acid and gas from lactose and liquefied gelatin were identified as C. perfringens.

Staphylococcus aureus was enumerated by direct surface plating on Baird-Parker agar (Difco). Duplicate plates were inoculated for each dilution and incubated at 35°C for 48 hours.

The logarithms (base 10) of bacterial counts were analyzed statistically by analysis of variance and Duncan's (1955) multiple range test.

C. Selection of Transport Media

The cryoprotective influence of various suspending media, added to ground beef samples prior to freezing and storage, on the survival of coliforms, E. coli and the total aerobic microflora was evaluated. The purpose of this preliminary investigation was to select the most

cryoprotective test solutions for more detailed study in later exper-

iments.

1.   Test Agents

Three compounds were evaluated as potential cryoprotectors:

dimethylsulfoxide (J.T. Baker Chemical Co., Phillipsburg, N.J.),

glycerin (Fisher Scientific Co., Fair Lawn, N.J.), and sucrose (Fisher

Scientific Co.).   Each agent was evaluated at five levels of concen-

tration (5, 10, 20, 30 and 50% wt/vol).   Both 25 and 50 ml volumes of

each suspending menstruum were tested.   These compounds were selected

for study on the basis of their reported ability to provide cryopro-

tection to a variety of both gram-positive and gram-negative bacteria.

2.   Preparation of Suspending Media

For each experiment stock solutions of each agent at all

concentrations were prepared on a weight-to-volume basis with phos-

phate-buffered diluent and adjusted to pH 7.0 ± 0.1.   Stock solutions

of dimethylsulfoxide (DMSO) and glycerin were autoclaved at $121^{o}C$ for

15 minutes.   Stock sucrose solutions were filter-sterilized using a

Nalgene filter unit (Sybron Corp., Rochester, N.Y.), equipped with

a 0.20 micron membrane.

3.   Preparation of Samples for Frozen Storage

Chub packs of ground beef were obtained from local retail

supermarkets.   Three locations were sampled aseptically within a chub

pack to obtain each 25 gram sample.   All samples for each experiment

replicate were drawn from the same chub pack.   Each sample was weighed

into a sterile polyethylene Whirl-Pak bag (NASCO Inc., Ft. Atkinson,
Wisc.). A volume (25 or 50 ml) of suspending media was aseptically
pipetted in with the sample and the bag was sealed. A total of 30
suspending treatments (2 vol x 3 agents x 5 conc) were evaluated in
each experiment. This experimental procedure was repeated a total of
three times with one sample per test treatment per experiment. Test
treatments were evaluated against control samples for their effective-
ness in maintaining initial (e.g., pre-freezing) microbial levels.
Control samples were drawn and packaged without added suspending
media. Samples were frozen and stored at $-11^{\circ} \pm 1.0^{\circ}C$ in a Philco
upright freezer (Model 24FV42; Philco Corp., Philadelphia, Pa.).

4. Thawing Procedure

Samples were removed and thawed after five days of frozen storage.
Each sample was first held under tap water for one minute to facilitate
removal. Samples were then aseptically removed from the whirl-pak
bags and placed into tared, sterile Mason jars. The whirl-pak bags
were rinsed with 25 ml volumes of phosphate-buffered diluent and an
appropriate volume of diluent was added to the Mason jar to achieve
a 1:10 dilution. After diluting, samples were allowed to thaw for
two minutes prior to blending. Samples were assayed as described in
Section B for total aerobes, coliforms and E. coli. Aerobic plate
counts were determined at 5, 20 and $35^{\circ}C$. Three sets of plates were
prepared for each dilution; all counts were performed in duplicate.

Based on results obtained in this study two agents (DMSO, glycerin)
at three levels of concentration (10, 20 and 30% wt/vol) were selected

for more detailed study in further experiments (Section H).

D. Selection of a Plate Incubation Temperature for Aerobic Plate
    Count Determinations

In conjunction with the previous study (Section C), plate counts

for all ground beef samples were determined at three incubation tem-

peratures: $5^{\circ}C$, $20^{\circ}C$, and $35^{\circ}C$. The purpose of this study was to

compare cell recoveries at these temperatures and to select a plate

incubation temperature which would most rapidly and accurately esti-

mate the total spoilage microflora. This would be useful in monitor-

ing the response of the spoilage microflora to transport test condi-

tions. Three sets of plates were prepared for each dilution. One

set was incubated at $5^{\circ}C$ for 10 days; a second at $20^{\circ}C$ for 5 days;

the third at $35^{\circ}C$ for 48 hours. Two criteria were evaluated: total

cell recoveries, and the time required to obtain satisfactory colony

outgrowth.

Based on these results a plate incubation temperature of $20^{\circ}C$

was selected for experiments described in Section H, but plates were

then counted after an incubation period of 72 hours.

E. Test Organisms

A culture of Clostridium perfringens, type A, Hobbs serotype

(HT) 2 was obtained from the V.P.I. and S.U. Anaerobe Laboratory,

Blacksburg, Virginia. The Hobbs serotype used has been implicated

in food poisoning outbreaks.

A culture of coagulase-positive Staphylococcus aureus was ob-

tained from the Department of Food Science and Technology, V.P.I. and

S.U., Blacksburg, Virginia.

F. Culture Maintenance and Preparation

C. perfringens was maintained in cooked meat (CM) medium (Difco).
Each tube contained about 1.0 g of cooked meat medium plus 8 ml of
CM diluent (10.0 g of tryptone [Difco]; 1.0 g of sodium thioglycol-
late; 1.0 g of soluble starch [Difco]; 2.0 g of dextrose; and 1 liter
of distilled water). Stock cultures were transferred at three week
intervals, incubated at 35°C overnight (16-18 hours), then stored at
room temperature. Room temperature rather than refrigerated storage
was used because a rapid loss in viability of C. perfringens at refrig-
eration temperature has been noted by Canada et al. (1964). "Working
stock" cultures were maintained in a similar manner but fresh "working
stock" cultures for use in experiments were prepared at weekly inter-
vals using an inoculum from the stock culture.

S. aureus was maintained on slants of Trypticase Soy Agar
(Baltimore Biological Laboratory). Stock cultures were transferred at
three week intervals, incubated at 35°C overnight (16-18 hours),
then stored at 5°C. "Working stock" cultures were maintained in
Trypticase Soy Broth (Baltimore Biological Laboratory) and held at
room temperature. Fresh "working stock" cultures for use in experi-
ments were prepared at weekly intervals using inoculum from the stock
slants.

C. perfringens cultures were prepared for use in experiments in
the following manner: a transfer (0.1 ml) was made from the "working
stock" culture to a tube of Fluid Thioglycollate Medium (FTM;

Baltimore Biological Laboratory) which was incubated at 35°C for 12 hours. This 12 hour FTM culture was transferred (0.1 ml) to another tube of FTM which was incubated at 35°C for 3 hours. This 3 hour FTM culture was used to inoculate (0.1 ml) the FTM growth tubes.

S. aureus cultures were prepared for use in experiments in the following manner: a transfer (0.1 ml) was made from the "working stock" culture to a tube of TSB which was incubated at 35°C for 12 hours. This 12 hour TSB culture was transferred (0.1 ml) to another tube of TSB which was incubated at 35°C for 3 hours. This 3 hour TSB culture was used to inoculate (0.1 ml) the TSB growth tubes.

G.   Growth Curve Procedures

Cultures were prepared for growth curve experiments as described in Section F. C. perfringens was grown in FTM; S. aureus in TSB. Cells were grown at 35°C and sampled at hourly intervals. C. perfringens was assayed by pour plating with TSA. Plates were overlaid with 5 to 7 ml of TSA and incubated in Gas Pak 100 anaerobic systems (BBL) at 35°C for 24 hours. S. aureus was assayed by pour plating with TSA. Plates were incubated at 35°C for 24 hours. All plate counts were performed in duplicate.

C. perfringens, grown under these conditions, reached late logarithmic phase at 4 hours; S. aureus at 6 hours. Cells in the late logarithmic phase of growth were used to inoculate ground beef (Section H).

H.   Study of Transport Methodologies

Conditions for minimizing cell losses in ground beef samples

during transport were evaluated.

1. Inoculation, Mixing and Packaging Procedures

Approximately 2,000 grams of ground beef were removed from a 5-lb-chub pack and placed into a Hobart Model 4612 meat cutter (Hobart Manufacturing Co., Troy, Ohio) for inoculation. The cutter was equipped with a 4.76-mm (3/16 inch) plate.

Inocula were prepared in the following manner: Cultures of C. perfringens and S. aureus were grown as described in Section F. A 3 hour FTM culture of C. perfringens was transferred (0.1 ml) to another tube of FTM medium. A similar transfer (0.1 ml) of S. aureus was made into TSB. Cultures were incubated at 35°C for an appropriate period of time (Section G) to obtain cells in the late logarithmic phase of growth. Each culture was then transferred (1.0 ml) to separate tubes containing 9 ml of phosphate-buffered diluent. From each tube an inoculum (5.0 ml) of diluted cells was added dropwise to the meat mass. The meat was then passed twice through the meat cutter to obtain a final distribution of approximately 10^4 inoculated cells per gram for each cell species.

After mixing, a total of forty-five 25-g samples were drawn. Three samples were assayed to obtain initial cell counts for total aerobes (20°C, 72 hours), coliforms, E. coli, C. perfringens and S. aureus. Thirty-six samples were weighed into sterile Whirl-Pak bags and randomly assigned to each of twelve treatments representing combinations of 2 standard transport methodologies (described below), and 25 ml volumes of 2 agents (DMSO, glycerin), each at 3 levels of

concentration (10, 20 and 30% wt/vol). Six control samples were
weighed into sterile Whirl-Pak bags, packaged without added suspending
media, and assigned to each of the two transport procedures. A sche-
matic representation of this sampling procedure is shown in Figure 1.
This experimental procedure was repeated a total of 5 times.

   2. Simulated Transport Procedures

Two methods of sample transport were tested: one using dry ice:
the second, a commercially manufactured Trans Temp insulator (Kay
Laboratories, Inc., Moberly, Mo.) equipped with canisters of a eutectic
salts mixture which function as a temperature controllant during sam-
ple shipment. Immediately after packaging, samples were frozen for a
period of approximately 24 hours. One set (21 samples) was frozen in
dry ice; the second (21 samples) in a freezer at $-11^{o}C$. Samples
frozen in dry ice were then transferred to a "Freeze-Safe" Model
3960-F02 shipping container (Polyfoam Packers Corp., Chicago, Ill.).
The condition of these samples was checked once after 36 hours and
then repacked with additional dry ice if necessary. Samples frozen
at $-11^{o}C$ were transferred to a polyurethane Trans Temp shipping unit
(Model 312) equipped with 4 pre-conditioned temperature controllant
canisters. The temperature of Trans Temp held samples rose gradually
during storage from $-11^{o}C$ initially, to approximately $-3 \pm 1.0^{o}C$,
when removed 72 hours later. Throughout this study both shipping
containers were held at an ambient temperature of approximately $65^{o}F$.
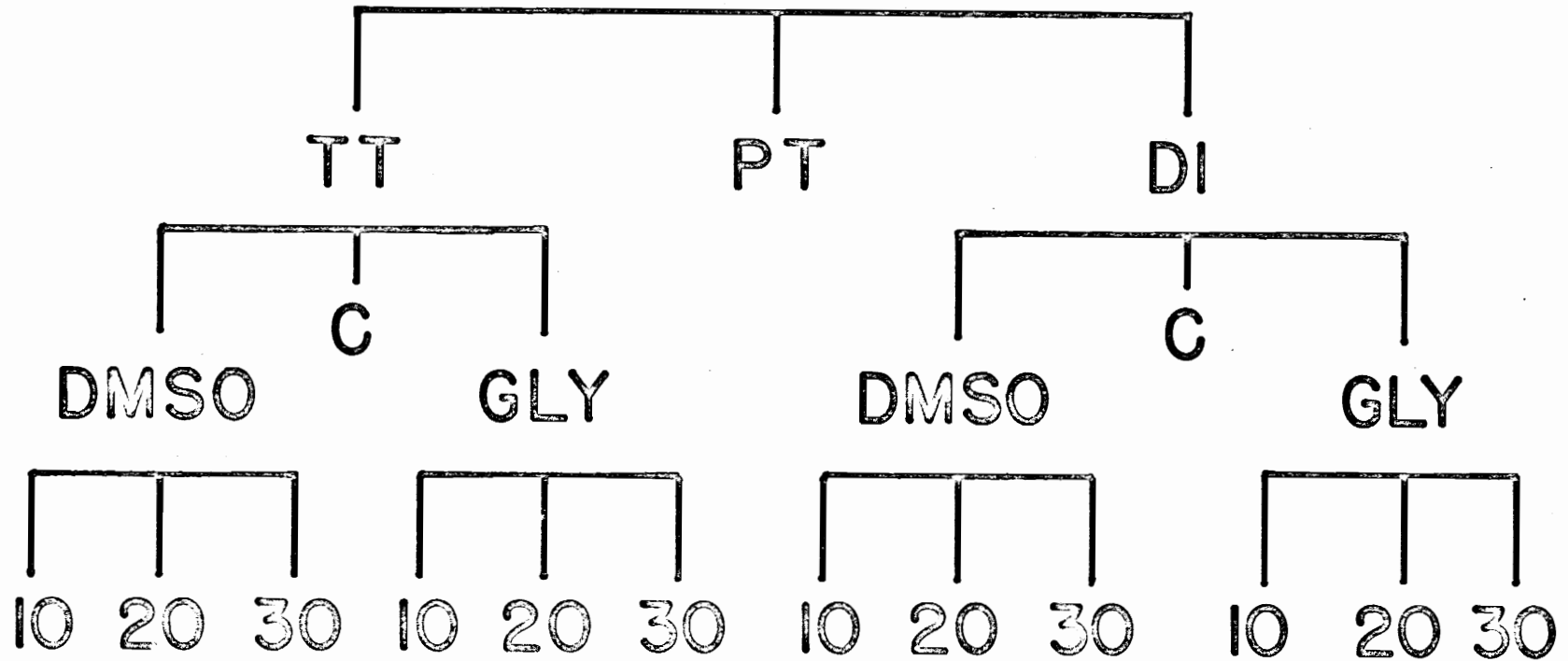
Fig. 1. Scheme used to package ground beef samples using twelve combinations of agent, agent concentration and transport methodology. TT = Trans Temp; PT = pre-transport (initial) samples assayed; C = control; DI = Dry Ice; DMSO = dimethylsulfoxide; GLY = glycerin; 10, 20, 30 = agent concentrations % (wt/vol). Each test cell was represented by triplicate 25 g samples per experimental replicate.

3. Thawing Procedures

After 65 hours of storage, samples were removed and thawed as described in Section C. Coliforms, E. coli, S. aureus, and C. perfringens were assayed as previously described (Section B). Cell recoveries from each transport treatment, including controls, were determined and compared to determine conditions which minimized cell losses during transport.

RESULTS AND DISCUSSION

A. Comparative Study of Plate Incubation Temperatures for Determining an Aerobic Plate Count

In conjunction with the suspending media study (Section B), plate counts for all ground beef samples were determined at 3 incubation temperatures: $5^{O}C$, $20^{O}C$ and $35^{O}C$. Although an incubation temperature of $35^{O}C$ has generally been recommended for estimating a total bacterial count of fresh or frozen meats (AOAC, 1975; USDA, 1974) some researchers now favor a lower temperature. Goepfert (1976) compared $20^{O}C$ plate counts of ground beef samples against $35^{O}C$ counts. On the average a $20^{O}C$ incubation temperature resulted in ten-fold higher counts. Westhoff and Feldstein (1976) used incubation temperatures of $28^{O}C$ and $35^{O}C$ to determine total counts for 140 ground beef samples. Consistently higher counts were obtained by incubating plates at $28^{O}C$. Both studies concluded that a lower incubation temperature would result in counts which more accurately estimate the total spoilage microflora. The American Public Health Association (1966), it should be noted, has recommended that total counts for fresh meats be determined at $21^{O}C$.

An incubation temperature of $5^{O}C$ has generally been used to obtain a "psychrophilic" plate count. The chief disadvantage of determining counts at this incubation temperature is the time factor. Plates must generally be incubated 10 days to obtain satisfactory colony outgrowth; too excessive a period of time for routine quality control work. Tompkin (1973) has noted that the predominant spoilage

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microflora of raw, refrigerated foods is not strictly psychrophilic.
On the contrary, a majority of bacteria associated with the spoilage
of perishable meat were found to have optimum temperatures for growth
at 20°-35°C. Consequently, the term "psychrotrophic," denoting the
microflora capable of growth under refrigerated conditions but showing
optimal growth at higher temperatures, was suggested for counts deter-
mined at 5°C.

The influence of plate incubation temperature on the Aerobic
Plate Count (APC) of raw ground beef is presented in Table 1. Plate
counts obtained at both 5° and 20°C incubation temperatures were con-
sistently and significantly ($P \leq 0.01$) higher than counts determined
at 35°C. On the average, a count 0.7 log/g greater was obtained by
incubating plates at 20°C rather than at 35°C. This was somewhat less
than the ten-fold difference reported by Goepfert (1976), but consis-
tent with the general conclusion that a higher and more accurate
estimate of the true spoilage microflora may be obtained by incubating
plates at a lower temperature. Plate counts determined at both 5°
and 20°C differed slightly but not significantly, and this was in
agreement with the study of Ayres (1960) who reported no significant
differences in plate counts determined by incubating plates at both
4° and 25°C. The chief advantage of incubating plates at 20°C rather
than at 5°C is the considerable period of time saved in obtaining
maximum colony outgrowth at the higher incubation temperature.

Based on the data presented above, an incubation temperature
of 20°C was selected for subsequently determining Aerobic Plate Counts
of ground beef samples.

Table 1. Influence of plate incubation temperature on the Aerobic Plate Count (APC) of raw ground beef.

Incubation Temp. (^oC)	APC/g [1,2]
5 (10 days)[3]	6.5×10^4 a
20 (5 days)	8.2×10^4 a
35 (48 hr)	1.7×10^4 b

[1]Each value is the average of 105 samples from three 5-lb-chub packs.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \le 0.01$.

[3]Numbers in parentheses represent the incubation period of plates at the respective temperatures.

B. Effect of Suspending Media on the Recovery of Selected Bacteria
from Frozen Ground Beef.

Several studies have reported the recovery of coliform organisms
and E. coli from frozen food products (Burton, 1949; Brown and Gibbons,
1950; Kereluk and Gunderson, 1959; Larkin et al., 1955; Ray and Speck,
1973b). Generally, these indicator groups have been found sensitive
to freezing and thawing although the extent of lethality may vary
with the food product. Estimates of the total aerobic microflora,
however, may remain relatively unaffected by short term freezing
treatments (Ray and Speck, 1973a). To assess the combined effects
of freezing, frozen storage and thawing on the recovery of coliforms,
E. coli, and the total aerobic microflora of ground beef samples as
determined at three incubation temperatures of 5°, 20° and 35^oC,
ground beef was sampled prior to, and after 5 days of frozen storage
at -11^oC. A storage period of 5 days was selected to represent the
maximum period of time thought necessary to transport frozen food
samples from one location to another prior to analysis.

These results are presented in Table 2. The data indicates no
significant reductions in any of the microbiological indices tested.
Recoveries of the total aerobic microflora measured at plate incu-
bation temperatures of 5°, 20°, and 35^oC were 57, 50 and 78%, respec-
tively. Since the recovery medium employed, Plate Count Agar (Difco),
represents a nonselective, nutritionally rich medium, apparent
reductions in counts could either be due to death or injury. It is
suggested, however, that the lower recovery rate obtained at a
plate incubation temperature of 5^oC is due, in part, to the inability

Table 2. Survival of the total aerobic microflora, coliforms and
E. coli in ground beef frozen and stored at $-11^{\circ}C$ for 5 days.

Organism	Number of cells/g[1,2]	
	Pre-freezing	Post-thawing
APC ($5^{\circ}C$, 10 days)[3]	9.4×10^4 a	5.4×10^4 a
APC ($20^{\circ}C$, 5 days)	1.0×10^5 a	7.0×10^4 a
APC ($35^{\circ}C$, 48 hr)	1.8×10^4 a	1.4×10^4 a
Coliforms	2.6×10^3 a	2.1×10^3 a
E. coli	1.3×10^3 a	8.8×10^2 a

[1]Each value is the average of nine samples.

[2]Numbers in the same row having the same lower-case letters attached
are not significantly different from one another at the level
$P \leq 0.01$.

[3]Numbers in parentheses represent the temperature and length of plate
incubation, respectively.

of sublethally injured cells to repair at this low temperature.
Janssen and Busta (1973) reported the effect of incubation tempera-
ture on the repair of freeze-injured Salmonella anatum cells. Repair
was defined as the ability of injured cells to form colonies on a
selective plating medium (xylose-lysine-peptone agar with 0.2% sodium
desoxycholate added) after a period of incubation in sterile recon-
stituted nonfat dry milk with 10% solids not fat. Freeze-injured
cells repaired rapidly when incubated between 25° and 42°C. At lower
temperatures the repair process was extremely slow and progressively
fewer cells were subsequently able to form colonies on the selective
medium as the incubation temperature approached 5°C.

Recovery rates for coliforms and E. coli cells after freezing
were 81 and 68%, respectively. Similar recovery rates have been re-
ported by Ray and Speck (1973a) for E. coli cells suspended and frozen
in meat broth, crab meat or whole egg liquid. Although the reductions
noted in these indicator groups were not statistically significant they
may be important from a regulatory viewpoint. The various microbiolog-
ical criteria proposed to date (Johnson, 1975) by state and federal reg-
ulatory agencies all specify low tolerance levels for coliforms and
E. coli cells in fresh or frozen meat products. Frozen transport of
samples intended for microbiological analysis may result in reductions
which would give an inaccurate assessment of a company's compliance
with proposed tolerance levels.

Freeze-injured bacterial cells may be recovered if the cells are
first allowed to repair in a nutritionally rich, nonselective medium.
In cases where the preservation of quantitative relationships are

not important, as in determining the presence of absence of <u>Salmonella</u>
organisms, the use of noninhibitory pre-enrichment media is recom-
mended (FDA, 1976; USDA, 1974). Quantitative recovery of selected
bacteria from a mixed population, though, requires the use of selec-
tive media and/or restrictive growth conditions. The reported
ability of a number of compounds to confer protection when added to
pure culture suspensions prior to freezing suggested that the addition
of such an agent to food samples prior to frozen transport might re-
duce the extent of lethal and sublethal injury and allow improved
recovery of frozen cells with selective media. To test this hypoth-
esis various concentrations and volumes of three reported "cryoprotec-
tive" agents were added to ground beef samples prior to freezing.
After 5 days storage at $-11^{o}C$, recoveries of coliforms, <u>E. coli</u>,
and total aerobic microflora were determined and compared against
pre-freezing levels as well as against recoveries from samples frozen
and stored without added suspending media. Protection was assumed
to be the maintenance of numbers of cells at approximately the
original levels.

Fifteen of the thirty suspending treatments were found to improve
the recovery of coliform cells from frozen ground beef samples as com-
pared to control (Table 3). A 50 ml volume of 10% dimethylsulfoxide
(DMSO) yielded the highest estimate of coliform content; an estimate
one log greater than that obtained prior to frozen storage. No explan-
ation other than experimental error or the inherent variability of
the MPN procedure can be suggested for this and similar discrepancies.
Similar inconsistencies using the MPN procedure have been reported.

Table 3. Influence of suspending media on survival of coliforms in ground beef samples stored 5 days at $-11^{o}C$.

Agent Conc. (% w/v)	Most Probable Number/g[1,2]					
	Vol Suspending Treatment (ml)					
	DMSO		GLYCERIN		SUCROSE	
	25	50	25	50	25	50
5	2.7×10^3abc	2.2×10^3bc	1.6×10^3bc	1.3×10^3bc	1.8×10^3bc	2.3×10^3abc
10	4.3×10^3abc	2.7×10^4a	4.4×10^3abc	1.8×10^3bc	6.2×10^2c	3.7×10^3abc
20	3.9×10^3abc	6.3×10^3ab	1.6×10^3bc	2.8×10^3abc	1.3×10^3bc	4.9×10^3abc
30	1.7×10^3bc	1.6×10^3bc	3.2×10^3abc	2.5×10^3abc	1.8×10^3bc	7.3×10^2bc
50	1.0×10^3bc	1.3×10^3bc	2.2×10^3bc	2.9×10^3abc	1.7×10^3bc	7.6×10^2bc

Average count (n=9) before freezing 2.6×10^3abc

Average count (n=9) of controls 2.1×10^3bc

[1]Each value is the mean of three samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at $P \leq 0.01$.

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by other investigators (Fanelli and Ayres, 1959; Kereluk and

Gunderson, 1959; Nickerson and Pollack, 1971; Ray and Speck, 1973a;

Warsack et al., 1973). Although the MPN assay generally yielded the

highest estimate of coliform recovery from frozen foods, results were

so variable and unreproducible that many investigators preferred a

more selective pour plating technique. For the purposes of this pre-

liminary experiment the relative efficacy of suspending treatments

in improving the recovery of frozen-thawed microorganisms will be

emphasized.

DMSO at concentrations of 5, 10 and 20% improved the survival of

coliforms, while 30 and 50% treatments, which remained unfrozen through-

out the storage period, yielded recoveries lower than obtained in con-

trol samples. It is possible that the apparent lower recoveries

observed with the latter two DMSO treatments simply reflect a sen-

sitivity by coliform organisms to high concentrations of this specific

solute. Ground beef samples packaged with 50% concentrations of gly-

cerol also remained unfrozen, but with these treatments the coliform

population remained relatively stable. Glycerin treatments at the 5%

level yielded, on the average, 31% lower coliform recoveries than

observed in control samples. Survival rates generally improved,

however, at higher concentrations. Hollander and Nell (1954) reported

a similar concentration effect. These researchers found that as the

concentration of glycerol in a suspending medium was raised from 0 to

15%, the ability of Treponema pallidum to survive (e.g., remain motile)

multiple freeze-thaw cycles also increased from 36 to 100 percent.

Within glycerin treatments reported here a 25 ml volume of a 10%

suspending solution yielded the greatest recovery of coliform cells.

Ground beef samples packaged with 25 ml volumes of sucrose all resulted in decreased coliform survival rates as compared to control. These reductions ranged form 16 to 70%. The lower concentration range of 50 ml volume treatments generally improved survivals, but 30 and 50% sucrose levels resulted in an average 65% reduction in the recovery of coliform organisms as compared to control.

Improved recoveries of E. coli cells from frozen ground beef samples were demonstrated with thirteen of thirty suspending treatments (Table 4). DMSO enhanced survival rates when applied at 10 and 20% levels prior to frozen storage. A 50 ml volume of 20% DMSO yielded the highest estimate of E. coli content among all treatments tested, while a 25 ml volume at the 5% level gave the lowest estimate; the latter DMSO treatments resulted in counts significantly reduced from pre-freezing determinations. Glycerin protected E. coli at 20 and 30% concentration levels; survival rates decreased appreciably at lower solute concentrations. Sucrose treatments gave highly variable results. Three treatments effectively improved the survival of E. coli, while two treatments resulted in recoveries significantly below pre-freezing determinations.

Thirteen suspending treatments improved survival of the aerobic microflora determined at a plate incubation temperature of 5°C (Table 5). A 25 ml volume of 10% DMSO performed best within this group of solute treatments and only a 50 ml volume of 50% DMSO gave lower recoveries than obtained from control samples. Among all treatments tested, ground beef samples suspended in a 25 ml volume of 10%

Table 4. Influence of suspending media on survival of _Escherichia_ _coli_ in ground beef samples stored 5 days at $-11^{\circ}C$.

Agent Conc. (% w/v)	Most Probable Number/g[1,2]					
	Vol Suspending Treatment (ml)					
	DMSO		GLYCERIN		SUCROSE	
	25	50	25	50	25	50
5	3.3×10^2d	5.7×10^2bcd	7.6×10^2bcd	7.7×10^2bcd	1.3×10^3abc	6.5×10^2bcd
10	1.5×10^3ab	9.8×10^2bcd	7.5×10^2bcd	8.0×10^2bcd	5.4×10^2bcd	1.2×10^3abc
20	9.6×10^2bcd	3.3×10^3a	1.3×10^3abc	9.0×10^2bcd	4.6×10^2cd	1.5×10^3ab
30	8.0×10^2bcd	7.8×10^2bcd	1.4×10^3ab	1.6×10^3ab	9.8×10^2bcd	3.3×10^2d
50	$6.2 \times x0^2$bcd	7.6×10^2bcd	9.2×10^2bcd	7.1×10^2bcd	6.4×10^2bcd	7.6×10^2bcd

Average count (n=9) before freezing 1.3×10^3ab

Average count (n=9) of controls 8.8×10^2bcd

[1] Each value is the mean of three samples.

[2] Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.05$.

Table 5. Influence of suspending media on survival of aerobic microflora (APC 5°C) in ground beef samples stored 5 days at −11°C.

Agent Conc. (% w/v)	Number of Cells/g[1,2]					
	Vol Suspending Treatment (ml)					
	DMSO		GLYCERIN		SUCROSE	
	25	50	25	50	25	50
5	7.8×10^4abc	7.8×10^4abc	6.4×10^4bc	6.7×10^4abc	5.3×10^4bc	4.6×10^4c
10	1.0×10^5ab	7.8×10^4abc	1.5×10^5a	5.4×10^4bc	7.8×10^4abc	5.6×10^4bc
20	7.7×10^4abc	8.4×10^4abc	7.3×10^4abc	5.4×10^4bc	6.0×10^4bc	4.3×10^4c
30	8.8×10^4abc	7.1×10^4abc	6.8×10^4abc	6.8×10^4abc	4.6×10^4c	5.7×10^4bc
50	5.9×10^4bc	5.3×10^4bc	6.0×10^4bc	4.4×10^4c	5.7×10^4bc	4.0×10^4c

Average count (n=9) before freezing 9.4×10^4ab

Average count (n=9) of controls 5.4×10^4bc

[1]Each value is the mean of three samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.05$.

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glycerin gave the highest APC at 5°C. As with DMSO treatments, only a 50 ml volume of 50% glycerin gave lower recoveries than observed in control samples. This particular treatment resulted in a reduction significantly below the APC obtained prior to freezing. A 25 ml volume of 10% sucrose improved the APC at 5°C by 26% as compared to control. Five other sucrose treatments, however, did not improve the survival rate and four of these resulted in counts significantly below pre-freezing determinations.

Among all suspending treatments tested, a 25 ml volume of 10% DMSO resulted in the highest post-thawing APC at 20°C (Table 6). Counts with this treatment averaged 46% higher than those obtained in untreated control samples. Among all treatments tested, a 50 ml volume of 50% sucrose offered the least degree of protection. Counts with this treatment represented only 49% of the original microbial levels. Of all DMSO treatments, only a 50 ml volume with 50% solute failed to improve the survival of the aerobic microflora as measured at 20°C. With the nine remaining DMSO treatments an average 22% improvement in recovery rates was observed with counts ranging from 1 to 46% above control recoveries.

Eight of ten glycerin suspending treatments improved the APC at 20°C. Within these treatments counts ranged from 9 to 36% above control counts with an average 22% improvement in the survival rate. A 25 ml volume of 10% glycerin conferred the highest degree of protection during frozen storage within this treatment group. Six of ten sucrose suspending treatments improved the post-thawing APC at 20°C. A 25 ml volume of 10% sucrose protected as well as the best

Table 6. Influence of supending media on survival of aerobic microflora (APC 20°C) in ground beef samples stored 5 days at -11°C.

Agent Conc. (% w/v)	Number of cells/g[1,2]					
	Vol Suspending Treatment (ml)					
	DMSO		GLYCERIN		SUCROSE	
	25	50	25	50	25	50
5	7.7×10^4ab	9.2×10^4ab	7.7×10^4ab	9.0×10^4ab	9.4×10^4ab	8.4×10^4ab
10	1.3×10^5a	9.0×10^4ab	1.1×10^5ab	9.8×10^4ab	1.1×10^5ab	7.3×10^4ab
20	1.0×10^5ab	9.6×10^4ab	8.8×10^4ab	5.3×10^4b	9.8×10^4ab	6.2×10^4ab
30	8.4×10^4ab	8.6×10^4ab	1.0×10^5ab	8.0×10^4ab	5.3×10^4b	7.7×10^4ab
50	7.1×10^4ab	6.2×10^4ab	8.2×10^4ab	5.1×10^4b	5.8×10^4ab	4.9×10^4b

Average count (n=9) before freezing 1.0×10^5ab

Average count (n=9) of controls 7.0×10^4ab

[1]Each value is the mean of three samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.01$.

glycerin treatment.

Fifteen of thirty suspending treatments improved the survival of the aerobic microflora determined at 35°C (Table 7). Within the solute concentration range of 5 to 30%, glycerin treatments appeared to offer the most consistent protection among the three agents tested. A 25 ml volume of 20% DMSO produced the highest survival rate among DMSO treatments, resulting in 33% higher counts, on the average, than obtained from control samples. A 25 ml volume of both 10 and 20% sucrose, and a 50 ml volume of 10% sucrose, appeared to protect about equally and produced the best survival rates among sucrose treatments. A 50 ml treatment at the 50% level for all three agents reduced counts below the control level and this was consistent with results described above for APC's at 5° and 20°C.

Two factors contributed to both the lack of significant differences obtained among test treatments and to the variability noted within test treatments: the low number of experimental replicates, and an insufficient number of samples per replicate. For these reasons no one application could be singled out as the best cryoprotective treatment. But this investigation was designed primarily to test the protective influence, if any, of the various suspending treatments, and to select from these a group for more detailed study.

Two concentration levels for all agents, the 5 and 50% levels, were excluded from further study as these generally offered less protection than more intermediate concentrations. It is suggested that low recovery with 5% treatments was a function, primarily, of

Table 7. Influence of suspending media on survival of aerobic microflora (APC 35°C) in ground beef samples stored 5 days at -11°C.

	Number of cells/g[1,2]					
Agent Conc. (% w/v)	Vol Suspending Treatment (ml)					
	DMSO		GLYCERIN		SUCROSE	
	25	50	25	50	25	50
5	1.6×10^4ab	1.8×10^4ab	2.0×10^4ab	2.2×10^4a	1.5×10^4ab	1.7×10^4ab
10	1.9×10^4ab	2.0×10^4ab	2.0×10^4ab	2.1×10^4ab	2.1×10^4ab	2.2×10^4a
20	2.1×10^4ab	1.6×10^4ab	1.8×10^4ab	2.2×10^4a	2.3×10^4a	1.6×10^4ab
30	1.7×10^4ab	1.6×10^4ab	2.3×10^4a	2.2×10^4a	1.3×10^4ab	1.8×10^4ab
50	1.4×10^4ab	1.1×10^4ab	1.8×10^4ab	1.2×10^4ab	1.5×10^4ab	1.0×10^4ab

Average count before freezing 1.8×10^4ab

Average count of controls 1.4×10^4ab

[1]Each value is the mean of three samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.01$.

the solute concentration tested and that higher levels are needed, under these experimental conditions, to be effective. In many cases recovery rates for samples packaged with 50% solutions of DMSO and glycerin were seen to decrease as compared to more intermediate treatments and, in some cases, these recoveries were less than observed in control samples. This may reflect an adverse response by microorganisms to an environment which is too hypertonic.

Volume effects were not easily identifiable except in the 5°C Aerobic Plate Count assay. In this one instance 50 ml volumes of DMSO and glycerin at the 50% level resulted in counts significantly reduced from pre-freezing determinations. Over the entire concentration range for all three agents no clear, consistent advantage could be seen for choosing between 25 and 50 ml volume treatments. Rather than continue both volume treatments only 25 ml volumes were studied further.

Based on the results of this preliminary investigation, 25 ml volumes of 10, 20 and 30 percent DMSO and glycerin solutions were selected as representative of the most effective suspending treatments tested, and were therefore chosen for use in further experiments.

C. Growth Curves

Growth curve data for $\underline{C}$. $\underline{perfringens}$ HT_2 and for $\underline{S}$. $\underline{aureus}$ were used to determine the culture age for inoculated studies and in the standardization of laboratory procedures. $\underline{C}$. $\underline{perfringens}$ HT_2 grown in FTM at 35°C had a generation time of approximately 20 minutes and reached late log phase within 4 hours (Fig. 2). $\underline{S}$. $\underline{aureus}$ grown
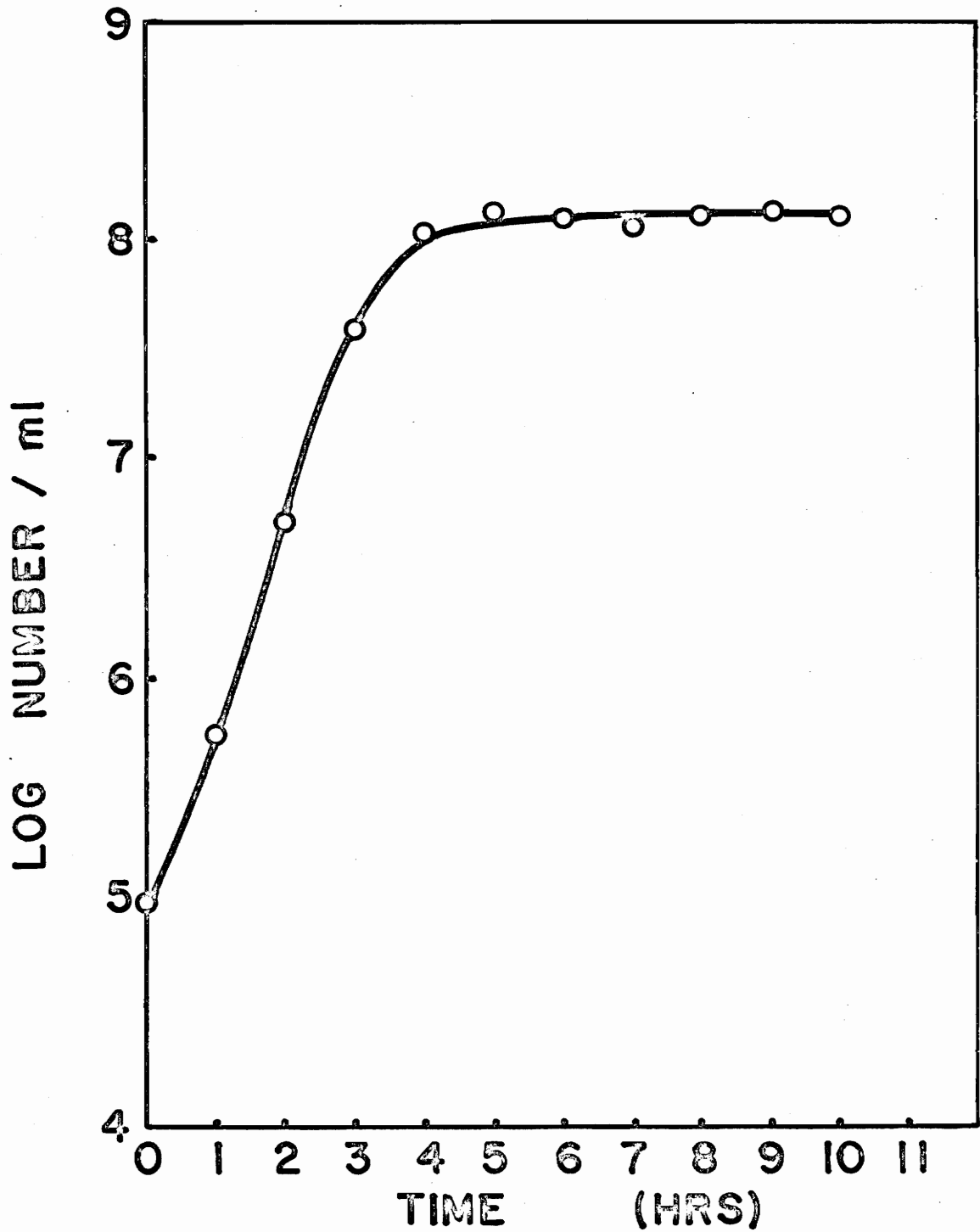
Fig. 2. Growth curve for C. perfringens, Hobbs Type (HT) 2 at 35°C in Fluid Thioglycollate Medium (FTM).

in TSB at 35°C had a generation time of approximately 27 minutes and reached late log phase within six hours (Fig. 3). Late logarithmic phase cells of both microbial species were used to inoculate ground beef prior to sampling, freezing and storing in further recovery experiments.

D. Study of Transport Methodologies

Two procedures are presently used by governmental regulatory agencies to transport perishable food samples intended for microbiological analysis. The Animal and Plant Health Inspection Service (APHIS), USDA, uses Trans Temp shipping units. Generally, samples are drawn, packaged in Whirl-Pak bags, then placed in a freezer at approximately -10°C. Samples are removed after 24 hours and placed into Trans Temp units which are equipped with canisters of a pre-conditioned eutectic salts mixture. These mixtures function as a temperature controllant during sample shipment and are formulated to maintain a temperature of -3°C for approximately 72 hours. The U.S. Food and Drug Administration (FDA) recommends the use of insulated containers with dry ice as the temperature controllant. In accordance with this procedure samples are drawn, frozen for a period of approximately 24 hours at from -55° to -90°F, then packed in dry ice for shipment.

Several reports indicate that the number of viable bacterial cells recovered from a frozen suspension may vary with the temperature of frozen storage. Weiser and Osterud (1945) noted that the rate of storage death of E. coli cells was much greater above -30°C than at temperatures of -30°C and below. Similar observations were
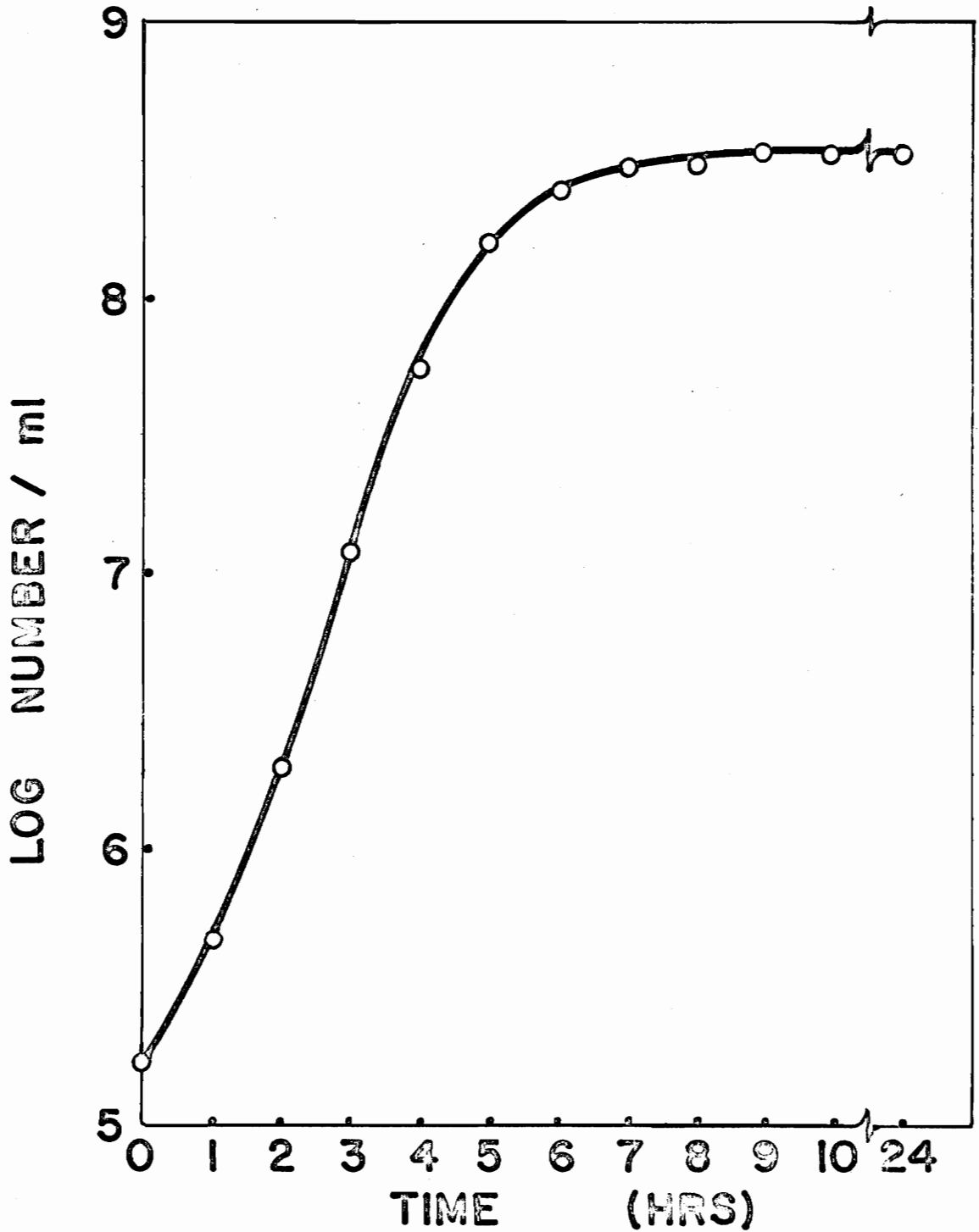
Fig. 3. Growth curve for S. aureus at 35°C in Trypticase Soy
Broth (TSB).

reported with salmonellae (Hartsell, 1951), $\underline{S}$. $\underline{aureus}$ (Haines, 1938),
and $\underline{C}$. $\underline{perfringens}$ (Barnes $\underline{et}$ $\underline{al}$., 1963). To assess and compare the
effects of the shipping procedures described above on the recovery
of $\underline{C}$. $\underline{perfringens}$, coliforms, $\underline{E}$. $\underline{coli}$, $\underline{S}$. $\underline{aureus}$ and Aerobic Plate
Counts (20°C), ground beef was assayed prior to, and after, a com-
bined freezing and storage operation which would simulate the two
transport methods. These results are presented in Table 8. Percen-
tage recoveries are presented in Table 9.

Both transport procedures significantly decreased the number
of viable $\underline{C}$. $\underline{perfringens}$ cells in ground beef samples. This was not
unexpected. $\underline{C}$. $\underline{perfringens}$ is known to be extremely sensitive to
both refrigeration and suz-zero temperatures (Canada $\underline{et}$ $\underline{al}$., 1964;
Strong and Canada, 1964). Samples frozen and stored in dry ice,
however, demonstrated significantly greater recoveries of $\underline{C}$.
$\underline{perfringens}$ than samples "transported" in Trans Temp units. A
combination of factors might have been responsible for this effect
including the slightly lower freezing rate and the period of sub-zero
temperature variation when Trans Temp samples were transferred
after freezing at -11°C to storage at -3°C. Although no one explan-
ation is entirely satisfactory similar results were reported by
Hauschild and Hilsheimer (1974); losses of viable $\underline{C}$. $\underline{perfringens}$ in a
variety of frozen meat and turkey products were always greater when
stored at -18°C than in dry ice (-55° to -60°C).

Coliform and $\underline{E}$. $\underline{coli}$ cells suffered less drastic losses than $\underline{C}$.
$\underline{perfringens}$, although significant reductions did occur. Higher re-
coveries were obtained from samples transported by the Trans Temp

Table 8. Recovery of selected bacteria from frozen ground beef as influenced by transport procedure.

Organism	Cells/g[1,2]		
	INITIAL	TRANS TEMP	DRY ICE
C. perfringens	1.1×10^4a**	4.3×10^1c**	1.8×10^3b**
Coliforms (MPN)	8.4×10^2a*	4.4×10^2b*	3.7×10^2b*
E. coli (MPN)	3.8×10^2a*	2.0×10^2ab*	1.6×10^2b*
S. aureus	6.8×10^4a*	5.8×10^4b*	6.5×10^4ab*
APC (20°C)	3.8×10^6a*	2.3×10^6b*	3.0×10^6ab*

[1]Each value represents the mean of fifteen samples.

[2]Numbers in the same row having different lower-case letters are significantly different from one another at the levels *P $\leq$ 0.05 and **P $\leq$ 0.01.

Table 9. Per cent recovery of selected bacteria from frozen ground beef as influenced by transport procedure.

Organism	Transport Procedure	
	TRANS TEMP[1]	DRY ICE[2]
C. perfringens	<1	16
Coliforms	52	44
E. coli	53	42
S. aureus	85	96
APC (20°C)	62	80

[1]Samples frozen 24 hours at -11°C, then transferred to Trans Temp shipping units (-3°C) for 72 hours.

[2]Samples frozen 24 hours in dry ice (-55 to -60°C), then kept in dry ice for 72 hours.

technique (52%) than by the dry ice (44%). Both procedures pro-
duced significant losses from pre-transport determinations. E. coli
reductions were of a similar magnitude. Maximum cell death (58%)
occurred as a result of dry ice transport. Ray and Speck (1975)
also recovered less viable E. coli after freezing in dry ice (44%)
than at a higher temperature (62% at -20°C). But these results are
not supported by earlier investigations. Haines (1938), Weiser and
Osterud (1945), and Straka and Stokes (1959) concluded that as the
temperature of freezing and storage was progressively lowered, recov-
ery rates for E. coli generally improved. This discrepancy may be
due to either differences in quantitative technique, or, more likely,
to differences in the cell suspending medium; a complex food sub-
stance as opposed to simplified suspending solutions.

S. aureus survived transport very well. Approximately 96% of
the initial population was recovered after dry ice transport. In
comparison, 85% survived Trans Temp transport. Ray and Speck (1973b)
reported similar losses. Twelve per cent of the S. aureus population
frozen in water at -20°C for 24 hours were subsequently unable to
form colonies on TSA. These researchers also found a high percentage
(77%) of nonlethally injured cells. Injury was defined as the inabil-
ity of frozen-thawed cells to form colonies on a selective medium
(Vogel and Johnson agar). Although the present study does not test
specifically for nonlethal injury, the high recoveries obtained on a
selective medium (Baird-Parker agar) indicate that S. aureus was
very tolerant to both transport procedures.

The Trans Temp procedure also resulted in significant reduc-
tions in the aerobic microflora (20°C). Only a sixty-two per cent
recovery rate was obtained. This was lower than the 70% recovery
rate observed with a similar freezing treatment in earlier experi-
ments (Table 2), although the previous treatment did not involve the
transfer of samples to a higher holding temperature. This period of
temperature variation, then, may account for the present lower re-
covery rate. As observed with C. perfringens and S. aureus, a
higher percentage (80%) of the aerobic microflora survived in samples
frozen and held in ice.

The results presented in Table 9 indicate that among these
bacterial groups S. aureus is the most resistant, and C. perfringens
the least resistant to transport procedures which involve a freezing
treatment. The nature of these differences, however, is not known.

The cryoprotective influence of various suspending solutions
added to ground beef samples prior to simulated transport was also
investigated. Although a range of concentration levels was tested,
packaging food samples with protective solutions in excess of 10 per
cent solute may not be an acceptable adjunct to Trans Temp transport.
In these cases samples were not in a frozen condition at the end of
the simulated transport period (72 hours) though the sample temper-
ature remained approximately -3°C. Control and samples mixed 1:1
with 10 per cent solute solutions were frozen as, of course, were
all treatments kept in dry ice.

The most dramatic effect was observed with C. perfringens
(Table 10). The survival of C. perfringens in Trans Temp held

Table 10. Recovery of <u>Clostridium</u> <u>perfringens</u> HT_2 from frozen ground beef as influenced by transport procedure and suspending medium.

Solute Conc. (% w/v)	Transport Procedure[1,2]			
	TRANS TEMP		DRY ICE	
control[3]	4.3×10^1d		1.8×10^3bc	
	DMSO	GLYCERIN	DMSO	GLYCERIN
10	1.5×10^3c	2.3×10^3bc	3.3×10^3bc	3.0×10^3bc
20	3.0×10^3bc	4.0×10^3bc	3.3×10^3bc	3.3×10^3bc
30	2.8×10^3bc	4.8×10^3ab	3.3×10^3bc	3.8×10^3bc
	Average count before transport 1.1×10^4a			

[1]Each value represents the mean/g of fifteen samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.01$.

[3]Mean of control samples; packaged, frozen and stored with no added suspending media.

samples was significantly improved by the presence of suspending

media. Log recoveries among this transport treatment group ranged

from 1.5 to 2.0 higher than control recoveries, with a 30% glycerin

treatment providing the highest degree of protection. Among glycerin

treatments for both transport procedures agent concentration appears

to directly influence cell survival. Lovelock (1954) suggested that

low molecular weight solutes protected cells during freezing on a

molar basis. Although the results obtained with glycerin treatments

seem to support this hypothesis the same effect was not observed with

DMSO treatments. Among samples frozen and held in dry ice, control

yielded 16% recovery of the initial inoculum, while yields from

treated samples averaged 29% and ranged from 27 to 33%. No signifi-

cant differences were observed among these cryoprotective treatments

and though all treatments improved cell survival the degree of pro-

tection conferred remained generally constant. Hauschild and

Hilsheimer (1974) have also reported that recovery of viable C.

perfringens from foods kept in dry ice could be improved when foods

were mixed 1:1 (wt/vol) with 20% glycerol.

Suspending treatments also generally improved the survival of

coliforms during simulated sample transport (Table 11). Among Trans

Temp held samples glycerin treatments did not give the same pattern

of protection as noted above with C. perfringens. In this case recov-

ery rates decreased with increasing solute concentrations. Whether

this discrepancy was due to the variability of the MPN assay or just

reflected a sensitivity to solute under these experimental conditions

could not be determined. In the absence of protective media minimal

Table 11. Recovery of coliforms from frozen ground beef as influenced by transport procedure and suspending medium.

Solute Conc. (% w/v)	Transport Procedure[1,2]			
	TRANS TEMP		DRY ICE	
control[3]	4.4×10^2abc		3.7×10^2c	
	DMSO	GLYCERIN	DMSO	GLYCERIN
10	5.2×10^2abc	6.2×10^2abc	6.7×10^2abc	5.6×10^2abc
20	6.4×10^2abc	4.9×10^2abc	6.6×10^2abc	6.8×10^2ab
30	4.9×10^2abc	4.4×10^2bc	4.9×10^2abc	7.5×10^2ab

Average count before transport 8.4×10^2a

[1]Each value represents the mean MPN/g of fifteen samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.05$.

[3]Mean of control samples; packaged, frozen and stored with no added suspending media.

coliform losses were obtained from samples handled by the Trans Temp
procedure. In the presence of solute the reverse was true. Between
similar suspending treatments higher cell recoveries were generally
obtained from samples kept in dry ice. Two treatments, 20 and 30%
glycerin, significantly improved survival rates to 81 and 89%, respec-
tively.

Postgate and Hunter (1961) reported 95% recovery of viable $\underline{A}$.
$\underline{aerogenes}$ after freezing in glycerol (10% w/v). The higher rate of
recovery obtained by these researchers may reflect their use of a
non-selective assay medium. Warseck $\underline{et}$ $\underline{al}$. (1973) demonstrated that
freeze-injured coliforms were sensitive to lauryl sulfate, the medium
used in the present study to detect, on a presumptive basis, coliform
survival.

The influence of protective media on the recovery of $\underline{E}$. $\underline{coli}$
from ground beef samples after transport is shown in Table 12.
Minimal losses occurred when samples were mixed 1:1 with 10% DMSO
and kept in dry ice. Eighty-seven per cent of the $\underline{E}$. $\underline{coli}$ population
was recovered with this treatment; significantly higher than obtained
from control (42%). The addition of 20% concentrations of either
solute improved recoveries an average 27% although these differences
were not significant. The protection offered Trans Temp held samples
was more variable; two treatments failed to improve recoveries.
Minimal losses occurred in the presence of a 20% DMSO solution where
74% of the $\underline{E}$. $\underline{coli}$ population was recovered. This was not considered
a significant improvement over control.

Table 12. Recovery of _Escherichia coli_ from frozen ground beef as influenced by transport procedure and suspending medium.

Solute Conc. (% w/v)	Transport Procedure[1,2]			
	TRANS TEMP		DRY ICE	
control[3]	2.0 x 10^2abc		1.6 x 10^2c	
	DMSO	GLYCERIN	DMSO	GLYCERIN
10	1.9 x 10^2bc	2.3 x 10^2abc	3.3 x 10^2ab	2.1 x 10^2abc
20	2.8 c 10^2abc	1.8 x 10^2bc	2.6 x 10^2abc	2.7 x 10^2abc
30	2.5 x 10^2abc	2.4 x 10^2abc	2.1 x 10^2abc	2.1 x 10^2abc

Average count before transport 3.8 x 10^2a

[1]Each value represents the mean MPN/g of fifteen samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.05$.

[3]Mean of control samples; packaged, frozen and stored with no added suspending media.

The influence of suspending medium-transport procedure inter-
actions on the recovery of $\underline{S}$. $\underline{aureus}$ is shown in Table 13. The addi-
tion of 10% DMSO allowed 100% recovery of cells from samples shipped
by both transport procedures. Control recovery rates, however, were
generally high and though some cryoprotective treatments improved cell
survival, the magnitude of these differences may not be important.

Suspending solutions generally improved the survival of aerobes
(APC 20°C) in ground beef samples during Trans Temp transport (Table
14). Treatment recovery rates ranged from 67 to 100% and averaged
80%. A 10 per cent DMSO solution significantly improved counts (100%
recovery). This was also consistent with previous experimental
results (Table 6) where this treatment was judged to protect very
well. Counts from dry ice samples were relatively unaffected by
these treatments. Differences between counts were slight and not
significant although the overall recovery rate (76%) was similar to
Trans Temp treatments.

Mazur (1960, 1966, 1970) suggested that two factors might be
involved in the freezing death of bacteria: solution effects, which
were responsible at low cooling velocities; and intracellular
freezing, which occurred upon rapid freezing. The electron microscopy
studies of Nei $\underline{et}$ $\underline{al}$. (1967, 1969) tend to support this hypothesis.
Lovelock (1953) found that damage of red blood cells occurs between
-3 and -40°C and that this critical range of temperature corresponds
to that region in which cells are exposed to concentrated salt solu-
tions during freezing. In the present study cooling velocities were
only roughly known but were assumed to be in the range where

Table 13. Recovery of <u>Staphylococcus</u> <u>aureus</u> from frozen ground beef as influenced by transport procedure and suspending medium.

Solute Conc. (% w/v)	Transport Procedure[1,2]			
	TRANS TEMP		DRY ICE	
control[3]	5.8×10^4c		6.5×10^4abc	
	DMSO	GLYCERIN	DMSO	GLYCERIN
10	6.8×10^4ab	6.0×10^4bc	6.8×10^4ab	6.3×10^4abc
20	5.8×10^4c	6.0×10^4bc	6.3×10^4abc	6.0×10^4bc
30	6.3×10^4abc	5.8×10^4bc	7.0×10^4a	6.0×10^4bc

Average count before transport 6.8×10^4ab

[1]Each value represents the mean/g of fifteen samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.05$.

[3]Mean of control samples; packaged, frozen and stored with no added suspending media.

Table 14. Recovery of aerobic microflora (APC 20°C) from frozen ground beef as influenced by transport procedure and suspending medium.

Solute Conc. (% w/v)	Transport Procedure[1,2]			
	TRANS TEMP		DRY ICE	
control[3]	2.3×10^6c		3.0×10^6abc	
	DMSO	GLYCERIN	DMSO	GLYCERIN
10	3.8×10^6a	3.0×10^6abc	2.8×10^6abc	2.5×10^6bc
20	3.0×10^6abc	3.0×10^6abc	3.5×10^6ab	2.8×10^6abc
30	2.5×10^6bc	2.8×10^6abc	2.8×10^6abc	3.0×10^6abc

Average count before transport 3.8×10^6a

[1]Each value represents the mean/g of fifteen samples.

[2]Numbers having different lower-case letters attached are significantly different from one another at the level $P \leq 0.05$.

[3]Mean of control samples; packaged, frozen and stored with no added suspending media.

solution effects were the predominant lethal factor. C. perfringens, S. aureus and aerobic microflora, in the absence of suspending media, were more tolerant of dry ice transport. This might reflect a favorable response to a more optimum cooling velocity and, comcommitantly, a shorter exposure to debilitating salt concentrations. One observation argues against this conclusion. Calcott and MacLeod (1975) found that in the low cooling range, E. coli showed a peak of survival at $8^{o}C$/min with survival decreasing as the cooling rate was increased or decreased from this value. In the present study E. coli exhibited an atypical response; cells survived better when the cooling rate least approached this optimum. Furthermore, both coliforms and E. coli demonstrated a higher rate of survival under simulated Trans Temp transport conditions; conditions which, presumably, extended the exposure of cells to high solute concentrations. It would be interesting to investigate the recovery of these cells further using a more direct quantitative method. Previous investigators (Fanelli and Ayres, 1959; Kereluk and Gunderson, 1959; Warseck et al., 1973) have noted the variable recovery of coliforms from frozen foods obtained by the MPN method. A direct plating procedure may give a clearer and more reproducible assessment of coliform survival.

Suspending media mixed with ground beef samples prior to freezing and simulated transport generally improved cell recovery rates. Suspending media exerted their most pronounced cryoprotective effect on C. perfringens. Approximately 2 log (base 10) higher concentrations of these cells survived transport in some cases although the overall recovery rates were still low. Of particular

interest was that cryoprotective treatments yielded similar cell recoveries regardless of the method used to simulate sample transport. Straka and Stokes (1959) observed that the choice of freezing menstruum affected not only the extent of cell death but also the extent of nonlethal cell injury. No effort was made in the present study to directly quantitate sublethally injured cells. The ability of cryoprotective treatments, then, to mitigate cell injury can only be inferred. Ray and Speck (1973b) found that only 12% of a population of S. aureus cells was lethally injured by freezing but that a high percentage (77%) of the surviving population was "structurally injured" and could not be detected in a selective medium (Vogel and Johnson agar). In some cases the present study noted a recovery rate of 100% for S. aureus frozen and transported in samples mixed with suspending solutions. Since the assay medium (Baird-Parker agar) was selective perhaps the use of cryoprotective solutes offers a means to aid quantitative recovery of frozen cells from a mixed bacterial population.

SUMMARY AND CONCLUSIONS

A study of existing procedures used to transport food samples was undertaken and modifications to minimize bacterial cell losses during transport were investigated. Ground beef was chosen as the food for this study.

In a preliminary study plate counts for ground beef were determined at three incubation temperatures: $5^{\circ}C$, $20^{\circ}C$, and $35^{\circ}C$. The purpose of this study was to compare cell recoveries at these three temperatures and to select a plate incubation temperature which would most rapidly and accurately estimate the total spoilage microflora. Plate counts obtained at both 5° and $20^{\circ}C$ were significantly higher than at $35^{\circ}C$. Plate counts obtained at 5° and $20^{\circ}C$ differed slightly but not significantly. Countable plates were obtained at $20^{\circ}C$ within 72 hours; a 10 day incubation period was required for satisfactory colony outgrowth at $5^{\circ}C$. A plate incubation temperature of $20^{\circ}C$ was selected for estimating the total spoilage microflora of ground beef.

A second preliminary investigation was conducted to determine the cryoprotective influence of various low molecular weight solutes on the recovery of naturally occurring coliforms, E. coli and aerobic microflora from ground beef after 5 days storage at $-11^{\circ}C$. Dimethylsulfoxide (DMSO), glycerin, and sucrose were each tested at 5 levels of concentration (5, 10, 20, 30 and 50% w/v). Each suspending treatment was mixed 1:1 and 2:1 with ground beef prior to freezing. Post-thawing recovery rates were determined and compared against pre-freezing cell levels as well as against recoveries from samples frozen

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and stored without added suspending media.

Recovery rates for coliforms and E. coli in the absence of suspending media averaged 81 and 68%, respectively. Aerobic micro-flora recoveries varied with the temperature of plate incubation. Recovery rates at 5°C (57%) were lower than at 20°C (70%) or 35°C (78%). In many cases 100% recovery of microbial groups was obtained from treated samples. Agent concentrations of 5 and 50% were generally less effective than more intermediate concentration levels. Volume effects were not important. DMSO and glycerin, each at 10, 20 and 30% levels of concentration were most effective in preserving pre-freezing levels of contamination and were selected for more detailed study.

Further experiments were conducted on ground beef inoculated with Clostridium perfringens and Staphylococcus aureus. Growth curves for both organisms were determined to standardize inoculation pro-cedures and to determine cell age. C. perfringens, type A, Hobbs serotype (HT)2 grew quite rapidly at 35°C and reached late log phase of growth within 4 hours in Fluid Thioglycollate Medium. A coagulase-positive strain of S. aureus grown at 35°C reached late log phase of growth within 6 hours in Trypticase Soy Broth. Late log was the stage of growth at which cells were inoculated into ground beef. Cells were diluted and mixed into ground beef so as to obtain a final dis-tribution of approximately 10^4 inoculated cells per gram for each cell species.

Two basic methods of sample transport were compared: Trans Temp shipping units, which employ canisters of a eutectic salts solution to maintain transport temperature, were compared against samples

packed and transported with dry ice as the temperature controllant. Trans Temp samples were first frozen for a period of 24 hours at $-11^\circ C$, then transferred to preconditioned shipping units commercially designed to maintain a temperature of $-3^\circ C$ during transport. A second set of samples was frozen in dry ice for 24 hours, then repacked with additional dry ice in an appropriate shipping container. Samples were removed after 72 hours of simulated transport and assayed for total aerobes, coliforms, E. coli, C. perfringens and S. aureus.

C. perfringens, the most freeze-sensitive organism tested, suffered significant losses in samples transported by both methodologies but recovery rates were significantly higher in samples frozen and kept in dry ice. Recovery rates for S. aureus and total aerobes were also higher after dry ice transport, though these differences were not significant. Slightly higher coliform and E. coli recoveries were obtained from Trans Temp held samples.

Cell recoveries could be improved by mixing samples 1:1 with concentrations of either DMSO or glycerin prior to freezing and transport. Suspending treatments allowed similar post-thawing recovery rates from samples regardless of the transport procedure employed. Mixing samples with cryoprotective solutions, however, may be a more appropriate adjunct to dry ice transport. Trans Temp held samples mixed with media in excess of 10% solute did not remain solidly frozen throughout the transport period although the temperature of the samples remained at $-3^\circ C$.

Two basic transport procedures were studied and modifications for minimizing bacterial cell losses during transport were investigated.

Further study of these transport methodologies with food substances other than ground beef is warranted. The influence of cryoprotective solutions on improving quantitative recovery of selected bacteria from mixed microbial populations especially needs more thorough investigation. Until appropriate recovery procedures are developed proper selection of transport conditions remains the most promising means for controlling the extent of bacterial cell death and injury during frozen sample transport. In the absence of a better understanding of the mechanism of freezing death, a choice of optimum transport conditions can only be based on practical experience gained through repeated experimentation.

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John R Guilfoyle

EFFECT OF TRANSPORT METHODS ON RECOVERY
OF BACTERIA FROM GROUND BEEF SAMPLES

by

JOHN ROHAN GUILFOYLE

(ABSTRACT)

A comparison of 2 basic methods presently used to transport
perishable food samples intended for microbiological analysis is
presented. A transport system using an appropriate, pre-chilled con-
tainer with dry ice as the temperature controllant resulted in a signif-
icantly higher (P $\leq$ 0.01) survival rate of <u>Clostridium</u> <u>perfringens</u>
vegetative cells in ground beef samples as compared to survivals in
samples shipped in Trans Temp containers using canisters of a commer-
cially formulated eutectic salts mixture as temperature controllant.
Dry ice kept samples also resulted in greater recoveries of
<u>Staphylococcus</u> <u>aureus</u> and the aerobic microflora. Slightly higher
recoveries of coliforms and <u>Escherichia</u> <u>coli</u> were obtained from samples
held by the Trans Temp procedure. Mixing samples 1:1 with 10, 20 or
30% (wt/vol) buffered solutions of either dimethylsulfoxide or glycerol
prior to freezing generally improved the survival of all microorganisms
assayed regardless of the transport system tested. The results indicate
that packaging perishable food samples with a volume of cryoprotective
solute may be a useful adjunct to frozen transport systems thereby
improving survival and allowing more complete recovery of selective
microorganisms with existing assay procedures.