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Preservation of lactic cultures

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PRESERVATION OF LACTIC CULTURES

by

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INTRODUCTION

The manufacture of cheese, buttermilk, sour cream and other cultured dairy products is a large and expanding industry. One of the most important functions in this industry is the development, maintenance, preservation and use of active starter cultures. A plant producing cultured dairy products is dependent upon the starters that are used. Slow or contaminated cultures lead to serious defects in the finished product and to financial loss.

To prevent losses from starter failures, or the manufacture of inferior products attributable to poor starter, the modern plant must employ rigid sanitation and proper techniques of culture propagation and transfer. Usually, a plant must maintain several cultures in order to minimize the risk of being without a suitable starter should one or more cultures become inactive. The task of maintaining cultures properly involves considerable expense because of the labor, equipment and supplies required. For this reason, much interest is expressed in the preservation of starter cultures. If cultures could be stored for extensive periods and retain their initial activity, much time and labor involved in culture transfer could be eliminated and the manufacturer would be more certain of having active cultures available.

With this in mind, the present work was undertaken to examine some of the factors involved in preserving lactic cultures and to develop preservation techniques which would guarantee maximum retention of acid-producing ability. This study also was made to help to satisfy an

apparent lack of information on various factors affecting lactic streptococci during freezing, especially at extremely low temperatures.

REVIEW OF LITERATURE

Preservation of microorganisms has long been a subject of investigation. As early as 1887, Prudden (62) studied the effect of freezing on bacteria found in the ice supply of New York City. Liquid air and liquid hydrogen were used to freeze microorganisms in 1900 (48, 49, 50).

Fry (21) briefly reviewed the literature on factors influencing survival of frozen or dried bacteria: the nature of the organism, the suspending medium, conditions of growth and age of the culture, cell concentration of the suspension, degree of vacuum and drying temperature, atmosphere of storage, temperature of storage, residual moisture and absorption of water.

This work deals with the effects of freezing and frozen storage; therefore, preservation of bacteria by drying will be discussed only briefly.

Variables involved in the preservation of bacterial cultures can be grouped into three general categories: (i) conditions of growth and age of the culture, (ii) preparation for preservation and (iii) the method of preservation and storage.

Conditions of Growth and Age of the Culture

Comparatively little work has been done on the effect of growth conditions upon survival of an organism during preservation. This is largely because the type of organism under investigation usually determines a certain growth medium and a fairly definite growth temperature. Lactic cultures are generally grown in milk, although Lamprech (39) grew cultures

in broth containing Tryptone, yeast extract, glucose and magnesium phosphate. Other types of organisms are usually grown in broth. Chance (9) and Weiser and Hennum (86) preserved cultures of bacteria grown on agar.

The temperature of incubation prior to freezing or drying is generally that which is optimal for the organism being studied. This is rarely done with lactic cultures. Foster et al. (20) state that the optimum temperature for Streptococcus lactis is 30 C. Since lactic cultures, unless single strain, consist in part of Leuconostoc sp. a lower incubation temperature is used to encourage flavor development. The lower incubation temperature is also favored because the corresponding longer incubation time of 14 to 16 hr readily fits into a work-day schedule. Lamprech (39) reported incubating lactic cultures at 25 C before freezing. Anderson (2) incubated single strain cultures at 72 F (22.2 C) before freezing.

Age of the culture at the time of freezing has been found to be an important factor in the survival of an organism. Prudden (62) studied the ability of bacteria found in the New York City ice supply to withstand freezing. The count per ml of an active culture of Staphylococcus pyogenes aureus was reported as "innumerable" before freezing to 14 to 30 F (-10 to -1 C). After 18 days storage the count was 220,000/ml; 49,000/ml survived for 66 days. An old culture of the same organism failed to survive 7 days. He concluded that the number of cells killed during freezing depends upon the vitality of the organism.

Effect of age of the cells in a lactic culture upon survival is confounded by the effect of higher amounts of acid in older cultures. Cardwell and Martin (8) grew a culture in reconstituted non-fat-dry-milk and froze after 12, 16 and 20 hr incubation. After storage for 30 days

the 12-hr cultures produced slightly less acid than the 16- and 20-hr cultures. Anderson (2) froze cultures varying from 0.22 to 0.98% titratable acidity, calculated as lactic acid. Samples were thawed after storage for 2, 6 and 12 months at less than 8 F (-13.3 C). Cultures with higher titratable acidities produced more acid when inoculated at a rate of 5% into milk and incubated for 4 hr at 90 F (32.2 C). The activities of the cultures before freezing were not reported; the acidity resulting in the maximum percentage survival was not determined.

After storing freshly inoculated, coagulated and fully ripened cultures, Swartling and Lindgren (82) found that the most active cultures were those frozen immediately after inoculation. Simmons and Graham (75) recommended freezing in less than 6 hr after inoculation to obtain minimum loss of activity.

Lamprech (39) grew S. lactis cells in broth, centrifuged, resuspended in skimmilk and froze at -20 C. Cells older than 8 hr at the time of harvesting and freezing lost activity during storing. Eight-, 10- and 12-hr cultures were more active initially than younger or older cells.

Fry and Greaves (22) freeze-dried a strain of a paracolon bacillus. They reported that young cultures, 4.5 to 6 hr old, were more sensitive to drying than were 18- to 24-hr cultures.

Effect of aeration of a culture of Escherichia coli upon survival of cells during freezing was studied by Harrison and Cerroni (26). Aerated E. coli cultures survived freezing better than non-aerated cultures.

Preparation for Preservation

Adjustment of pH

Arpai (5) stated that metabolic freezing injury is affected by the pH of the fluid in which the culture is frozen. Johns (35) neutralized lactic cultures to 0.16% titratable acidity with 40% NaOH before freezing. After frozen storage, cultures were considered sufficiently active as long as the count remained above 500,000/ml. The unneutralized culture dropped from a count of 91 million/ml to a count of 160,000/ml after 1 week of frozen storage. The neutralized culture, which had an initial count of 120 million/ml, had a count of 570,000/ml after 12 weeks and 220,000/ml after 15 weeks.

Lamprech (39) adjusted lactic cultures to pH values of 5, 6 and 7, and held them at 4 C and -20 C. At both temperatures, storage at pH 5 was detrimental to the cultures; pH 7 was slightly superior to pH 6 at -20 C.

Straka and Stokes (81) froze cultures of E. coli and three Pseudomonas species. At pH 5 and pH 8 the least number of cells was injured, and the death rate was high. At pH 6 and pH 7, the number of cells killed was low, but more cells were injured.

Neutralization appears to be injurious to cultures which are to be dried. Rogers (68) found that neutralizing with K_3PO_4 decreased the activity of freeze-dried cultures. $CaCO_3$ did not increase culture activity when used as a neutralizer. The same results were obtained by Sapp and Hedrick (70) who indicated that $Ca(OH)_2$ was more detrimental to dried cultures than was NaOH.

Cell concentration

Little evidence as to the effect of cell concentration upon survival has been presented in the literature. Liska (42) freeze-dried 1:10 dilutions of starter cultures. After 150 days storage at -10 to -15 C there were sufficient viable organisms to result in satisfactory culture activity. Richardson (64) condensed a milk culture of S. lactis in a vacuum pan to 25-29% solids. The resulting cell paste was then lyophilized. The activity of the lyophilized cell paste was reported to be comparable with that of an uncondensed lyophilized culture.

Straka and Stokes (81) reported that cell concentration did not affect the survival of E. coli or three species of Pseudomonas. However, Major et al. (51) stated that with E. coli, Ps. aeruginosa, Salmonella gallinarum, Serratia marcescens, Staphylococcus aureus and Bacillus pumilis the percent survival varied in proportion to the initial cell concentration. They noted that the effect of cell concentration was not evident when cultures were thawed without storage. It was pointed out that their results could have been affected by the occurrence of cells in clumps or chains. Breaking of clumps or chains would result in an increase in plate count; all cells in a clump would have to be killed to prevent a colony from being formed.

Protective additives and suspensory medium

The value of protective additives was recognized as early as 1911 when Hammer stated:

> Lactic acid organisms are preserved by adding milk sugar, milk powder or starch to liquid cultures of the organisms and drying at low temperatures. The dry starters thus produced are admitted to have better keeping qualities than

liquid starters (25, p. 527).

Milk has been shown to provide protection to frozen organisms. Hilliard and Davis (31) found that milk and cream protected Bacterium coli during freezing and thawing. Freezing in cream with 30% fat was much better than the use of distilled or tap water, according to Hilliard et al. (32). Keith (36) reported that, in milk, the largest proportion of organisms survived freezing in the least diluted milk.

Generally, lactic cultures are frozen or dried in milk. Simmons and Graham (75) favored the use of fresh skim plus 2% added dry skim for freezing cultures. Anderson (2) froze bulk starter in skimmilk with 9% total solids. This level of solids was found, by Cardwell and Martin (8), to offer slightly less protection to frozen cultures than 14 and 18% solids. Lattuada and Foster (40) reported satisfactory survival of S. lactis when the cells were suspended in 5% solids reconstituted skimmilk prior to drying.

Although the preservative action of glycerol has been studied extensively, little work of this nature has been done with lactic cultures. Heinemann (29) added 0, 10 and 20% glycerol to ripened cheese cultures. Cultures containing 20% glycerol were active after storage at 35 F (1.7 C) for up to 2 months and at 5 F (-15 C) and -20 F (-28.9 C) for up to 6 months. Cultures to which no glycerol had been added showed a considerable decrease in activity at all temperatures of storage. Lamprech (39) froze concentrated lactic cultures in skimmilk, water and 0.1% peptone broth. After 24 hr at -20 C, the cultures were thawed and plated. Glycerol was found necessary for survival of the organisms frozen in water or peptone broth, but was not necessary for survival of organisms frozen in milk.

Richardson (64) found 10% glycerol to be beneficial in maintaining the activity of a frozen culture. He reported that glycerol was no better than milk solids in protecting against repeated freezing and thawing.

The protective action of glycerol has been demonstrated in other types of microorganisms. Keith (36) reported that less than 1% of Bacterium coli cultures survived freezing 5 days in tap water, but that a "very large" percentage survived at least 6 months when frozen in water plus 5 to 42% glycerol. Nakamura et al. (57) held cultures of Shigella sonnei at -20 C for 5 weeks. Less than 1% of the organisms survived without glycerol in the suspending medium. The optimum level of glycerol was found to be in the vicinity of 10%, where 91% of the organisms survived. They also found, as did Hollander and Nell (33) working with E. coli and Diplococcus pneumoniae, that glycerol protected cells against repeated freezing and thawing. Postgate and Hunter (60) compared glycerol with diethylene glycol, l-erythritol, glucose, sucrose and polyethylene glycol. Use of each of these additives resulted in 100% survival of Aerobacter aerogenes when frozen in liquid nitrogen, but only glycerol offered enough protection to permit extended storage at -20 C.

Peptones and carbohydrates have frequently been recommended as additives for use in bacterial preservation. Annear (3) compared the use of 10% peptone, 10% peptone plus 10% glucose and 10% peptone plus 10% sorbitol as protective agents. The peptone-carbohydrate mixtures were comparable in protective action; both were superior to peptone alone. Of the crystalline additives, Heller (30) found the use of sucrose to result in the lowest death rate in lyophilized cultures of Staph. pyogenes and E. coli. He noted that a crystalline compound, to be effective, must have

high solubility and be easily assimilated by the organisms. Peptone resulted in the lowest death rate of colloidal additives, 1% peptone having the same protective effect as 10% sucrose. The most effective colloidal substances were those with high hydrophilic property and protective colloid effect. Mixtures of fermentable crystalline substances and hydrophilic colloids resulted in lower death rates than did either group singly. Zimmerman (89) also found that the protective effect of a sugar was correlated with the ability to penetrate the cell, being in this instance, freeze-dried Serratia marcescens. Hilliard and Davis (31) found glucose to offer no protection at -10 C. On the other hand, Fry and Greaves (22) stated that 5 to 10% glucose greatly increased the survival rate of freeze-dried bacteria. Hilliard and Davis worked primarily with Bacterium coli; the latter authors used one strain of a paracolon bacillus. Luyet and Keane (47) found that with chick embryo heart, frozen in liquid nitrogen, 1.5 M glucose offered maximum protection. In the same work, ethylene glycol and NaCl were compared with glucose. Maximum protection from NaCl resulted with the use of a 1 M solution. The loss of efficiency of NaCl and glucose at higher levels was believed to be caused by too rapid dehydration and to the toxicity of the NaCl. Ethylene glycol was found to be better than either NaCl or glucose, offering high protection in concentrations of from 20 to 100%.

Weiser and Hennum (86) lyophilized cultures of E. coli which had been suspended in 1% peptone, inactivated rabbit serum and skimmilk. The initial mortality in peptone was 85 to 95%, while 50 to 65% of the cells suspended in rabbit serum died during drying. Skimmilk was intermediate in protective effect, but the results were highly variable. Human serum

albumin has been used successfully by Schreiner et al. (71) to protect human erythrocytes stored at -196 C.

Gelatin appears to offer little protection to frozen or dried cells. Squires and Hartsell (79) found little advantage in adding gelatin to cultures of E. coli before freezing. Gelatin had no effect on survival of cheese cultures dried by Sapp and Hedrick (70).

Benedict et al. (6) reported that urea and salts of tricarbonylic acids were of some benefit in protecting lyophilized cultures of Serratia marcescens. Urea was also beneficial to Leuconostoc mesenteroides and Ps. aureofaciens.

The use of dimethyl sulfoxide has been reported recently in the literature, but none of these reports are concerned with bacterial preservation. Kite and Doebbler (37) stated that dimethyl sulfoxide was as effective as glycerol in protecting HeLa cells against freezing. Dougherty (15), Nagington and Greaves (56) and Porterfield and Ashwood-Smith (59) reported that addition of dimethyl sulfoxide resulted in higher recovery of frozen tissue cultures than the use of glycerol.

The Method of Preservation and Storage

Freezing

Freezing is generally employed at some stage during preservation of a bacterial culture. The freezing temperature appears to be of considerable importance in the survival of a particular species of microorganism. Lactic cultures have been held at temperatures varying from slightly above freezing to -196 C. Cultures stored by Heinemann (29) were active after 6 months at 5 F (-15 C) and -20 F (-28.9 C), but were active for

only 2 months at 35 F (1.7 C). Antila and Peltola (4) reported that starter cultures stored at -12 C kept their acid producing ability for approximately 1 month. Rudnick and Glenn (69) were able to make Cottage cheese from cultures which had been held at -18 F (-27.8 C) for up to 150 days. Anderson (2) stored cultures at less than 8 F (-13.3 C) and was able to make Cottage cheese after storing for 12 months. Little loss in activity of a freshly inoculated culture placed at -20 C occurred in 6 months, according to Simmons and Graham (75). Nilsson and Wass (58) inoculated Lactobacillus bulgaricus into milk immediately before freezing. After 16 weeks at -18 to -22 C, only slight weakening of the cells was noted. Cowman and Speck (12) stored lactic streptococci at 3, -20 and -196 C. After 3 days, the cultures stored at -196 C retained most of their acid-producing ability. Cultures held at -20 C showed the greatest loss in activity. Cultures were subjected to extended storage at the two lower temperatures. Acid-producing ability of cultures stored at -196 C was equivalent to that of the original fresh culture. Cultures held at -20 C showed considerable decrease in activity.

Keith (36) held Bacterium coli cultures at temperatures ranging from 37 C to -20 C. He noted a high death rate at 37 C, which diminished as the temperature approached 0 C. At slightly below 0 C, the death rate was little different from that at -20 C, but was considerably slower than above 0 C. Haines (24) stored cells of six bacterial species, one yeast and spores of two bacterial species at -1 and -20 C. The most rapid death rate was reported to be at the higher temperature. However, Hilliard et al. (32) found -15 C to be more lethal to microorganisms than -2 C. Straka and Stokes (81) froze cells of Ps. fluorescens and stored them at

-7, -18 and -29 C. As the storage temperature was lowered, the proportion of injured cells was found to increase while the proportion of killed cells decreased. Similar findings were reported by Arpai (5) in that Ps. fluorescens sustained more injury at -18 C than at -7 C. Little difference in cell sensitivity was found between -18 and -30 C. Experiments with E. coli produced similar results.

When the storage temperature is dropped still further, survival rates appear to be higher. Treponema pallidum was stored by Hollander and Nell (33) for 2 months at -70 C without loss of virulence. At -40 and -15 C, virulence decreased rapidly. Weiser and Osterud (87) observed that the rate of storage death of E. coli is greater above -30 C than below. These authors stated that storage death at -195 C either does not take place or was not evident during the period studied. The longest storage period studied was 10 hr, which may not have been long enough to allow valid conclusions about storage death to be drawn. Smith and Swingle (77) froze bacteria at -17.8 C and in liquid air at -190 C. At both temperatures over 99% of a culture of Bacillus typhosus was killed. They felt, however, that if an organism could pass 0 C safely, even absolute zero would not harm the organism. Macfadyen (48) and Macfadyen and Rowland (49) found "no impairment in the vitality" of ten species of bacteria after storage at both 20 hr and 1 week at -190 C. Later, the same authors (50) held microorganisms in liquid hydrogen at -252 C with no apparent loss in activity.

The rate of freezing or thawing influences the lethality of the freezing treatment. Haines (24) stated that rapid freezing results in the death of a constant proportion of cells. He found that these pro-

portions varied from 80% of the cells in a culture of Bacillus pyocyaneus (Ps. aeruginosa) to 0% of a suspension of spores of either B. mesentericus or B. megatherium. He observed, however, that the temperature or rate of freezing had little effect upon survival of microorganism within "specified limits." Mazur (52) reported cooling rates of less than 50 C/min to result in maximum survival of Saccharomyces cerevisiae, Pasteurella tularensis and Aspergillus flavus. Slow freezing combined with rapid thawing gave the highest survival of Pasteurella tularensis. Doebbler and Rinfret (14) investigated the effect of ultrarapid freezing and thawing upon microorganisms. Cell suspensions were sprayed onto the surface of liquid nitrogen with a syringe. The resulting frozen droplets were thawed by immersion in a liquid medium at 37 C. Survival rates varied from 100% with E. coli to 22% with Aspergillus niger.

Lamprech (39) froze concentrated lactic cultures in tubes placed in a refrigerator at -20 C and immersed in methyl alcohol at -20 C. Cultures were thawed by placing tubes in a 4 C water bath for 2 hr and a 30 C water bath for 5 min. No significant effect of either freezing or thawing rate upon survival was found. Cardwell and Martin (8) reported no difference in culture activity after freezing in a refrigerator at 0 F (-17.8 C) or in front of a blower at -20 F (-28.9 C). Cultures were thawed by dumping directly into milk, holding in a 110 F (43.3 C) water bath for 1 hr and holding at 40 to 44 F (4.4 to 6.7 C) for 8 hr. They noted that rapid thawing greatly decreased culture activity. Moss and Speck (55) compared the effect of freezing rates in buffered distilled water and in 10% solids skimmilk. The rate of freezing had little influence on the injury or death of cells frozen in milk. In buffered distilled water,

however, cell injury was more pronounced with fast freezing and a higher proportion of cells were killed with slow freezing.

Drying

Various methods of drying bacterial cultures have been reported in the literature. Miller and Simons (53) reported on the survival of bacteria dried at room temperature in vacuum over $CaCl_2$. Cultures were rehydrated after storage for 21 years at 10 C. Only 13 of 202 cultures, representing 67 species, failed to grow. Fisher (19) dried cultures in vacuum over P_2O_5. During the process of drying, 18 to 99% of the organisms (2724 strains) were killed. After 14 years, 83% of these strains were found by Rhodes (63) to be viable. Both $CaCl_2$ and P_2O_5 were used by Stark and Herrington (80) in vacuum drying seven species of microorganisms, including several cultures of S. lactis and S. paracitrovorus. Two-thirds of streptococcal cultures grew readily after storage for 97 days. Annear (3) successfully dried approximately 30 species of microorganisms by placing a drop of a culture suspension on a cotton-wool tuft and drying under vacuum with P_2O_5 as a desiccant.

Generally, bacterial cultures are dried from the frozen state. Proom and Hemmons (61) reported that drying at -78 C resulted in survival of a higher proportion of cells than drying at room temperature. Weiser and Hennum (86) found that 10 to 15% fewer E. coli survived drying at 22 C than at -15 C.

Swift (83) froze cultures in test tubes immersed in a salt-ice mixture. The cultures were then placed in a desiccator and were dried under vacuum in 12 hr. Fifteen strains of non-hemolytic streptococci

frozen in this manner were reported to have survived 22 to 40 months. Hammer (25) soaked paper strips in culture, then vacuum dried, with a salt-ice mixture for refrigeration and H_2SO_4 as a desiccant. Rogers (68) dried lactic cultures by freezing and exposing to vacuum over H_2SO_4. One part of the resulting powder would coagulate 1 million parts of milk in 17 hr at 30 C.

Fry and Greaves (22) described a method of freeze-drying in which the cultures were frozen during centrifugation. Apparatus for freeze-drying using liquid air was also discussed. Wagman and Weneck (84) dried cultures by blowing air or nitrogen gas over frozen pellets of bacterial suspensions.

Richardson (64) and Richardson and Calbert (65) held a frozen and a lyophilized culture of S. lactis at -28 C. After 6 months, the frozen culture was less active than the lyophilized culture. However, the former author reported that at the end of 6 and 12 weeks there was no significant difference in activity after one transfer.

Rogers (68) found a constant decrease in numbers of bacteria when dried in a warm air current. The loss of cells was reduced when the cultures were spray dried. In recent years, spray drying has been investigated as a means of preserving lactic cultures. Richardson (64) found low activity in spray-dried concentrated cultures. Sapp and Hedrick (70) and Lattuada and Foster (40) have attempted to spray dry lactic cultures. The latter authors reported that 50 to 60% of the cells were viable immediately after drying.

As with frozen cultures, the temperature of storage of dried cultures is important to the survival of the organisms. Proom and Hemmons

(61) found 4 C to be a better storage temperature than 37C. Horter (34) was able to store successfully freeze-dried cultures for 2 years at 4 C. About half the organisms in a culture of lyophilized E. coli died in 10 days at room temperature, according to Weiser and Hennum (86). At 10 C, 40 days elapsed before half the organisms died. Richardson (64) found that at 2 to 5 C, lyophilized cultures lost activity more rapidly than at -23 to -28 C.

The nature of the atmosphere during storage has been found to be of importance in certain instances. Proom and Hemmons (61) reported that death of cells proceeded ten times faster when dried cultures were stored under oxygen or air than when under nitrogen or in vacuo. Stark and Herrington (80) reported that exposure of dried bacterial cultures to free oxygen resulted in pronounced killing of cells. Cultures dried by Hammer (25) survived longer with vacuum desiccation than with air desiccation. Lattuada and Foster (40) observed that lactic cultures stored in air at 4 C had nearly a tenfold decrease in the number of cells during 30 days storage. Ninety percent of the cells stored under nitrogen at -18 C were viable after 60 days. However, the effect of temperature may have contributed greatly to these results. Richardson (64) could find no significant differences in survival of dried lactic cultures stored under air, vacuum and nitrogen, each at 2 to 5 C and -23 to -28 C.

Other methods of preservation

Hartsell (27), Gordon and Smith (23) and Morton and Pulaski (54) advocated preserving bacteria by storing agar slants or stabs under sterile paraffin oil. Lindgren and Swartling (41) found this method

inferior to freezing or lyophilizing of lactic cultures. Sears (72) placed paraffined corks in tubes containing agar slant cultures of E. coli-mutabile. The cultures were still viable after 14 years at room temperature. Chance (9) resuspended slant cultures, centrifuged and decanted the supernatant. To the remaining culture, he added 10 ml of a 1% NaCl solution. Organisms were viable after 3 years and 10 months, provided the salt solution did not evaporate to dryness.

Mechanism of Death During Freezing and Drying

A brief search of the literature reveals much disagreement and lack of knowledge concerning the causes of death of microorganisms during freezing or drying. Hammer (25) felt that the concentration of salts during drying was responsible for death of bacteria. Postgate and Hunter (60) stated that the concentration of solutes did not exert any lethal effect during freezing. Mazur (52) presented evidence that death was not due to a high concentration of solutes. Microorganisms survived cooling in 4.0 M $CaCl_2$ and 3.3 M $MgCl_2$. Death only occurred upon the formation of ice. Keith (36), Hilliard and Davis (31) and Weiser and Osterud (87) also observed a sharp increase in the death rate when the suspending medium was frozen. This fact led the former two authors and Hollander and Nell (33) to believe that death was due to a mechanical crushing of the cells by extracellular ice crystals. Harrison and Cerroni (26) compared survival of bacteria upon repeated freezing and thawing to survival upon disintegration by shaking with glass beads. Higher survival with freezing led them to conclude, as Haines (24) did earlier, that the cause of death was not mechanical.

Mazur (52) presented a convincing argument that death was not due to extracellular freezing, but to intracellular ice. He reasoned that slow freezing allowed water to flow out of the cell, thereby reducing the amount of ice which could be present. Rapid thawing was said to prevent growth of ice crystals in the cell. Weiser and Hargiss (85) found the presence of crystals in the cell less lethal than the absence of crystals resulting from rapid freezing and thawing. They felt that partial dehydration probably occurred during crystallization, making the cells more resistant to freezing and thawing. Smith and Swingle (77) attributed resistance to freezing to the absence of water in cells. However, Haines (24) believed that the removal of water from cells during freezing resulted in a concentration of solutes within the cell, a change in pH and the destruction of one or more proteins. Native cellular protein from Bacillus pyocyaneus (Ps. aeruginosa) coagulated rapidly at -2 C but changed little at -20 C. Similar views were expressed by Luyet and Gehenio (46).

The action of protective additives has been explained with the above theories in mind. Squires and Hartsell (79) found that phosphate buffer aided glycerol in protecting cells during frozen storage. The phosphate was thought to prevent denaturation or flocculation of certain cellular proteins. Sugars capable of penetrating the cell were thought by Zimmerman (89) to exert their protective effect through some modification of the intracellular water content. Luyet and Gehenio (45) and Smith et al. (76) attributed the protective effect of glycerol to its ease of penetration into the cell and efficiency in binding water, resulting in smaller ice crystals within the cell. Luyet and Keane (47) presented the same

explanation for the action of ethylene glycol in a frozen culture. However, Postgate and Hunter (60) found no correlation between the ability of a substance to penetrate cells and its protective action. They also felt there was no necessity for an additive to dehydrate partially cells in order to protect them during freezing. Sherman (73), working with unfertilized mouse eggs, questioned the requirement for glycerol to penetrate cells to protect them during freezing.

EXPERIMENTAL METHODS

Collection, Classification and Handling of Cultures

Twelve single-strain cultures of lactic streptococci, two mixed-strain, multiple-type lactic cultures and five strains of Leuconostoc sp. were obtained from the Dairy Microbiology culture collection at Iowa State University. One single-strain culture of S. lactis and seven mixed-strain, multiple-type lactic cultures were obtained from the Food Products Analysis Laboratory at Iowa State University.

The species classification of each single-strain lactic culture was established by the criteria of Sherman (74): growth at 40 C, in 0.3% methylene blue, in 4.0% NaCl and at pH 9.6. Methylene blue milk was made according to the method of Clark (10). Nutrient broth (13), adjusted to the desired salt level or pH, was used for the salt and pH tolerance tests.

Cultures were transferred at least three times weekly. Litmus milk, made from Matrix powder[1] reconstituted to 11% solids and autoclaved at 121 C for 12 min, was used for culture maintenance.

Cultures were grown in Matrix before freezing. The powder was reconstituted to 11% solids and dispensed into 6-oz prescription bottles or Erlenmeyer flasks. Since no other brand of powder was used, reconstituted Matrix will hereafter be referred to as "skimmilk". The reconstituted skimmilk was steamed 1 hr and cooled in flowing tap water. A 1% inoculum was used; cultures were incubated at 21 C for a specified length of time,

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[1] Galloway-West Co., Fond du Lac, Wis.

usually 16 hr.

In experiments where broth cultures were used, a medium of the following composition was used:[1]

Trypticase (BBL)[2]	20 g
Yeast extract (Difco)[3]	5 g
Gelatin	2 g
NaCl	4 g
Dextrose	5 g
Lactose	5 g
Distilled water	1 liter
	pH 6.8

The broth was autoclaved for 15 min at 121 C. Inoculation and incubation were as with the reconstituted skimmilk. To achieve maximum culture activity it was found necessary to neutralize developed acidity. The pH was maintained between 6.0 and 6.5 by the addition of sterile Na_2CO_3 to the broth cultures at selected intervals.

Enumeration and Activity Determination

Methods for estimating bacterial counts of cultures were those outlined in Standard Methods for the Examination of Dairy Products (1). Duplicate plates were poured with Eugon agar[2] which contained 100 ml filtered V-8 Cocktail Vegetable Juice[4] per 900 ml water. Plates were incubated 4 days at 21 C. Colonies were counted with the aid of a Quebec colony counter.

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[1]Baughman, R. W. Ames, Iowa. Media for lactic cultures. Private communication. 1962.

[2]Baltimore Biological Laboratory, Inc., Baltimore, Md.

[3]Difco Laboratories, Inc., Detroit, Mich.

[4]Campbell Soup Co., Camden, N. J.

For the determination of culture activity, 11% solids skimmilk was dispensed into 20 x 125 mm screw-capped test tubes at the rate of 20 ml/tube. The tubes and their contents were steamed 30 min and immediately cooled in tap water. The skimmilk was inoculated with 0.2 ml culture and incubated at 32 C for 6 hr ± 5 min. An 18-ml sample was titrated to the phenolphthalein end point with 0.1 N NaOH, using the equivalent of 20 drops of a 1% solution of phenolphthalein. Near the end of the titration, the pipette was rinsed with the culture-base mixture to remove culture remaining in the pipette. The titratable acidity of an uninoculated control was subtracted from that of the culture, giving the result in terms of developed acidity, calculated as lactic acid. Activities are reported as 100 x developed acidity.

Freezing and Thawing of Cultures

Freezing in tubes

Except where noted, cultures were frozen in 3-ml quantities in sterile 16 x 125 mm screw-capped test tubes. Breakage of tubes was experienced with larger quantities when tubes were immersed in liquid N_2. For storage at -20 C, tubes were immersed in liquid N_2 for 30 sec, then placed in a deep freeze at -20 C. For storage at -196 C, tubes were immersed in liquid N_2 for at least 2 min and were then transferred to an LNR-35 liquid N_2 refrigerator.[1]

In early experiments with storage at -196 C difficulty was encountered

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[1] Linde Company, Division of Union Carbide Corp., 270 Park Ave., New York 17, N. Y.

in removing caps from the tubes rapidly enough to prevent buildup of high pressure from the evaporating N_2. As a safety measure, cotton plugs were used in tubes stored in liquid N_2.

Cultures were thawed by placing the tubes in a water bath at 40 C until no evidence of ice was present. Samples for plate counts or activity tests were taken no more than 10 min after thawing.

Droplet freezing

Droplets of culture were dropped directly onto the surface of liquid N_2 from a syringe fitted with a 20-gauge needle. For small amounts of culture a 5-ml syringe graduated in units of 0.2 ml was used. A 30-ml syringe calibrated in 1-ml divisions was used for larger volumes of culture. Calibration of syringes was verified with bacteriological transfer pipettes meeting specifications listed in Standard Methods for the Examination of Dairy Products (1).

A modification of the droplet freezing apparatus used by Rinfret and Doebbler (67) was used initially (Figure 1). Rinfret and Doebbler found it necessary to have a moving surface of liquid N_2 to prevent clumping of droplets before they were frozen. A stream of liquid nitrogen was directed into the upper end of an inclined trough and allowed to flow to the opposite end. Droplets of culture were directed onto the surface of the N_2 stream. The droplets, frozen by the time they reached the lower end of the trough, were funneled into sterile cotton-stoppered 10 x 75 mm test tubes in 0.2 ml amounts and stored at -196 C.

Droplets were thawed by emptying the contents of a tube into 20 ml skimmilk or into 19.8 ml phosphate-buffered dilution blanks (1) tempered

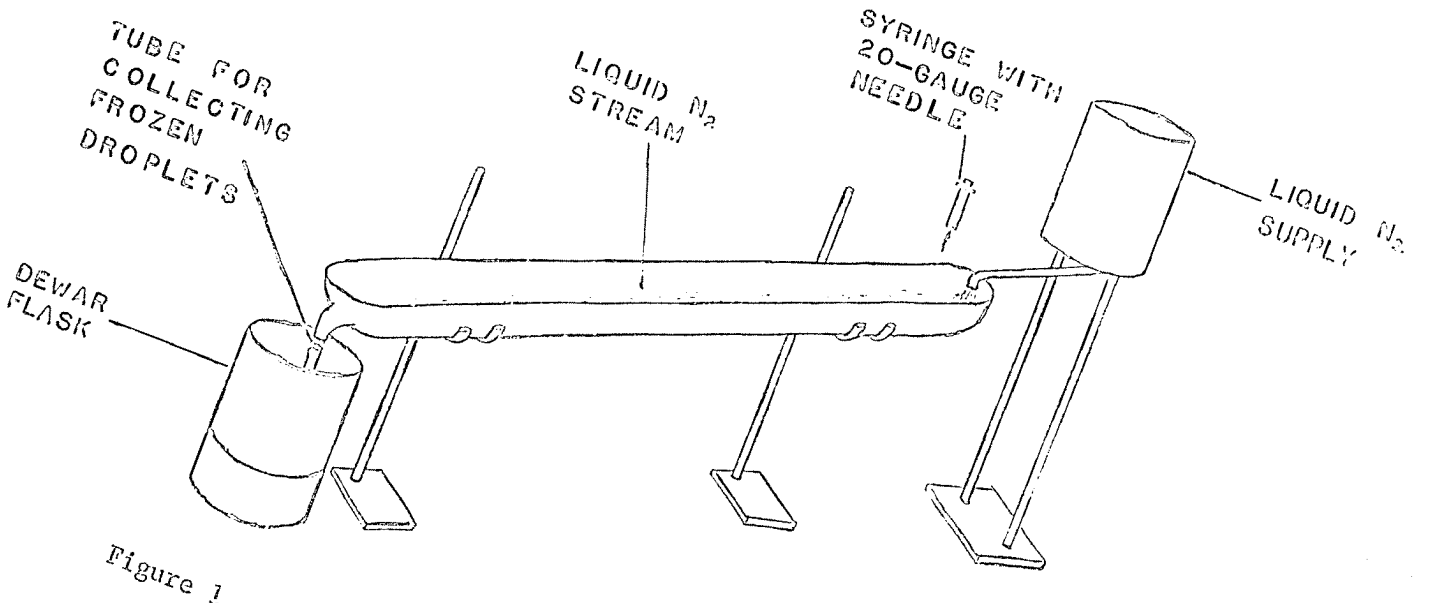

Figure 1. Apparatus for freezing of cultures in droplet form

to 40 C. The milk was placed in the 32 C incubator for activity determination; dilutions blanks were used for agar plate counts.

The apparatus shown in Figure 1 was found to be inconvenient for freezing large quantities of culture. Therefore, in later work, droplets of culture were simply directed into a 1900-ml Dewar flask containing liquid N_2. A syringe fitted with a 16-gauge cannula was used for this work. Frozen droplets were collected in a tea strainer and funneled into appropriate containers for storage at -196 C.

Rate of Freezing and Thawing

Rate of freezing of cultures placed in a deep freeze at -20 C was determined by inserting a thermometer in 5 ml skimmilk in a 16 x 125 mm threaded test tube. The temperature was recorded periodically until the frozen milk was at -20 C. All other freezing rates were determined using an iron-constantan thermocouple. The time required to reach a given potential was measured with a stop watch. The potentials, in millivolts, were converted to temperatures and plotted on a graph. Droplet freezing rates were taken from the data of Rinfret (66).

Eight rates of freezing were studied. Screw-capped test tubes containing 5 ml culture were placed in a deep freeze at -20 C, in the vapor over liquid N_2, or were immersed in liquid N_2 to the level of the culture in the tubes. Aluminum tubes were flattened to cross-sectional measurements of 10 x 25 mm. These, containing 2 ml culture, were suspended in the vapor phase above liquid N_2 or were immersed in liquid N_2. To achieve more rapid rates of cooling, methods similar to those reported by Cowley et al. (11) and Luyet (44) were employed. Aluminum tubes were dipped in

a 1:1 mixture of methanol and glycerol. The methanol was allowed to evaporate, leaving a thin layer of glycerol on the outer surfaces of the tubes. Tubes were then rolled in talcum powder until uniformly covered. Cultures were frozen in 2-ml amounts by immersing in liquid N_2 tubes coated with glycerol and glycerol plus talcum powder. Droplet freezing also was investigated. Methods for this have been described earlier.

Five rates of thawing were studied. Screw-capped test tubes containing culture, previously frozen to -20 and -196 C, were placed in a cooler at 4 C, at room temperature (approximately 26 C), or in a water bath at 40 C. Aluminum tubes were placed in a water bath at 40 C. Droplet frozen cultures were thawed by dumping into milk at 40 C, by placing tubes containing the frozen droplets at room temperature, or in a water bath at 40 C.

In all freeze-thaw studies, cultures were thawed within 2 hr of the time the desired freezing temperature was achieved.

Atmosphere of Cultures During Growth

The effect of growing cultures under CO_2 and aeration upon susceptibility to freezing was studied. One hundred ml broth or skimmilk was placed in each of two 125 ml Erlenmeyer flasks and one 125 ml suction flask. After inoculation, one flask was placed in an airtight incubator and the atmosphere adjusted to 10% CO_2. The second flask was placed under normal atmospheric conditions. The suction flask was fitted with cotton-stoppered glass tubing for aeration. Air was bubbled through the culture using the suction of an aspirator.

At the end of 16 hr incubation, 5 ml culture from each flask was

placed in screw-capped test tubes. The tubes were placed in a deep freeze at -20 C and held for 6 weeks. Activity and agar plate counts were determined before freezing and after storage.

Growth Temperature

Cultures were incubated at 15, 21, 26 and 32 C until activities were equivalent to those of cultures incubated 16 hr at 21 C. Cultures were grown in 20-ml quantities in screw-capped tubes. At selected intervals, tubes were removed from the incubator and samples frozen. Activity tests were run before freezing and after varying periods of storage.

Adjustment of pH

Cultures were neutralized to 0.16% titratable acidity with Na_2CO_3 to study the effect of pH in limiting death during freezing.

A study was made to determine the optimum pH for frozen storage. At the end of 16 hr incubation, cultures were placed in an ice-water bath at 0 C to stop acid development. Activities of the cultures were determined and representative samples were frozen before the pH of the remaining material was adjusted. The pH was determined using a Beckman Zeromatic pH meter. The pH was adjusted to 6, 7 and 8 with 40% NaOH. At each pH level, samples were taken for freezing and determination of activity before further addition of NaOH.

Four neutralizers, NaOH, Na_2CO_3, $Ca(OH)_2$ and $CaCO_3$, were compared to determine their efficiency in preventing death of cells during freezing. The amount of each neutralizer calculated to lower the titratable acidity to 0.16% was added to 100 ml quantities of culture. Samples were taken

for freezing and activity testing in the usual manner.

Precooling Before Freezing

Five-ml quantities of 16-hr cultures were placed in sterile screw-capped test tubes. One tube of each culture was frozen to -196 C. The remaining tubes were divided into two groups. One group was placed in a water bath at 5 C; the other was placed in an ice-water bath at 0 C. Cultures were removed from the baths and frozen after 5 min (the minimum predetermined time required to reach 0 C) and 1, 6, 12 and 24 hr. Cultures were stored at -20 and -196 C for 24 hr. Activities were determined before freezing and upon thawing.

Cell Concentration

Broth cultures were centrifuged in an International Model U centrifuge. Initially, a force of 3500 g was employed. In most experiments, cultures were centrifuged at 4000 g for 30 min. After centrifuging, the supernatant liquid was decanted. The cell paste was resuspended in skimmilk to the desired concentration using a glass stirring rod dipped in 95% ethyl alcohol and flamed. Cell concentrates were held in a 0 C water bath until frozen. Samples of the concentrated cultures were rediluted to known volumes approximately those of the uncentrifuged cultures. Activity tests were conducted on the diluted samples. The final volume of the sample with an activity equivalent to that of the uncentrifuged culture was used to calculate the effective concentration of the culture. The term "effective concentration" is used here to indicate that the concentration was not calculated from cell numbers but from the amount

of active resuspended culture which could be obtained from a given concentrated culture.

Cell concentrates were frozen in tubes at -20 and -196 C and were droplet frozen and stored at -196 C.

Age of Culture

After incubation for 6 and 16 hr, broth cultures were centrifuged and resuspended in milk to 10^8 to 10^9 cells/ml. To determine how much freezing damage could be attributed to cell age and to reduced pH as a result of developed acidity, 0.6% lactic acid was added to half of each culture. Cultures were frozen and stored at -20 and -196 C for 4 hr and 6 weeks.

In a second investigation, cultures were centrifuged immediately after inoculation into broth and after incubation for 4, 8, 12, 16 and 20 hr. The centrifuged cultures were resuspended in skimmilk to 10^8 to 10^9 cells/ml. To insure that all cultures were of the same concentration, a series of dilutions was made in the resuspending operations. Activity tests were run on each dilution and the plate count of the lower dilution was determined. Cultures were frozen and stored at -20 C for 1 day and 4 weeks.

Additives

Broth and milk cultures were frozen with 10% each of sucrose, glycerol and dimethyl sulfoxide. A 60% solution of sucrose was used for addition to cultures. Cultures were frozen at -20 and -196 C and were stored for 6 weeks.

The protective quality of protein hydrolysates was studied. A preliminary investigation was conducted to determine the stimulatory effect of these compounds. Cultures were grown in the presence of 0.1% of the following: Difco[1] yeast extract, Casitone, Proteose-peptone, Casamino acids, Soytone and Tryptone; BBL[2] Lactalysate, Gelysate and Phytone; Sheffield[3] N-Z Amines A, AS, B, E, NAK and YT, N-Z Case, Edamin, Edamin S, Hy-Case, Hy-Case SF and Copanase D. From these, yeast extract, N-Z Amine A and Edamin S were selected for further study. Ten and 50% suspensions of each in distilled water were autoclaved 15 min at 121 C. Preparations were added to cultures at the rate of 0, 1, 5 and 10%. Cultures were frozen at -20 C and stored 4 weeks. Cultures containing 10% glycerol were frozen as controls. In addition to the usual activity tests after thawing, activities were determined in skimmilk containing 0.1% of the additive under investigation.

The use of egg white, powdered egg albumin and casein as protective additives was studied. Egg white was obtained from fresh eggs. Eggs were dipped in alcohol and flamed. A hole was made in each end of the eggs with a sterile instrument, and the white was blown into a sterile screw-capped test tube.

Cultures were frozen with 0, 5 and 10% egg white and stored at -20 and -196 C. Powdered egg albumin and casein were reconstituted to 25% suspensions with distilled water. These were added to cultures to give

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[1]Difco Laboratories, Inc., Detroit, Mich.

[2]Baltimore Biological Laboratory, Inc., Baltimore, Md.

[3]Sheffield Chemical Co., Inc., Division of National Dairy Products Corp., Norwich, N. Y.

0, 1 and 5% albumin or casein in each culture. Cultures were neutralized to 0.16% titratable acidity with Na_2CO_3 before freezing with the above three additives. This was done to facilitate the mixing of casein with the cultures.

Storage Temperature

Storage temperature studies were integrated with studies of other factors and general methods for these have been presented. In one investigation, prior to freezing and after thawing, cultures were plated using Standard methods agar. Counts were also determined with Eugon agar containing V-8 Cocktail Vegetable Juice and activity tests were conducted.

To determine the effect of storage upon the lag phase of lactic organisms, growth curves based on rate of acid production were plotted. Cultures were frozen to -20 and -196 C and thawed after 0.5 hr and 6 weeks. Before freezing and after thawing, a series of inoculations were made for activity determination. Tubes of inoculated skimmilk were incubated at 21 and 32 C. A tube of each culture at 21 C was removed every 4 hr during a 24-hr period and the titratable acidity determined. Cultures incubated at 32 C were titrated every 2 hr for 12 hr.

Storage Atmosphere

Five-ml quantities of 16-hr cultures were placed in screw-capped test tubes. One set of tubes was placed at -20 C. Additional tubes were placed in each of four desiccators. Each desiccator had previously been filled to a depth of 2 in. with glycerol and had been held at -20 C for 48 hr. Glycerol increased the rate of freezing and diminished temperature

fluctuation during handling of the desiccators. A vacuum was drawn on one desiccator until the pressure reached 1.0 mm Hg. One desiccator was flushed with CO_2, another with O_2, each for 3 min. A beaker containing 100 ml liquid N_2 was placed in the fourth desiccator and the N_2 allowed to evaporate. All desiccators were immediately placed at -20 C and held 4 weeks.

Manufacture of Cheese

Three comparisons were made involving the manufacture of Cheddar cheese. Cultures were droplet frozen and stored at -196 C and frozen in bulk at -20 C to be used for each comparison. On two occasions, the culture held at -20 C was frozen in 12-oz Cottage cheese containers. The filled containers were placed in $CaCl_2$ brine at -20 C to increase the cooling rate. Concentrated cultures were stored in test tubes for the third comparison. One vat of cheese in each comparison was made using fresh, unfrozen culture which had been transferred 3 times weekly during the 6-week storage period.

Two vats of cheese were made using culture stored at -20 and -196 C with no other treatments involved. For two vats, culture had been neutralized to 0.16% titratable acidity and had 2.5% yeast extract added before freezing. For the third set of two vats, 10 liters of broth were added to a 20-liter carboy. The broth was autoclaved in 2-liter flasks for 15 min at 121 C and aseptically pumped into the carboy with a vacuum pump. The culture was neutralized at intervals with Na_2CO_3 to maintain the pH between 6.0 and 6.7. The culture was centrifuged with a Sharples centrifuge at 32,000 rev/min. Undiluted cell paste was droplet frozen at

-196 C. Cell paste diluted with an equal volume of skimmilk was stored at -20 C.

Each vat of cheese was made from 250 lb milk containing 3.5% milk fat. A conventional make procedure was used, with 4.5 hr elapsing from the addition of rennet to milling of the curd.

Analyses of Data

All data, with the exception of that from the cheese-making and cell concentration experiments, were analyzed statistically using methods outlined by Snedecor (78). Duncan's (16) multiple range test was used to compare treatment means. Error mean squares for the analyses are listed in the Appendix.

RESULTS

The classification of single-strain cultures used in this study is shown in Table 1.

Rate of Freezing and Thawing

Freezing rates studied are shown in Figures 2, 3, 4 and 5. Figure 5 was plotted from data reported by Rinfret (66). Values plotted in Figures 2, 3 and 4 are the averages of four determinations. Table 2 shows the maximum and average cooling rates depicted by each curve. Droplet freezing data given are for water droplets 1 mm in diameter. The freezing rate in milk may vary somewhat from the rate given.

Thawing rates for metal and glass tubes placed in a water bath at 40 C are presented in Figure 6. The temperature of the center of the culture was assumed to be 0 C at the time the culture was completely thawed. Cultures in screw-capped tubes were thawed in approximately 2 min from -196 to 0 C. A slightly shorter time was required for tubes removed from the deep freeze where they had been held at -20 C. The time required for thawing frozen droplets in phosphate-buffered distilled water was observed to be approximately 1 sec. Exact times for thawing droplets of 1 mm diameter have been recorded by Rinfret (66). The average diameter of droplets frozen using the apparatus described in Figure 1 was approximately 2 mm. Droplets frozen in a stationary liquid N_2 bath averaged 3-4 mm in diameter, because a larger needle was used. The thawing time for these droplets was 2-3 sec. Droplets contained in screw-capped test tubes immersed in a 40 C water bath melted in 45 sec.

Table 1. Classification of single-strain cultures of *Streptococcus* sp.

Culture no.	Growth in 0.3% methylene blue	Growth in 4.0% NaCl	Growth at pH 9.6	Growth at 40 C	Species
497	+	+	-	+	*lactis*
565	+	+	+	+	*lactis*
573	-	-	-	-	*cremoris*
712	+	+	+	+	*lactis*
A15-5	-	-	-	+	*cremoris*
DL	+	+	+	+	*lactis*
FD-2	-	+	-	-	*cremoris*
FS-103	+	-	-	-	*cremoris*
HI-1	+	+	+	+	*lactis*
HI-10	-	-	-	-	*cremoris*
K2	-	+	+	+	*lactis*
W2	+	+	+	+	*lactis*
W26	+	+	+	+	*lactis*[a]

[a]Culture W26 exhibited considerable aroma, suggesting classification as *S. diacetilactis*.

Cultures in screw-capped tubes melted in 20 min when placed at room temperature from storage at -20 C and 40 min was required for cultures removed from liquid N_2. Droplets in test tubes melted in 10 min at room temperature. Cultures from -20 C storage thawed in 3 hr at 4 C; those from -196 C thawed in 5 hr at 4 C.

The results of a comparison of seven freezing rates are shown in

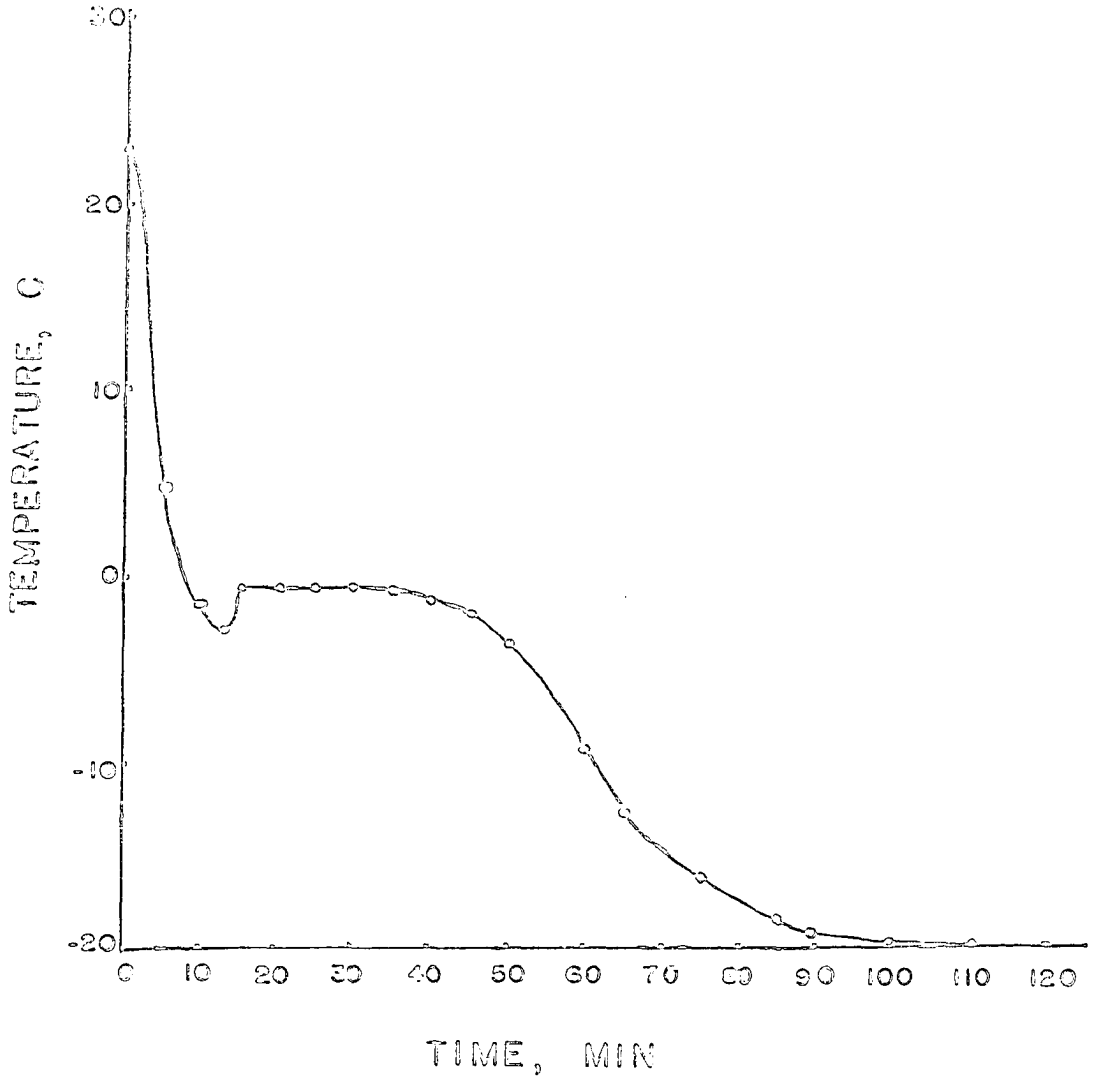

Figure 2. Cooling rate obtained in a screw-capped test tube containing 5 ml culture, placed at -20 C

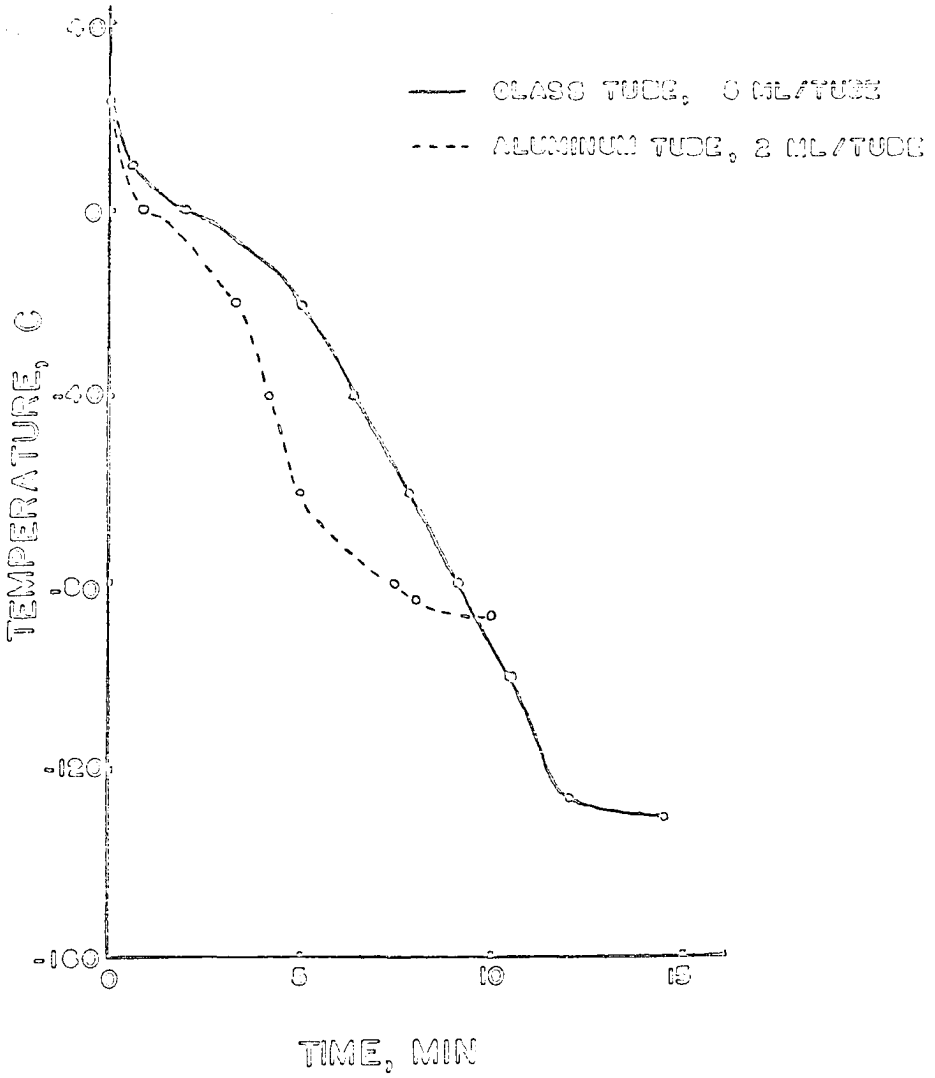

Figure 3. Cooling rates obtained at the center of glass and aluminum tubes suspended in N_2 vapor at -150 C

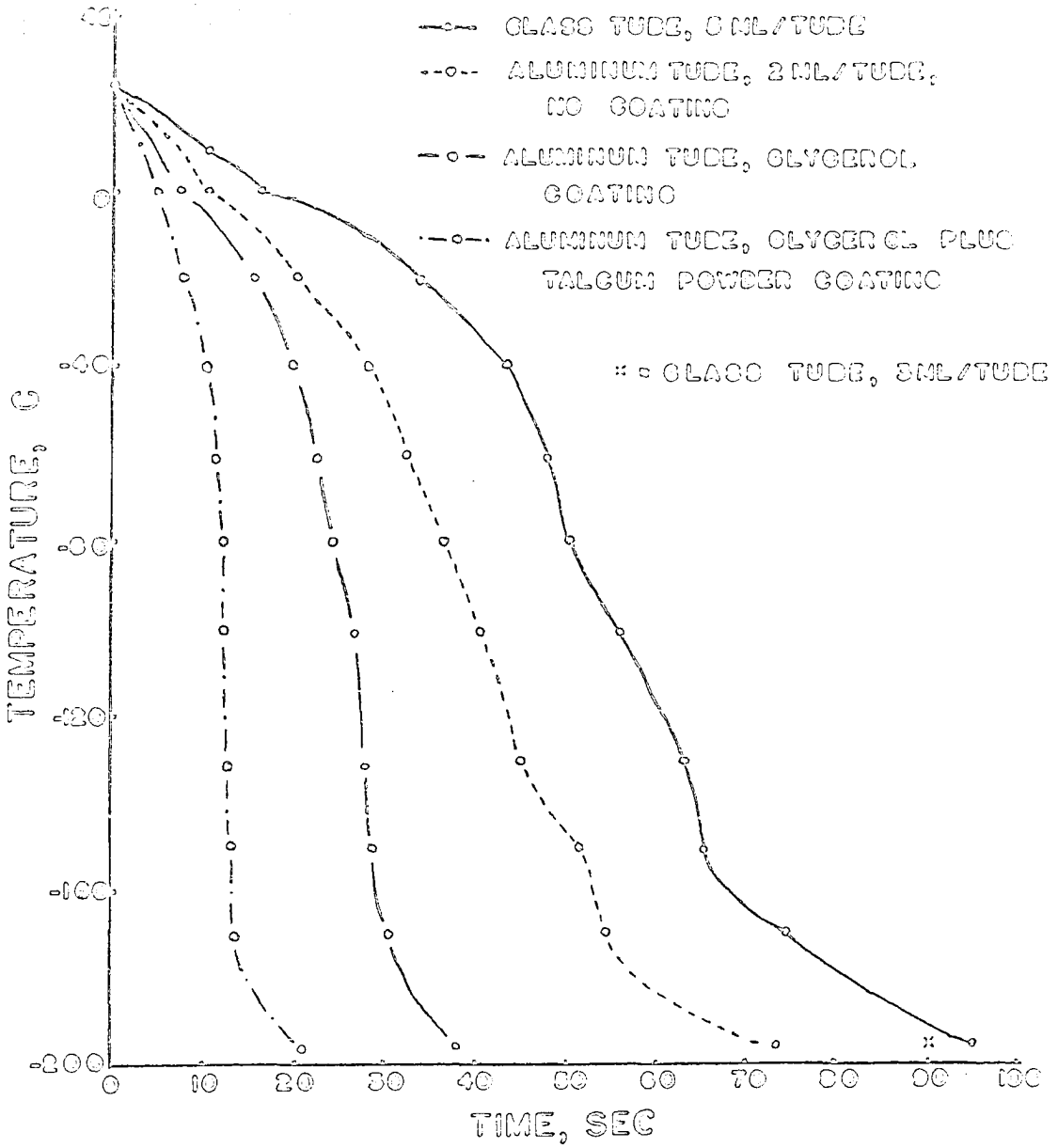

Figure 4. Cooling rates obtained at the center of glass and aluminum tubes immersed in liquid N_2

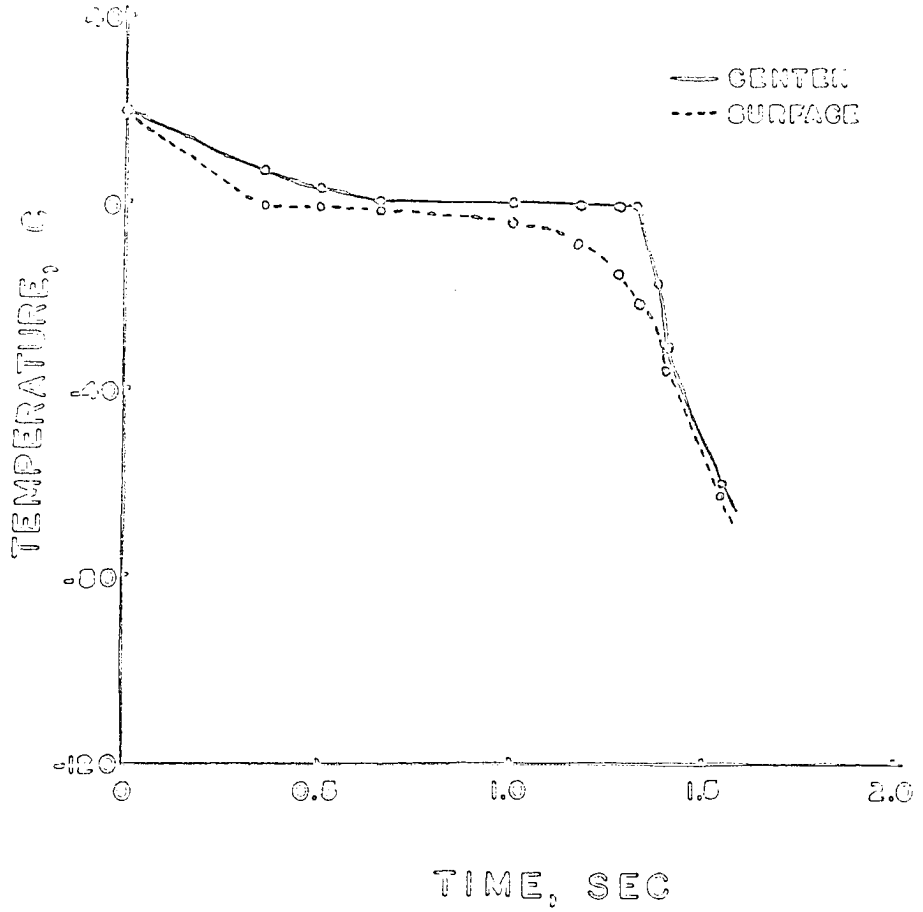

Figure 5. Theoretical cooling rate of a droplet of water 1 mm in diameter in liquid N_2

Table 2. Maximum and average freezing rates obtained by different methods of cooling

Freezing method	Maximum rate[a]	Average rate	Range over which average computed
5 ml/glass tube at -20 C	0.7 C/min	0.2 C/min	0 to -20 C
5 ml/glass tube in vapor phase at -150 C	15.3 C/min	12.4 C/min	0 to -126 C
2 ml/metal tube in vapor phase at -150 C	24.0 C/min	11.8 C/min	0 to -80 C
5 ml/glass tube immersed in liquid N_2	6.2 C/sec	2.5 C/sec 1.1 C/sec	0 to -196 C 0 to -20 C
2 ml/metal tube immersed in liquid N_2, no coating	4.3 C/sec	3.2 C/sec	0 to -196 C
2 ml/metal tube immersed in liquid N_2, glycerol coating	16.7 C/sec	6.7 C/sec	0 to -196 C
2 ml/metal tube immersed in liquid N_2, glycerol plus talcum powder coating	50 C/sec	11.7 C/sec	0 to -196 C
Droplet frozen		275 C/sec[b]	0 to -60 C

[a]Cooling rates above 0 C are not included.

[b]Center of droplet.

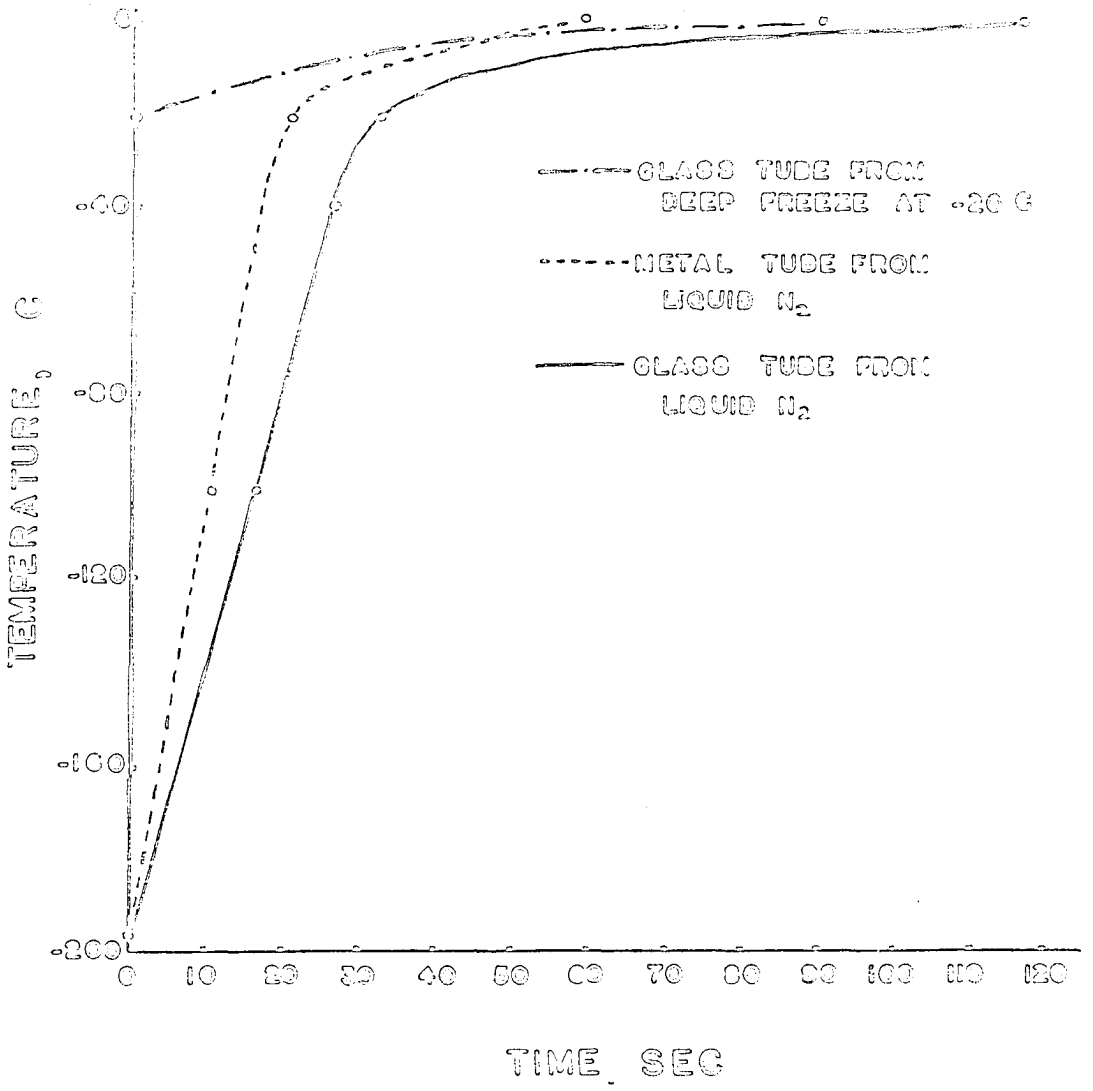

Figure 6. Thawing rates obtained in a 40 C water bath

Table 3. Cultures were thawed at 40 C. Recoveries of cultures subjected to these freezing rates did not differ significantly. Culture-freezing rate interaction was insignificant; no culture responded better to one rate than to another. Variation among cultures was significant.

The effect of varying the rate of warming from -20 and -196 C is shown in Table 4. Studies at each temperature were undertaken separately, so no comparison of temperature effect can be made. Cultures thawed slowly from -20 C showed higher activities than those thawed rapidly. The reverse was true for cultures thawed from -196 C, although less markedly so. Activities of cultures after being subjected to the slowest thawing rate were significantly lower than those obtained at the highest thawing rate.

These results suggested the need for further investigation to determine if the cause of the discrepancy was the difference in freezing temperature or freezing rate. Table 5 shows the results of a temperature comparison; rates of freezing remained as shown in Table 4. Cultures were more active when thawed slowly from -20 C or rapidly from -196 C. The results in Table 6 were obtained using similar freezing and thawing rates for each temperature. Freezing rate was found to have a significant effect on survival of the cultures ($P < 0.01$). No difference in percentage survival of cultures was found when thawed slowly or rapidly. A significant freeze-thaw interaction was found ($P < 0.01$): slow freeze-slow thaw or fast freeze-fast thaw combinations resulted in higher survival than did slow freeze-fast thaw or fast freeze-slow thaw combinations. Effect of freezing temperature was not significant. A three-way interaction between freezing and thawing rates and freezing temperature

Table 3. Survival of single-strain Streptococcus sp. after exposure to different rates of freezing, expressed as per cent of initial activity[a]

Average freezing rate[b]	% Recovery	Culture no.	% Recovery[c]
0.2 C/min	92.7	497	91.3
12.4 C/min	93.1	565	87.0
11.8 C/min	92.7	573	94.0
3.2 C/sec	94.5	712	95.5
6.7 C/sec	95.6	A15-5	99.4
11.7 C/sec	94.0	DL	92.1
275 C/sec	92.1	FD-2	88.9
		FS-103	92.8
		HI-1	95.3
		HI-10	98.6
		K2	99.5
		W2	88.0

[a]Average of two replications.

[b]Frozen to temperatures recorded in Table 2.

[c]Averaged over all freezing rates.

was found to be significant ($P < 0.01$). At -20 C, survival was lower for slow freeze-slow thaw and for fast freeze-fast thaw treatments than at -196 C. Survival was higher at -20 C than at -196 C for the remaining treatments.

Recoveries of Leuconostoc cultures after being subjected to different

Table 4. Effect of varying thawing rate upon survival of single-strain lactic cultures

Thawing time from -20 C	Thawing time from -196 C	% of initial activity when thawed from -20 C[a]	% of initial activity when thawed from -196 C[b]
45 sec	58 sec	85.5	95.1
90 sec	117 sec	91.2	93.5
20 min	40 min	93.6	89.6
3 hr	5 hr	97.3	92.1
Cultures:[c]			
497		97.2	91.1
565		97.9	91.4
573		89.5	89.1
712		97.0	89.5
A15-5		92.2	95.2
DL		91.2	94.5
FD-2		87.2	92.7
FS-103		87.2	96.3
HI-1		93.9	92.2
HI-10		89.2	93.8
K2		88.1	94.3
W2		97.1	91.2

[a]Average of two replications; cultures frozen at 0.2 C/min.

[b]Average of three replications; cultures frozen at 2.5 C/sec.

[c]Averaged over all thawing rates.

Table 5. Effect of varying thawing rate from -20 and -196 C upon survival of single-strain lactic cultures[a]

Thawing time from -20 C	Thawing time from -196 C	% of initial activity when thawed from -20 C	% of initial activity when thawed from -196 C
90 sec	117 sec	90.5	98.0
20 min	40 min	99.8	91.6
3 hr	5 hr	103.4	92.0

[a]Average of cultures 497, 565, 573, 712, FD-2, FS-103, HI-10 and W2.

Table 6. Effect of varying freezing and thawing rates upon survival of single strain lactic cultures frozen to -20 and -196 C, expressed as percent of initial activity[a]

Freezing temperature	Slow freeze[b]		Fast freeze		Avg
	Slow thaw[c]	Fast thaw	Slow thaw	Fast thaw	
-20 C	93.1	87.4	90.6	98.0	92.3
-196 C	95.8	84.6	88.6	105.3	94.2
				Slow freeze	90.9
				Fast freeze	95.6
				Slow thaw	92.7
				Fast thaw	93.8

[a]Average of cultures 497, 565, 573, 712, FD-2, FS-103, HI-10 and W2.

[b]Slow freeze, -20 C: deep freeze at -20 C for 2 hr; -196 C: deep freeze at -20 C for 2 hr, N_2 vapor at -150 C for 5 min, immersed in liquid N_2 for 1 min. Fast freeze, both temperatures: immersed in liquid N_2.

[c]Slow thaw: tubes placed at room temperature; fast thaw: tubes placed in water bath at 40 C.

freezing and thawing rates are given in Table 7. Slow freeze-slow thaw and fast freeze-fast thaw combinations yielded higher proportions of surviving cells at -20 C. These differences were not significant when the data were subjected to statistical analysis.

Atmosphere of Cultures During Growth

The effect of growing cultures under CO_2 and aeration upon resistance to freezing is shown in Table 8. Cultures were frozen in broth with 10% sucrose, glycerol or dimethyl sulfoxide. Variations in atmosphere of growth had no significant effect upon the final activity of the cultures, nor upon the final plate counts of the cultures. Counts upon thawing averaged 19, 21 and 18 x 10^7/ml for the controls, aerated cultures and cultures grown under CO_2, respectively. However, before freezing, counts of aerated cultures were higher than those of cultures grown under CO_2, resulting in significant differences ($P < 0.01$) when recoveries were expressed as percent of initial plate count.

The data in Table 9 were obtained to determine if survival rates of aerated cultures could be increased by removing gases (primarily O_2) dissolved during aeration. No significant difference in counts was obtained after 6 weeks storage at -20 C. Cultures evacuated before freezing exhibited significantly higher ($P < 0.05$) activities after frozen storage than those frozen without evacuation.

Cultures grown in milk were subjected to the same comparisons. These results (Table 10) showed no significant differences in survival rates on the basis of either activity or plate count. Recovery rates were notably higher for cultures frozen in skimmilk than for those of

Table 7. Effect of varying freezing and thawing rates upon survival of single-strain cultures of Leuconostoc sp.[a]

Freezing temperature	Storage time	Slow freeze[b]		Fast freeze		Avg
		Slow thaw	Fast thaw	Slow thaw	Fast thaw	
-20 C	4 hr	104.6[c]	88.7	96.6	100.5	97.4
	6 weeks	85.2	58.1	78.0	91.6	77.5
-196 C	4 hr	84.2	99.8	91.8	95.3	92.2
	6 weeks	97.9	107.0	116.1	101.3	105.5
Avg		92.8	86.9	95.6	97.5	

[a]Average of one culture of L. dextranicum, one culture of L. citrovorum and three cultures of Leuconostoc sp.

[b]Freezing and thawing rates same as given in Table 6.

[c]Percent of initial plate count.

Table 8. Effect of growth atmosphere and additives upon survival of lactic cultures stored in broth for 6 weeks at -20 C[a]

Additive	Normal atmosphere	Aeration	10% CO_2	Avg
None	20.2[b] (42.1)	15.5 (19.1)	29.3 (50.8)	21.7 (37.3)
Sucrose	32.0 (58.9)	36.5 (56.9)	28.2 (62.0)	32.2 (59.3)
Glycerol	54.2 (53.6)	43.3 (53.7)	49.8 (84.9)	49.3 (64.1)
Dimethyl Sulfoxide	49.3 (77.5)	47.5 (65.1)	48.5 (87.8)	48.8 (76.8)
Avg	38.9 (58.0)	35.7 (48.7)	39.0 (71.4)	37.9 (59.4)

[a]Average of cultures 497, 565, 573, 712, A15-5, DL, FD-2, FS-103, HI-1, HI-10, K2 and W2.

[b]Upper no. = percent of initial activity; no. in parentheses = percent of initial plate count.

cultures frozen in broth (Tables 8, 9 and 10).

Growth Temperature

Activities of cultures frozen after growth at 15 and 21 C are shown in Tables 11, 12 and 13. Longer incubation times at 15 C were used in the trials reported in Table 12 because there was no indication that maximum activity had been attained in 26 hr (Table 11). At -20 C, cultures grown at 15 C for longer incubation periods showed higher activities after freezing than those with shorter incubation times. Differences were less marked for cultures grown at 21 C. Cultures incubated at 15 C for the

Table 9. Effect of additives and aeration followed by vacuum treatment upon survival of lactic cultures stored in broth for 6 weeks at 20 C[a]

Additive	Initial activity or plate count/ml x 10^{-7}	Aerated, no vacuum	Aerated, vacuum[b]	Avg
None	22.6[c]	9.9[d]	12.1	11.0
	(43)	(19.9)	(23.0)	(21.4)
Sucrose	20.2	27.7	30.3	29.0
	(33)	(71.4)	(62.4)	(66.9)
Glycerol	20.7	27.3	39.6	33.4
	(36)	(40.3)	(58.4)	(49.4)
Dimethyl sulfoxide	20.7	34.0	40.9	37.4
	(36)	(67.0)	(65.6)	(66.3)
Avg	21.0	24.7	30.7	
	(37)	(49.6)	(52.4)	

[a]Average of cultures 497, 565, 573, FD-2, FS-103, H1-10 and K2.

[b]Vacuum treatment of 1.0 mm Hg applied to aerated cultures for 5 min and released immediately before freezing.

[c]Upper no. = initial activity; no. in parentheses = initial plate count/ml x 10^{-7}.

[d]Upper no. = percent of initial activity; no. in parentheses = percent of initial plate count.

Table 10. Effect of growth atmosphere and additives upon survival of lactic cultures stored in skimmilk for 6 weeks at -20 C[a]

Additive	Normal atmosphere	Aeration, no vacuum	Aeration, vacuum[b]	10% CO_2	Avg
None	66.2[c]	58.2	60.5	62.4	61.8
	(83.6)	(79.4)	(71.4)	(76.6)	(77.8)
Sucrose	60.0	57.8	52.9	52.8	56.1
	(82.2)	(75.3)	(79.9)	(79.2)	(79.2)
Glycerol	61.8	59.2	55.3	61.0	66.4
	(88.2)	(80.6)	(86.4)	(85.1)	(85.1)
Dimethyl sulfoxide	67.7	55.7	64.0	64.3	63.2
	(84.3)	(75.5)	(89.1)	(84.4)	(83.3)
Avg	63.9	56.8	57.1	59.1	
	(84.6)	(77.7)	(81.7)	(81.3)	

[a]Average of cultures 497, 565, 573, 712, A15-5, DL, FD-2, FS-103, HI-1, HI-10, K2 and W2.

[b]Vacuum treatment same as given in Table 9.

[c]Upper no. = percent of initial activity; no. in parentheses = percent of initial plate count.

shorter incubation times had higher percentage survivals at -196 C. The maximum activity after storage at -196 C was observed in cultures incubated at 15 C for 28 hr. Maximum survival rates were not significantly different for cultures grown at either 15 or 21 C.

Table 11. Effect of storage at -20 and -196 C upon cultures grown at 15 and 21 C[a]

Growth temperature	Incubation time	Initial activity	-20 C		-196 C	
			1 day	6 weeks	1 day	6 weeks
15 C	18 hr	17.2	60.4[b]	21.3	129.0	145.4
			(8.0)	(1.9)	(18.8)	(20.8)
	22 hr	26.2	74.2	36.5	106.4	126.9
			(19.7)	(7.1)	(28.6)	(22.6)
	26 hr	31.5	102.8	50.6	108.1	118.1
			(32.0)	(14.4)	(34.1)	(41.0)
21 C	14 hr	33.6	94.2	37.0	119.1	120.6
			(32.1)	(10.2)	(37.5)	(36.6)
	16 hr	37.1	94.4	27.1	108.3	108.2
			(34.5)	(9.7)	(38.5)	(38.6)
	18 hr	38.2	92.2	45.0	93.4	99.7
			(35.2)	(16.3)	(36.6)	(43.3)

[a]Average of cultures 497, 573, FD-2, FS-103, W2 and W26.

[b]Upper no. = percent of initial activity; no. in parenthesis = final activity.

Table 12. Effect of storage at -196 C upon cultures grown at 15 and 21 C[a]

Growth temperature	Incubation time	Initial activity	Storage time	
			1 day	6 weeks
15 C	24 hr	48.5	94.4[b] (45.9)	107.5 (52.6)
	28 hr	53.3	86.9 (46.1)	100.9 (54.5)
	32 hr	50.4	92.4 (46.1)	97.4 (49.6)
21 C	16 hr	52.8	91.7 (48.2)	107.1 (56.8)

[a]Average of cultures 6, 28, 60, 122, FL, FD, M9 and Xi.

[b]Upper no. = percent of initial activity; no. in parentheses = final activity.

Recoveries of cultures frozen after growth at 26 and 32 C are compared with those of cultures grown at 15 and 21 C in Table 13. No significant difference in recoveries of cultures grown at 15 and 21 C was noted after storage at -196 C. Cultures grown at 21 C survived storage at -20 C better than did cultures grown at 15 C. Highest final activities were obtained with cultures grown at 26 C for 10 hr. Cultures grown at 32 C for 7 and 8 hr had highest percentage recoveries, but final activities were little greater than those of other cultures because of low initial activities.

To verify the results shown in Table 13, cultures were grown at 21, 26 and 32 C and stored at -20 C as shown in Table 14. Highest recoveries were obtained with cultures grown at 32 C although these cultures were

Table 13. Effect of storage for 4 weeks at -20 and -196 C upon cultures grown at 15, 21, 26 and 32 C[a]

Growth temperature	Incubation time	Initial activity	-20 C	-196 C
15 C	28 hr	39.7	37.3[b]	118.4
			(14.3)	(46.7)
21 C	16 hr	41.6	48.2	121.6
			(18.5)	(50.3)
26 C	10 hr	40.3	70.3	126.3
			(26.8)	(50.2)
	12 hr	43.2	56.4	112.5
			(23.2)	(48.2)
	14 hr	41.6	52.2	114.4
			(20.9)	(47.5)
32 C	6 hr	42.4	60.2	114.0
			(26.3)	(47.7)
	7 hr	38.5	75.8	128.1
			(27.5)	(47.8)
	8 hr	35.0	78.6	135.8
			(26.0)	(46.5)

[a]Average of cultures 573, FD-2, FS-103, 60, FD and M9.

[b]Upper no. = percent of initial activity; no. in parentheses = final activity.

least active before freezing.

pH of Cultures

Cultures were frozen at different pH values at -20 and -196 C. Average recoveries of these cultures are listed in Table 15. Recoveries after 4 weeks are plotted in Figure 7. Of the pH values studied, greatest survival occurred at pH 6. Unneutralized cultures showed the greatest decrease in activity.

No significant difference in survivals was found after storage for 1 day. At the end of 4 weeks, activities of cultures held at -20 C at pH values of 6 and 7 were significantly higher than those of cultures held at pH 4.6 or 8 ($P < 0.005$). No significant differences were found between activities of cultures held at pH 6 and 7 at -20 C or at any pH at -196 C.

The effect of neutralizing cultures to 0.16% titratable acidity with Na_2CO_3 before freezing is demonstrated in Tables 29, 31 and 34. In every instance higher activities after freezing were noted in the neutralized cultures.

Results of a comparison of the protective effects of four neutralizers are given in Tables 16 and 17. Average recoveries of cultures stored at -20 C appear in Table 16. After 4 hr, cultures with $Ca(OH)_2$ had activities significantly lower than those of cultures with NaOH, Na_2CO_3 and unneutralized cultures ($P < 0.05$). Survival rates after 6 weeks were significantly higher in cultures neutralized with Na_2CO_3 and NaOH than other cultures ($P < 0.005$).

Cultures used in the comparison shown in Table 17 were held at 0 C

Table 14. Effect of storage for 4 weeks at -20 C upon cultures grown at 21, 26 and 32 C[a]

Growth temperature	Incubation time	Initial activity	Recovery[b]
21 C	15 hr	59.2	41.1 (24.2)
26 C	9 hr	57.6	58.8 (33.9)
	10 hr	59.2	57.0 (33.6)
32 C	7 hr	53.1	71.9 (38.0)

[a]Average of cultures 6, 122, FL and Xi.

[b]Upper no. = percent of initial activity; no. in parentheses = final activity.

for 1 hr before freezing to allow the less soluble neutralizers to dissolve. Cultures used in the study reported in Table 16 were frozen within 10 min of neutralization. Na_2CO_3 and NaOH resulted in higher survival after 6 weeks than did $Ca(OH)_2$ and $CaCO_3$ ($P < 0.005$). These differences were more pronounced at -20 C. No significant differences in activity of cultures frozen with sodium or calcium neutralizers were noted after storage at -196 C.

Precooling Before Freezing

Average survival rates of single-strain cultures cooled from 21 C to 5 and 0 C and held up to 24 hr before freezing are shown in Table 18. Activities upon thawing did not differ significantly from those of

Table 15. Effect of pH of cultures upon survival after frozen storage[a]

pH	Initial activity	Storage time	-20 C	-196 C	Avg
4.6[b]	61.3	1 day	67.1[c]	86.7	76.9
			(42.2)	(53.4)	(47.8)
		4 weeks	24.3	94.3	59.3
			(14.8)	(57.1)	(36.0)
6.0	61.8	1 day	75.2	89.7	82.4
			(46.6)	(55.6)	(51.1)
		4 weeks	62.2	97.2	79.7
			(38.7)	(60.1)	(49.2)
7.0	61.3	1 day	74.5	85.0	79.8
			(44.3)	(52.7)	(49.2)
		4 weeks	56.1	91.9	72.3
			(34.5)	(56.3)	(45.3)
8.0	61.0	1 day	72.5	82.7	77.6
			(44.1)	(50.4)	(47.2)
		4 weeks	42.9	86.7	64.5
			(26.2)	(53.0)	(39.6)

[a]Average of cultures M26, FL and M9.

[b]Average pH before neutralization with 40% NaOH.

[c]Upper no. = percent of initial activity; no. in parentheses = final activity.

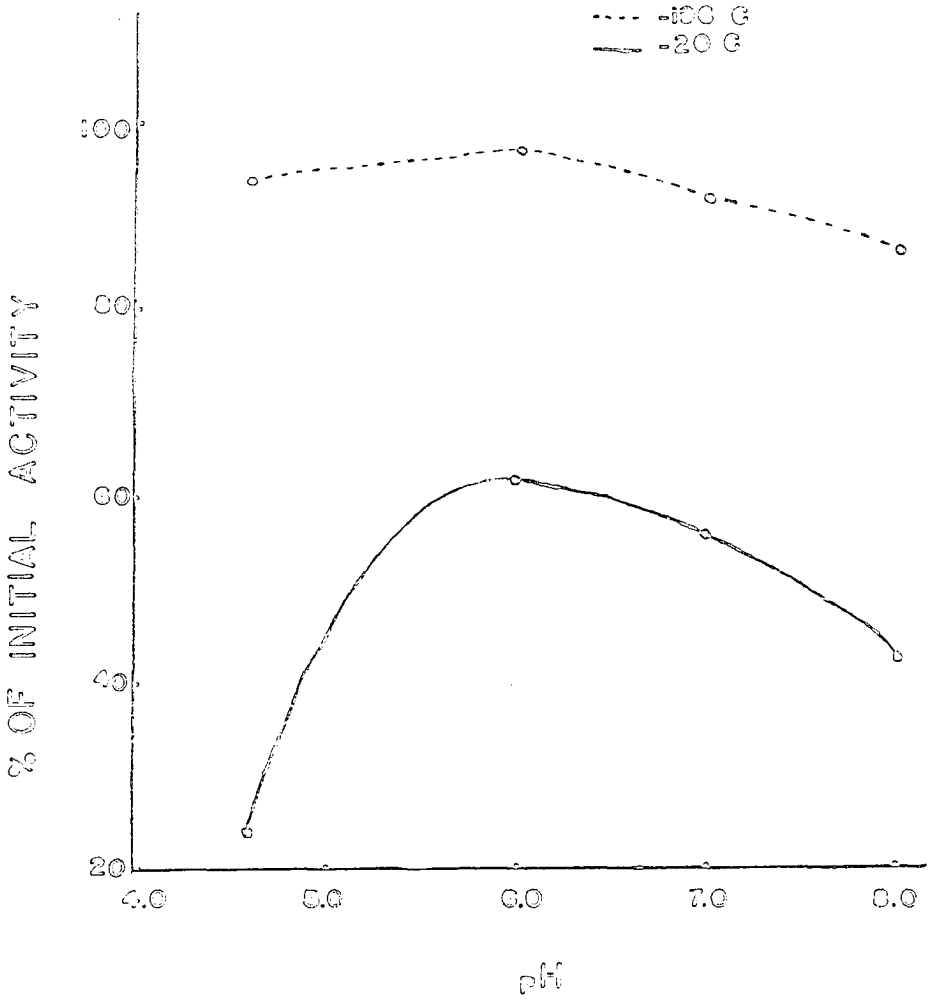

Figure 7. Recoveries of lactic cultures after storage for 4 weeks at different pH values

Table 16. Comparative efficiency of different neutralizers in protecting cultures against freezing damage at -20 C[a]

Neutralizer	pH at freezing	Initial activity	Storage time	Recovery[b]
None	4.58	56.9	4 hr	92.4 (52.6)
			6 weeks	4.2 (2.4)
NaCH	6.40	54.7	4 hr	95.6 (52.2)
			6 weeks	22.1 (12.1)
Na_2CO_3	6.24	56.2	4 hr	98.5 (55.4)
			6 weeks	19.2 (10.6)
$Ca(OH)_2$	5.91	56.2	4 hr	82.4 (46.6)
			6 weeks	0.7 (0.4)
$CaCO_3$	4.76	56.7	4 hr	90.2 (52.1)
			6 weeks	7.4 (4.2)

[a]Average of cultures W26, FD-2, FL and M9.

[b]Upper no. = percent of initial activity; number in parentheses = final activity.

Table 17. Comparative efficiency of different neutralizers in protecting cultures against freezing damage at -20 and -196 C[a]

Neutralizer	pH at freezing	Initial activity	Storage time	Recovery[b] -20 C	-196 C	Avg
None	4.55	67.2	1 day	80.8 (54.2)	96.2 (64.7)	88.5 (59.5)
			4 weeks	15.2 (9.9)	94.8 (63.7)	55.0 (35.1)
NaCH	6.12	68.8	1 day	93.8 (64.5)	100.4 (69.0)	97.1 (66.5)
			4 weeks	62.8 (43.1)	96.4 (66.5)	79.6 (36.5)
Na_2CO_3	5.99	67.6	1 day	91.2 (61.7)	98.7 (66.8)	94.9 (64.2)
			4 weeks	63.0 (42.6)	96.9 (65.6)	80.0 (54.1)
$Ca(CH)_2$	5.51	66.1	1 day	85.4 (56.1)	96.9 (64.0)	91.2 (60.0)
			4 weeks	26.9 (17.1)	97.5 (64.2)	62.2 (40.7)
$CaCO_3$	5.11	66.6	1 day	82.2 (54.3)	96.3 (64.1)	89.2 (59.2)
			4 weeks	44.3 (29.6)	93.3 (62.0)	68.8 (45.8)

[a]Average of cultures 60, 122 and FD.

[b]Upper no. = percent of initial activity; no. in parentheses = final activity.

Table 18. Effect of precooling cultures on survival after freezing for 24 hr[a]

Precooling temperature	Time held before freezing	-20 C[b]	-196 C[c]	Avg
Control[d]			105.0[e]	
5 C	0 hr	98.2	113.7	105.9
	1 hr	104.6	112.3	108.4
	6 hr	104.4	108.2	106.3
	24 hr	75.5	84.7	80.1
	Avg	95.7	104.7	100.2
0 C	0 hr	101.0	107.0	104.0
	1 hr	103.4	112.2	107.8
	6 hr	106.2	109.2	107.7
	24 hr	77.2	81.4	79.3
	Avg	96.9	102.5	99.7

[a]Average of two replications of cultures 497, 565, 573, 712, A15-5, DL, FD-2, FS-103, HI-1, HI-10, K2 and W2.

[b]Avg freezing rate = 0.7 C/min.

[c]Avg freezing rate = 6.2 C/sec.

[d]Frozen to -196 C at 6.2 C/sec without precooling.

[e]Percent of initial activity.

cultures frozen without precooling. Precooling temperature had no significant effect on survival. Survivals of cultures held 24 hr before freezing were lower than those of cultures held for shorter lengths of time ($P < 0.01$). Lower activities were noted in cultures frozen to -20 C than those frozen to -196 C.

Cell Concentration

The effect of neutralizing a broth culture upon the activity before centrifugation and after freezing is shown in Table 19. Preliminary experiments showed cultures neutralized after approximately 11 hr incubation were more active at the end of 16 hr than unneutralized cultures. Results in Table 19 show the effect of varying the amount of neutralization and the time of neutralization. Cultures containing higher amounts of Na_2CO_3 had greater activities before and after centrifuging and after freezing.

In subsequent studies a total of 0.50 g Na_2CO_3/100 ml culture was added to broth cultures in two equal portions at 11 and 15 hr incubation.

Recovery rates of drople. frozen concentrated cultures are given in Table 20. Percentages given are results of one determination per culture.

The effect of cell concentration upon survival after frozen storage is shown in Tables 21 and 22. Lower concentrations of culture FL (Table 21) survived storage at -20 C better than did higher concentrations. Higher concentrations of cultures FL and W26 survived storage at -196 C better than lower concentrations.

In Table 22 the effect of freezing a concentrated culture in the presence of 0, 10 and 25% egg white is shown. At the higher culture

Table 19. Effect of neutralization of developed acidity upon the activity of culture FL after concentration and freezing

Treatment	Subculture 1	2	3	4
	g Na_2CO_3/100 ml			
11-hr culture, Na_2CO_3 added	0.25	0.25	0.25	0.25
13-hr " " "	0.00	0.10	0.15	0.20
15-hr " " "	0.00	0.00	0.05	0.10
Total g Na_2CO_3	0.25	0.35	0.45	0.55
	Activity value for subcultures			
15 1/2-hr culture, before centrifuging	54.2	59.5	57.0	61.2
Centrifuged 30 min at 3500 g; 20:1 effective concentration	54.5	56.5	61.5	58.5
Held 20 hr at -20 C	42.5	45.5	52.8	54.5
	(78.0)[a]	(80.6)	(86.0)	(93.2)

[a]No. in parentheses is percent of activity after centrifugation.

concentration, initial activities decreased slightly with increased egg white concentration. At the lower cell concentration, initial activities were slightly higher with higher egg white concentration. Little difference in recovery was noted in samples stored at -196 C. Survival rates at -20 C diminished rapidly with increasing egg white concentration. The decrease was more noticable at higher cell concentration.

Table 20. Survival of concentrated commercial cultures after droplet freezing in liquid nitrogen

Culture[a]	Approximate effective concentration	% of initial activity after 1 hr at -196 C
60	80:1	98.3
FD	70:1	107.5
6	70:1	81.6
FL	60:1	96.7
FL	40:1	100.0
M9	30:1	87.9

[a]Centrifuged at 4000 g for 30 min except 40:1 concentration of FL which was centrifuged at 3500 g for 30 min.

Table 21. Survival of cultures after storage at -20 and -196 C at different concentrations, expressed as percent of initial activity

Effective concentration	Storage time	Culture FL		Culture W26
		-20 C	-196 C	-196 C
20:1	3 hr	74.3	91.3	81.9
	4 weeks	50.7	90.7	75.0
33:1	3 hr	66.7	75.7	98.4
	4 weeks	47.9	90.9	101.6
50:1	3 hr	53.2	85.6	100.0
	4 weeks	30.3	105.8	104.4

Table 22. Survival of a concentrated culture (122) containing different amounts of egg white after storage at -20 and -196 C

Effective concentration	Amount of egg white, %	Initial activity	Storage time	Recovery after storage at -20 C	Recovery after storage at -196 C
15:1	0	59.5	4 hr	101.7[a] (60.5)	103.8 (61.8)
			4 weeks	60.6 (36.0)	103.0 (61.2)
	10	61.0	4 hr	97.2 (59.2)	104.2 (63.5)
			4 weeks	54.5 (33.2)	100.0 (61.0)
	25	61.5	4 hr	97.2 (59.8)	100.4 (61.8)
			4 weeks	48.8 (30.0)	99.2 (61.0)
25:1	0	58.0	4 hr	95.2 (55.2)	100.4 (58.2)
			4 weeks	68.9 (40.0)	--[b]
	10	57.8	4 hr	99.6 (57.5)	103.9 (60.0)
			4 weeks	29.9 (17.2)	103.1 (59.5)
	25	56.0	4 hr	103.1 (57.8)	108.4 (60.8)
			4 weeks	20.5 (11.5)	108.1 (60.5)

[a]Upper no. = percent of initial activity; no. in parentheses = final activity.

[b]Sample lost.

Data in Tables 24, 25 and 26 show the effect of varying the concentration of cells upon the survival of frozen cultures. No significant effect of cell concentration was noted in recoveries shown in Table 24. An interaction between effects of cell concentration and age of cells was evident. The data in Tables 25 and 26 were obtained to evaluate this interaction. This was not possible in the earlier study because cell concentration varied considerably between age groups. Broth-grown cultures, Table 25, showed significantly higher ($P < 0.01$) recoveries with higher cell concentrations. Milk-grown cultures, Table 26, showed significantly higher ($P < 0.005$) recoveries with lower cell concentrations. No significant interaction between cell concentration and age of cells was found in either instance.

Age of Cells

Broth cultures were centrifuged and resuspended in skimmilk to the concentrations shown in Table 23. Survivals of cultures 8 and 16 hr old, frozen with and without 0.6% lactic acid, are given in Table 23. Older cells and cultures with no added acid had higher survival rates.

In the study reported in Table 24, varying concentrations of cells were used in an attempt to have a set of cultures with the same cell concentration and initial activity for all cell ages. Analysis of the data revealed a highly significant variation of survival rates among concentrations within age groups. Four- and 12-hr cultures had higher survival rates at higher cell concentrations. Lower cell concentration resulted in greater survival for 8- and 16-hr cultures. Results averaged over all cell concentrations showed highest survival with 8-hr cultures.

Table 23. Survival of 6- and 16-hr cultures stored at -20 and -196 C with added lactic acid[a]

Age of cells, hr	Initial activity or plate count/ml x 10^{-7}	Lactic acid added, %	Storage time	% of initial activity after storage at -20 C	% of initial activity after storage at -196 C
6	38.4[b]	0	4 hr	90.6	94.2
	(190)		6 weeks	46.0	89.9
		0.6	4 hr	84.7	87.0
			6 weeks	16.9	76.9
16	25.2 (46)	0	4 hr	108.3	112.6
			6 weeks	64.0	97.6
		0.6	4 hr	82.2	92.1
			6 weeks	32.0	72.8

[a]Average of cultures 497, 565, 573, 712, FD-2, FS-103, HI-10 and W2.

[b]Upper no. = initial activity; no. in parentheses = initial plate count/ml x 10^{-7}.

Table 24. Effect of age and concentration of cells upon survival at -20 C. Trial I[a]

Age of cells, hr	Agar plate count/ml x 10^{-8}	Initial activity	Recovery after storage for 1 day	Recovery after storage for 4 weeks
0	9.7	64.0	89.4[b] (57.1)	88.8 (56.8)
4	36	73.2	90.4 (66.0)	79.1 (57.8)

[a]Average of cultures 60, FD and Xi.

[b]Upper no. = percent of initial activity; no. in parentheses = final activity.

Table 24. (Continued)

Age of cells, hr	Agar plate count/ml x 10^{-8}	Initial activity	Recovery after storage for 1 day	Recovery after storage for 4 weeks
	18	66.3	81.6 (54.1)	58.6 (38.9)
8	68	75.3	93.7 (70.5)	89.9 (67.7)
	34	68.6	99.8 (68.4)	97.1 (66.6)
	23	65.0	103.6 (67.7)	98.4 (64.0)
12	45	68.6	100.9 (69.2)	94.4 (64.5)
	23	65.5	92.4 (62.7)	52.1 (34.2)
	16	64.2	90.6 (58.2)	68.0 (43.8)
16	15	66.9	98.0 (65.3)	52.9 (35.3)
	12	62.8	93.9 (58.6)	83.6 (52.2)
20	16	65.1	97.0 (63.1)	61.6 (40.4)
	13	60.4	100.7 (60.8)	76.5 (48.2)
	9.0	58.8	98.1 (57.7)	67.3 (39.7)
	7.0	55.3	102.2 (56.6)	66.1 (36.5)

Table 25. Effect of age and concentration of cells upon survival after 4 weeks at -20 C. Trial II[a]

Age of cells, hr	Initial agar plate count/ml x 10^{-8}	Initial activity	Initial activity/10^8 cells	Basis of determining survival[b]		
				Agar plate count/ml x 10^{-8}	Activity	Activity/10^8 cells
0	33	69.2	2.10	37.4 (12)	72.7 (50.7)	201.0 (4.22)
	22	64.0	2.91	24.4 (5.4)	52.5 (33.8)	215.5 (6.27)
	14	60.2	4.30	29.5 (4.3)	50.8 (31.8)	172.2 (7.40)
4	44	70.4	1.60	30.0 (15)	80.0 (56.8)	237.0 (3.79)
	33	67.4	2.04	22.3 (5.3)	64.4 (43.9)	406.0 (8.28)
	15	65.5	4.37	13.9 (2.2)	37.6 (24.8)	258.8 (11.3)
8	30	66.9	2.23	3.5 (0.9)	19.7 (13.1)	654.0 (14.6)
	20	61.2	3.06	5.1 (1.0)	34.4 (20.8)	680.0 (20.8)

[a]Average of cultures 60, 122 and FL.

[b]Upper no. = percent of initial count or activity ; no. in parentheses = final count or activity.

Table 25. (Continued)

Age of cells, hr	Initial agar plate count/ml x 10^{-8}	Initial activity	Initial activity/10^8 cells	Basis of determining survival[b]		
				Agar plate count/ml x 10^{-8}	Activity	Activity/10^8 cells
	15	59.8	3.99	11.4 (2.1)	17.5 (10.8)	128.8 (5.14)
12	19	66.5	3.50	36.3 (7.0)	64.8 (43.3)	176.7 (6.18)
	12	63.9	5.32	42.1 (5.3)	59.7 (39.6)	140.8 (7.48)
	10	58.2	5.82	47.6 (3.7)	70.7 (41.8)	194.4 (11.3)

Table 26. Effect of age and dilution of lactic cultures grown in milk upon survival after 4 weeks at -20 C[a]

Age of cells, hr	Initial agar plate count/ml x 10^{-7}	Initial activity	Initial activity/10^7 cells	Basis of determining recovery[b]		
				Agar plate count/ml x 10^{-7}	Activity	Activity/10^7 cells
10	74	54.9	0.74	16.8 (12)	26.9 (14.7)	165 (1.22)
	55	46.6	0.85	9.5 (5.1)	22.0 (10.3)	238 (2.02)
	37	39.8	1.08	14.8 (5.6)	26.8 (10.8)	179 (1.93)
	19	27.8	1.46	21.4 (4.1)	25.3 (7.1)	118 (1.73)
12	100	55.7	0.56	23.3 (23)	37.0 (20.8)	161 (0.90)
	76	49.5	0.65	21.9 (16)	38.6 (18.9)	166 (1.18)
	51	42.7	0.84	33.0 (16)	48.1 (20.7)	154 (1.29)
	25	30.7	1.23	40.7 (10)	53.7 (16.2)	132 (1.62)

[a]Average of cultures 60, FD and Ki.

[b]Upper no. = percent of initial count or activity; no. in parentheses = final count or activity.

Table 26. (Continued)

Age of cells, hr	Initial agar plate count/ml x 10^{-7}	Initial activity	Initial activity/10^7 cells	Basis of determining recovery[b]		
				Agar plate count/ml x 10^{-7}	Activity	Activity/10^7 cells
14	110	61.2	0.56	15.1 (18)	30.4 (18.7)	186 (1.04)
	82	53.9	0.66	31.2 (26)	48.8 (26.4)	155 (1.02)
	55	47.4	0.86	45.7 (26)	61.2 (28.6)	128 (1.10)
	27	37.4	1.38	52.2 (14)	52.9 (19.8)	102 (1.41)
16	100	58.8	0.59	24.7 (21)	37.4 (22.2)	180 (1.06)
	76	51.9	0.68	49.8 (38)	57.5 (29.8)	115 (0.78)
	51	44.2	0.87	61.5 (31)	66.8 (29.2)	108 (0.94)
	26	32.5	1.25	56.0 (14)	60.4 (19.3)	110 (1.38)

No significant differences in survival rates occurred with 4-, 12-, 16- and 20-hr cultures. Percentage survival of 0-hr cultures was slightly below that of 8-hr cultures.

Considerable variation in cell concentration between age groups was noted. This appeared to have some effect upon the results obtained, but the effect was not evident in the data in Table 23. Studies reported in Tables 25 and 26 were made in a further attempt to obtain equal cell concentrations between age groups, so that the effects of cell concentration and age of cells could be evaluated separately. Results in Table 25 show minimum recoveries occurred with 8-hr cultures. Recoveries increased with increasing culture age, from 10 to 16 hr, according to the data in Table 26.

Additives

Comparisons of the effects of sucrose, glycerol and dimethyl sulfoxide in protecting lactic cultures during frozen storage are shown in Tables 8, 9, 10 and 27. With the exception of the results in Table 10, cultures frozen with no additive had lower activities after freezing than those stored with additives. Recoveries after storage with glycerol or dimethyl sulfoxide were similar, although glycerol yielded higher survival rates in most cases. Sucrose appeared less beneficial than the other additives at -20 C. At -196 C (Table 27) no significant differences in recovery rates were found, although cultures containing dimethyl sulfoxide had somewhat lower activities after thawing than those with the other additives.

Protective and stimulatory effects of yeast extract, N-Z Amine A

Table 27. Effect of different additives upon survival of single strain lactic cultures stored for 6 weeks at -20 and -196 C[a]

Additive	% of initial activity after storage at -20 C	% of initial activity after storage at -196 C
None	56.9	95.0
Sucrose	62.7	98.5
Glycerol	73.6	93.6
Dimethyl sulfoxide	63.4	87.6

[a]Average of two replications of cultures 497, 565, 573, 712, A15-5, DL, FD-2, FS-103, HI-1, HI-10, K2 and W2.

and Edamin S are given in Table 28 and Figure 8. These additives were similar in ability to stimulate cultures into more rapid acid production. Little or no increase in stimulation occurred upon increasing the amount of additive in the culture above 5%. Maximum protection was offered by 10% N-Z Amine A. Yeast extract was slightly superior when 1% additive was used. The protection offered by 1% yeast extract was equivalent to that obtained from 10% glycerol in the cultures.

Since yeast extract appeared slightly superior at low levels, it was selected for further examination, as shown in Table 29. Recovery rates were significantly higher ($P < 0.01$) with higher levels of yeast extract. No interaction of effects of neutralization and level of yeast extract was found.

Efficiencies of fresh egg white and reconstituted powdered egg albumin and casein in protecting cultures against freezing damage are presented in Table 30. The addition of egg white to cultures resulted in

Table 28. Effect of yeast extract, N-2 Amine A and Edamin S in protecting single-strain lactic cultures during storage at -20 C[a]

Additive	Amount of additive, % in frozen culture	Amount of additive, % in activity test after thawing	Initial activity	Initial stimulation[b]	Storage time	Secondary stimulation[c]	Protective effect[d]	Thawing activity
Yeast extract	0	0	37.8	0	4 hr	0	2.6	35.1
					4 weeks	0	20.0	17.8
		0.1			4 hr	13.0		48.1
					4 weeks	13.6		31.4
	1.0	0		2.1	4 hr	0	2.1	37.8
					4 weeks	0	13.8	26.0
		0.1			4 hr	8.5		46.2
					4 weeks	6.9		32.8

[a]Average of cultures FD-2, FS-103, W2, W26.

[b]Increase in activity above that of initial activity due to carryover of additive in making inoculations for the activity test.

[c]Increase in activity due to 0.1% additive in skimmilk used for activity test.

[d]Decrease in activity during freezing; initial activity + initial stimulation + protective effect = thawing activity.

Table 28. (Continued)

Additive	Amount of additive, % in frozen culture	Amount of additive, % in activity test after thawing	Initial activity	Initial stimulation[b]	Storage time	Secondary stimulation[c]	Protective effect[d]	Thawing activity
	5.0	0		8.8	4 hr	0	0.3	46.2
					4 weeks	0	6.6	40.0
		0.1			4 hr	1.0		47.2
					4 weeks	3.9		42.6
	10.0	0		6.1	4 hr	0	1.4	42.4
					4 weeks	0	8.8	35.0
		0.1			4 hr	1.8		44.2
					4 weeks	6.1		35.9
N-Z Amine A	0	0	37.8	0	4 hr	0	2.6	35.1
					4 weeks	0	20.0	17.8
		0.1			4 hr	8.2		43.3
					4 weeks	5.8		23.6
	1.0	0		2.1	4 hr	0	1.5	38.4
					4 weeks	0	16.4	23.3

Table 28. (Continued)

Additive	Amount of additive, % in frozen culture	Amount of additive, % in activity test after thawing	Initial activity	Initial stimulation[b]	Storage time	Secondary stimulation[c]	Protective effect[d]	Thawing activity
		0.1			4 hr	4.7		43.4
					4 weeks	8.1		31.2
	5.0	0		7.9	4 hr	0	1.9	43.8
					4 weeks	0	4.4	41.1
		0.1			4 hr	4.6		48.4
					4 weeks	5.8		47.1
	10.0	0		8.1	4 hr	0	2.4	43.4
					4 weeks	0	3.4	42.4
		0.1			4 hr	3.4		46.8
					4 weeks	3.3		45.7
Edamin S	0	0	37.8	0	4 hr	0	2.6	35.1
					4 weeks	0	20.0	17.8
		0.1			4 hr	11.3		46.4
					4 weeks	10.4		28.2

Table 28. (Continued)

Additive	Amount of additive, % in frozen culture	Amount of additive, % in activity test after thawing	Initial activity	Initial stimulation[b]	Storage time	Secondary stimulation[c]	Protective effect[d]	Thawing activity
	1.0	0		1.1	4 hr	0	2.7	36.1
					4 weeks	0	17.1	21.7
		0.1			4 hr	7.6		43.7
					4 weeks	7.1		28.8
	5.0	0		7.9	4 hr	0	3.9	41.7
					4 weeks	0	12.1	33.5
		0.1			4 hr	3.1		44.8
					4 weeks	4.2		37.7
	10.0	0		8.5	4 hr	0	3.2	43.0
					4 weeks	0	11.8	34.5
		0.1			4 hr	0.2		43.2
					4 weeks	2.5		37.0
Glycerol	10.0	0	37.8	-1.5	4 hr	0	1.2	34.3
					4 weeks	0	13.8	22.4

Table 28. (Continued)

Additive	Amount of additive, % in frozen culture	Amount of additive, % in activity test after thawing	Initial activity	Initial stimulation[b]	Storage time	Secondary stimulation[c]	Protective effect[d]	Thawing activity
		0.1 (yeast extract)			4 hr	10.7		45.7
					4 weeks	14.1		35.8
		0.1 (N-Z Amine A)			4 hr	9.5		44.5
					4 weeks	9.1		31.6
		0.1 (Edamin S)			4 hr	9.0		44.0
					4 weeks	11.8		34.2

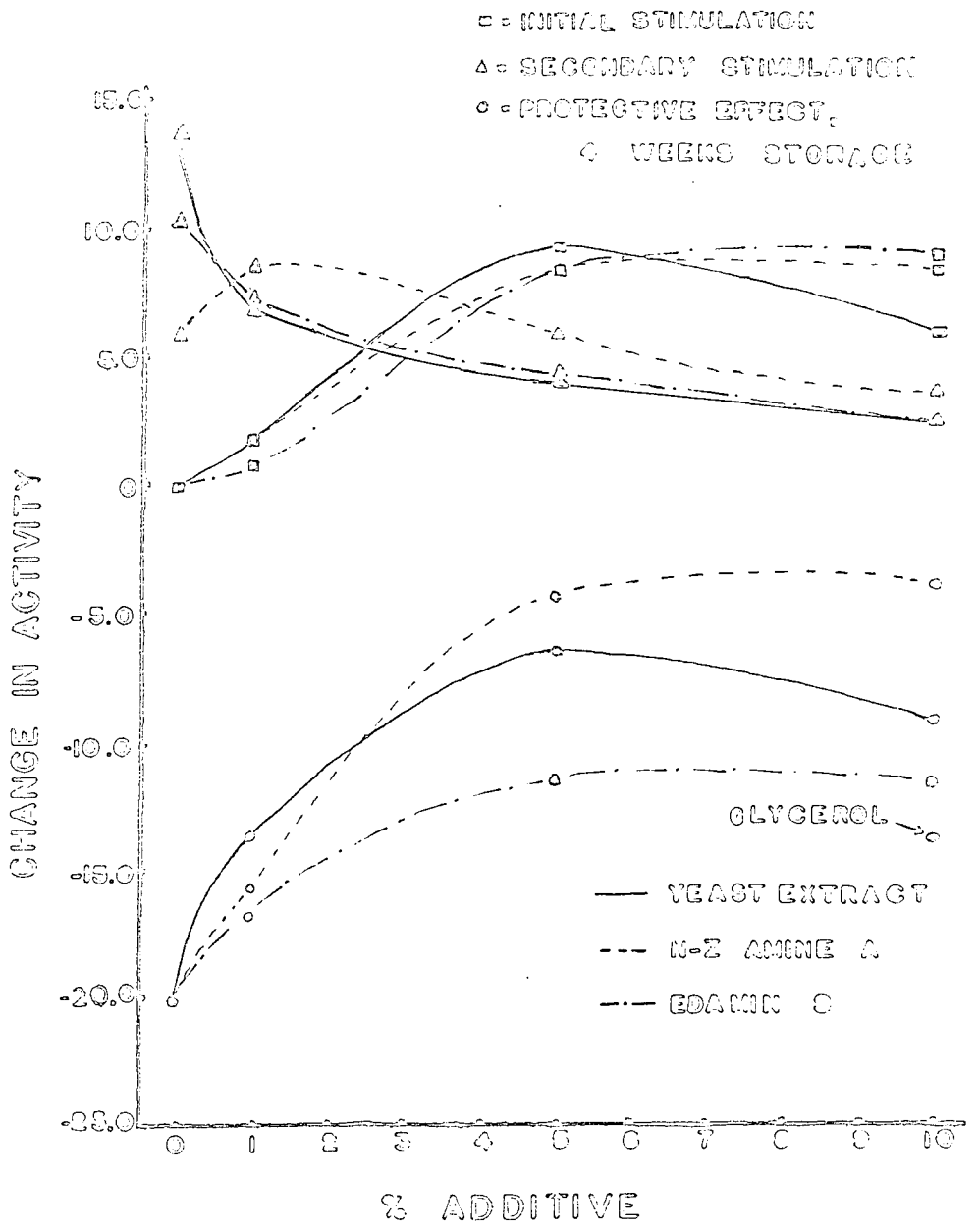

Figure 8. Protective and stimulatory effects of yeast extract, N-Z Amine A and Edamin S

Table 29. Survival of neutralized and unneutralized cultures after storage at -20 C with different levels of yeast extract[a]

Titratable acidity	Yeast extract, %	Initial activity	Storage time	Final activity	Recovery, %
0.392	0	37.6	4 hr	35.6	92.4
			4 weeks	8.9	26.5
	1	40.6	4 hr	38.2	94.4
			4 weeks	15.1	39.7
	10	49.6	4 hr	50.9	103.1
			4 weeks	26.3	53.1
0.16[b]	0	39.2	4 hr	36.9	93.7
			4 weeks	17.2	46.1
	1	42.8	4 hr	41.4	95.7
			4 weeks	18.4	43.7
	10	50.8	4 hr	50.0	98.1
			4 weeks	32.2	63.6

[a]Avg. of cultures FD-2, FS-103, W2 and W26.

[b]Assumed value: the amount of Na_2CO_3 calculated to reduce the titratable acidity to this level was added to each culture.

Table 30. Effect of egg white, egg albumin and casein upon survival of single-strain lactic cultures stored at -20 C[a]

Additive	Amount, %	Initial activity	Storage time	Final activity	Recovery, %
None		42.7	2 hr	40.3	95.1
			4 weeks	23.0	53.2
			8 weeks	15.2	35.4
Egg white	5	37.2	2 hr	37.8	101.3
			4 weeks	28.6	76.0
			8 weeks	18.9	50.8
	10	37.6	2 hr	39.4	105.0
			4 weeks	29.4	78.0
			8 weeks	22.5	59.0
Egg albumin	1	41.7	2 hr	40.6	97.4
			4 weeks	28.2	66.9
			8 weeks	24.6	57.3
	5	40.0	2 hr	39.9	99.8
			4 weeks	22.3	54.7
			8 weeks	15.8	38.1
Casein	1	45.8	2 hr	28.6	88.3
			4 weeks	24.8	55.5
			8 weeks	23.4	52.0
	5	40.8	2 hr	41.1	102.3
			4 weeks	24.9	60.6
			8 weeks	19.4	44.4

[a]Avg. of cultures 573, FD-2, FS-103, W26.

a significant decrease in activity before freezing ($P < 0.05$). No significant differences in percentage recovery were found after storage for 2 hr at -20 C. After 4 weeks cultures containing egg white had higher percentage recoveries ($P < 0.05$) than cultures containing casein. After 8 weeks, recovery rates for all additives were not significantly different. The presence of additives resulted in significantly higher recoveries than in cultures with no additive ($P < 0.005$).

Additives considered most effective in previous experiments were selected for the comparisons reported in Table 31. Levels of egg white, egg albumin and yeast extract were those corresponding to highest recoveries listed in Tables 28 and 29. All previous experiments with glycerol had involved 10% addition. Here 15 and 20% were the levels used. Cultures with glycerol had low initial activities but had significantly higher percentage recoveries than cultures with other additives ($P < 0.005$). Egg albumin resulted in lower recoveries than those of unneutralized cultures with no additive. These differences were considerably more pronounced ($P < 0.005$) at -20 C than at -196 C. A significant interaction between effects of additive and neutralization was found ($P < 0.05$): recovery rates of neutralized cultures with no additive, egg white and egg albumin were more nearly equal than the recovery rates of the corresponding unneutralized cultures. Neutralization was more effective in preserving cultures stored at -20 C than at -196 C ($P < 0.005$).

The survival of cultures containing no glycerol and 10% glycerol after extended storage is illustrated in Figures 9 and 10. Cultures stored at -20 C with glycerol had higher activities than those with no additive ($P < 0.05$), but at -196 C no significant differences were

Table 31. Survival of neutralized and unneutralized cultures after storage for 6 weeks at -20 and -196 C with different additives[a]

Titratable acidity	Additive and amount	Initial activity	Recovery after storage 6 weeks at -20 C	Recovery after storage 6 weeks at -196 C
1.038	None	52.1	28.6[b] (14.8)	111.9 (58.5)
	10% egg white	49.7	36.2 (18.4)	116.1 (57.6)
	1% egg albumin	51.8	24.2 (12.6)	102.8 (66.1)
	5% yeast extract	60.3	71.5 (43.2)	107.5 (65.1)
	15% glycerol	49.0	75.9 (37.4)	108.9 (53.7)
	20% glycerol	43.2	81.9 (35.4)	130.2 (55.2)
0.16[c]	None	52.0	69.0 (36.5)	115.2 (59.5)
	10% egg white	52.8	64.1 (33.9)	106.4 (56.2)
	1% egg albumin	53.8	66.4 (36.5)	102.3 (56.3)
	5% yeast extract	60.2	73.4 (44.2)	106.0 (63.8)
	15% glycerol	49.0	97.4 (47.8)	105.4 (51.5)
	20% glycerol	44.8	97.6 (42.9)	118.0 (52.2)

[a]Avg of cultures FD, W26 and M9.

[b]Upper no. = percent of initial activity; no. in parentheses = final activity.

[c]Assumed value: the amount of Na_2CO_3 calculated to reduce the titratable acidity to this level was added to each culture.

apparent.

Storage Temperature

Survival rates of Leuconostoc sp. after storage at -20 and -196 C are given in Table 7. Results of the effect of storage temperature upon Streptococcus sp. and mixed-strain, multiple-type cultures are shown in Tables 11, 13, 15, 17, 18, 21, 22, 23, 24, 27, 31, 32 and 33 and in Figures 7, 9, 10 and 11. In every instance storage at -196 C resulted in more active cultures than did storage at -20 C. Other variables studied had less effect on survival of cultures stored at -196 C than at -20 C.

Average recoveries of cultures 497, 573, 712, FD-2, FS-103 and W2 after storage for up to 28 weeks are plotted in Figures 9 and 10. An increase in activity upon extended storage was noted in cultures held in liquid N_2. Recoveries as determined by the agar plate count method

Table 32. Survival of culture FD concentrated 25:1 after storage at -20 and -196 C

Storage time	Recovery after storage at -20 C	Recovery after storage at -196 C: Culture in tubes	Recovery after storage at -196 C: Droplet frozen
1 hr	90.0[a] (54.9)	88.2 (53.8)	85.2 (51.9)
3 weeks	66.0 (40.2)	87.2 (53.2)	89.8 (54.5)
6 weeks	66.2 (40.4)	83.1 (53.6)	86.0 (52.5)

[a]Upper no. = percent of initial activity; no. in parentheses = final activity.

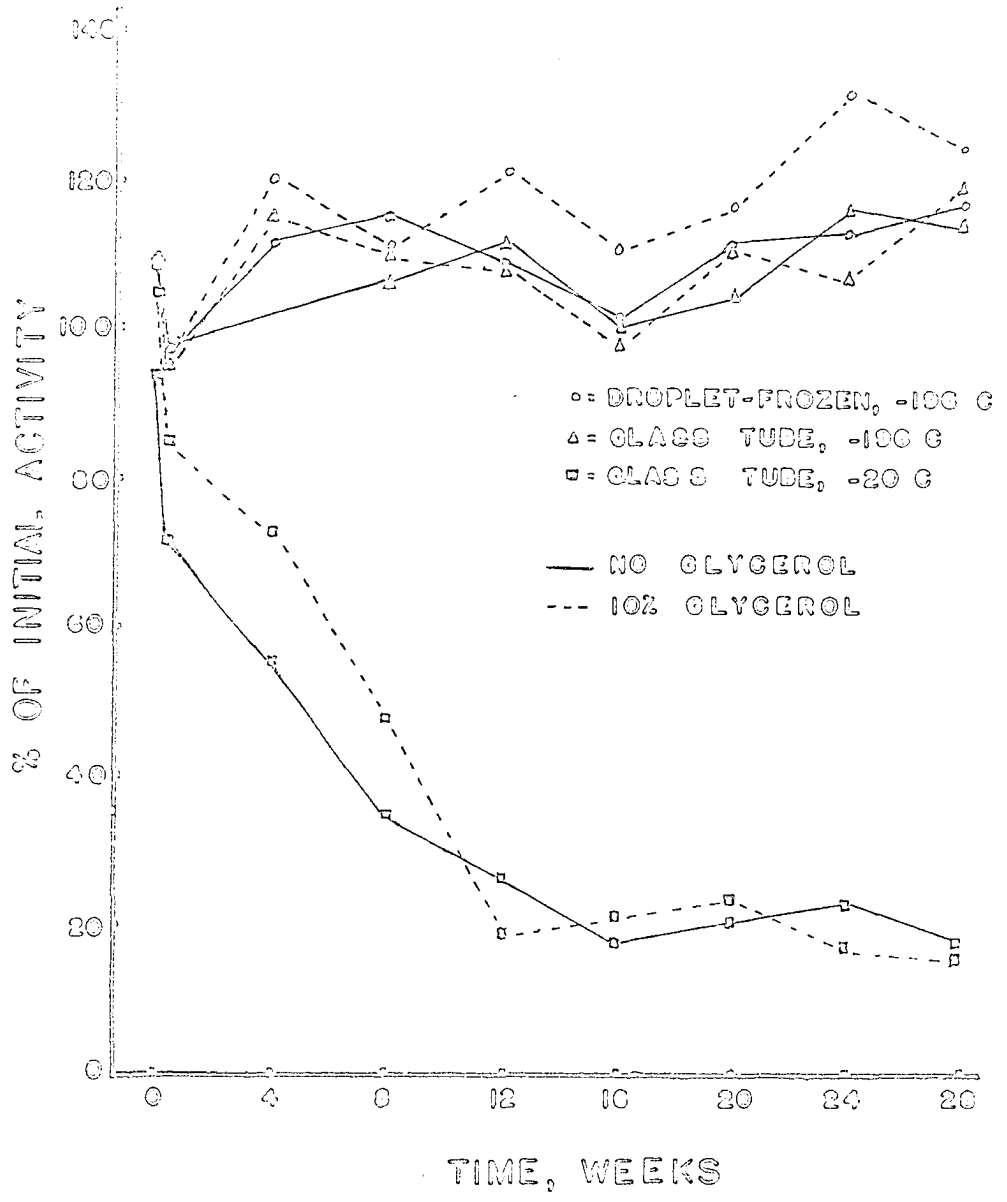

Figure 9. Effect of storage temperature and the presence of glycerol upon the activity of lactic cultures

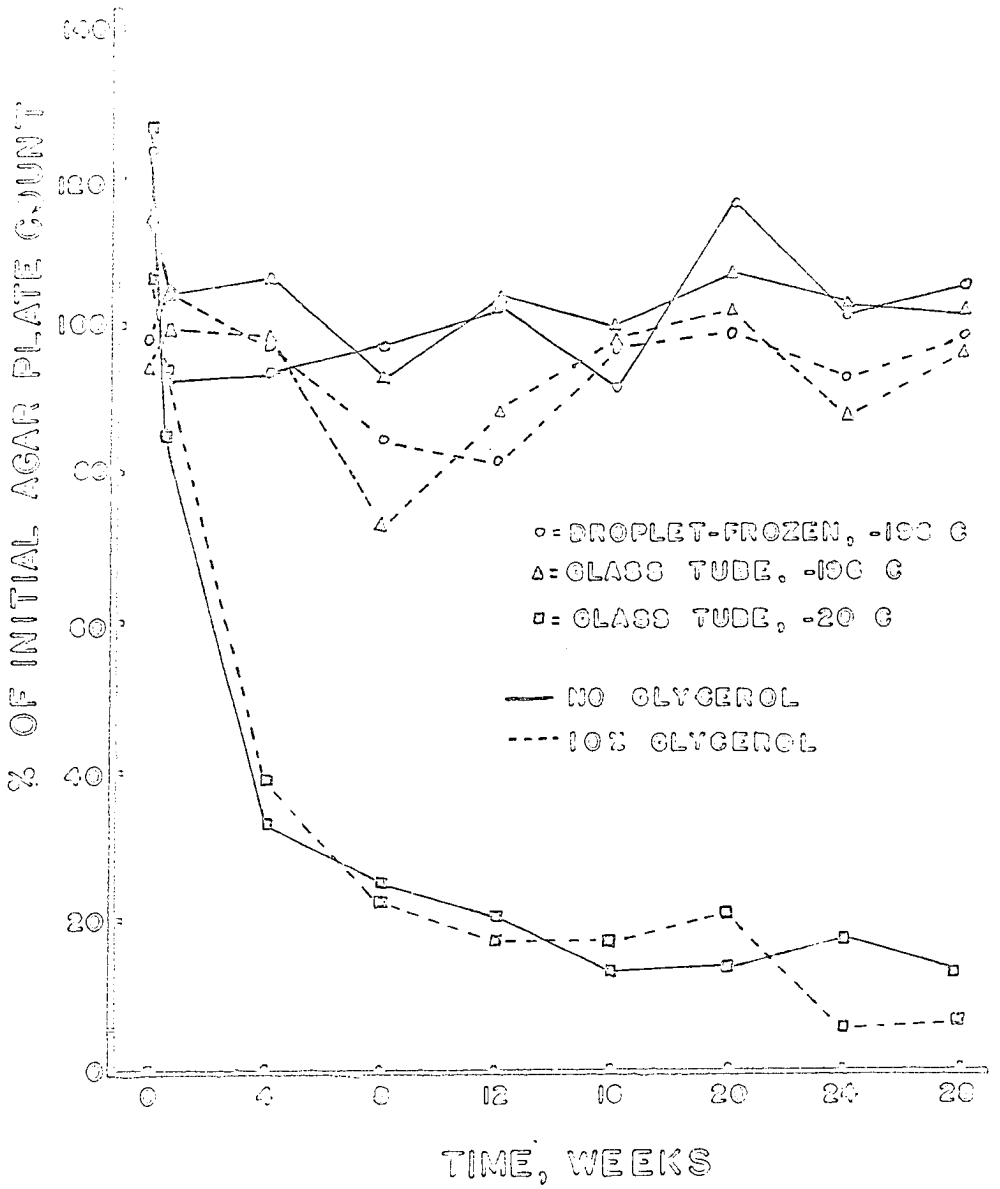

Figure 10. Effect of storage temperature and the presence of glycerol upon the agar plate count of lactic cultures

remained near 100% for the duration of the study. Cultures containing glycerol and stored at -196 C had lower counts than those stored without glycerol.

Droplet-frozen cultures were slightly, but significantly, higher in activity than cultures frozen in test tubes at -196 C. In a separate study, droplet-frozen commercial cultures (6, 28, 60, 122, FD, FL, M9 and Mi) averaged 104.1% and 98.9% of their initial activity after 2 hr and 6 weeks storage, respectively. Recoveries of droplet-frozen concentrated cultures are presented in Table 20.

A comparison of methods used to determine survival after storage at -20 and 196 C is given in Table 33. Culture activities and counts on both media were higher after storage at -196 C than after storage at -20 C. Percent recovery at -20 C using Standard methods agar approached being significantly lower ($0.05 < P < 0.10$) than recovery using acid-producing ability as a criterion.

In selecting the time and temperature of incubation for the activity test used in this study, results plotted in Figure 11 were obtained, using cultures FD-2, FS-103, M26, FD and FL. A secondary purpose in obtaining this information was to determine if the lag phase was shortened or unaffected by storage in liquid N_2. No appreciable lag in rate of acid development was noted in cultures stored at -196 C. Growth initiation appeared slower after storage at -20 C. Data obtained 0.5 hr after freezing (not plotted) showed no alteration of the growth curve upon thawing from either -20 or -196 C.

Table 33. Comparison of recovery rates after 6 weeks at -20 and -196 C as determined by acidity development and agar plate count[a]

Method of determining recovery	Percentage recovery after storage at -20 C	Percentage recovery after storage at -196 C
Culture activity	61.8	94.6
Agar plate count - Standard methods agar	51.3	97.5
Agar plate count - Eugon agar plus V-8 Juice	59.0	103.6

[a]Average of two replications of cultures 497, 565, 573, 712, A15-5, DL, FD-2, FS-103, HI-1, HI-10, K2 and W2.

Atmosphere of Storage

The effect of storage atmosphere upon neutralized and unneutralized cultures is shown in Table 34. Cultures stored under N_2 had significantly higher ($P < 0.05$) activities than cultures stored under CO_2, O_2 or vacuum. Storage under air resulted in significantly higher ($P < 0.05$) recovery than storage under CO_2. No other significant differences occurred. No interaction between atmospheric and neutralization effects were found.

Manufacture of Cheddar Cheese using Frozen Cultures

The ability of frozen cultures to function as starters for Cheddar cheese manufacture is shown in Table 35. Vats 1A, 2A and 3A were used as controls in each comparison. Cultures stored at -20 C showed considerable decrease in activity. Lower titratable acidities at milling were noted

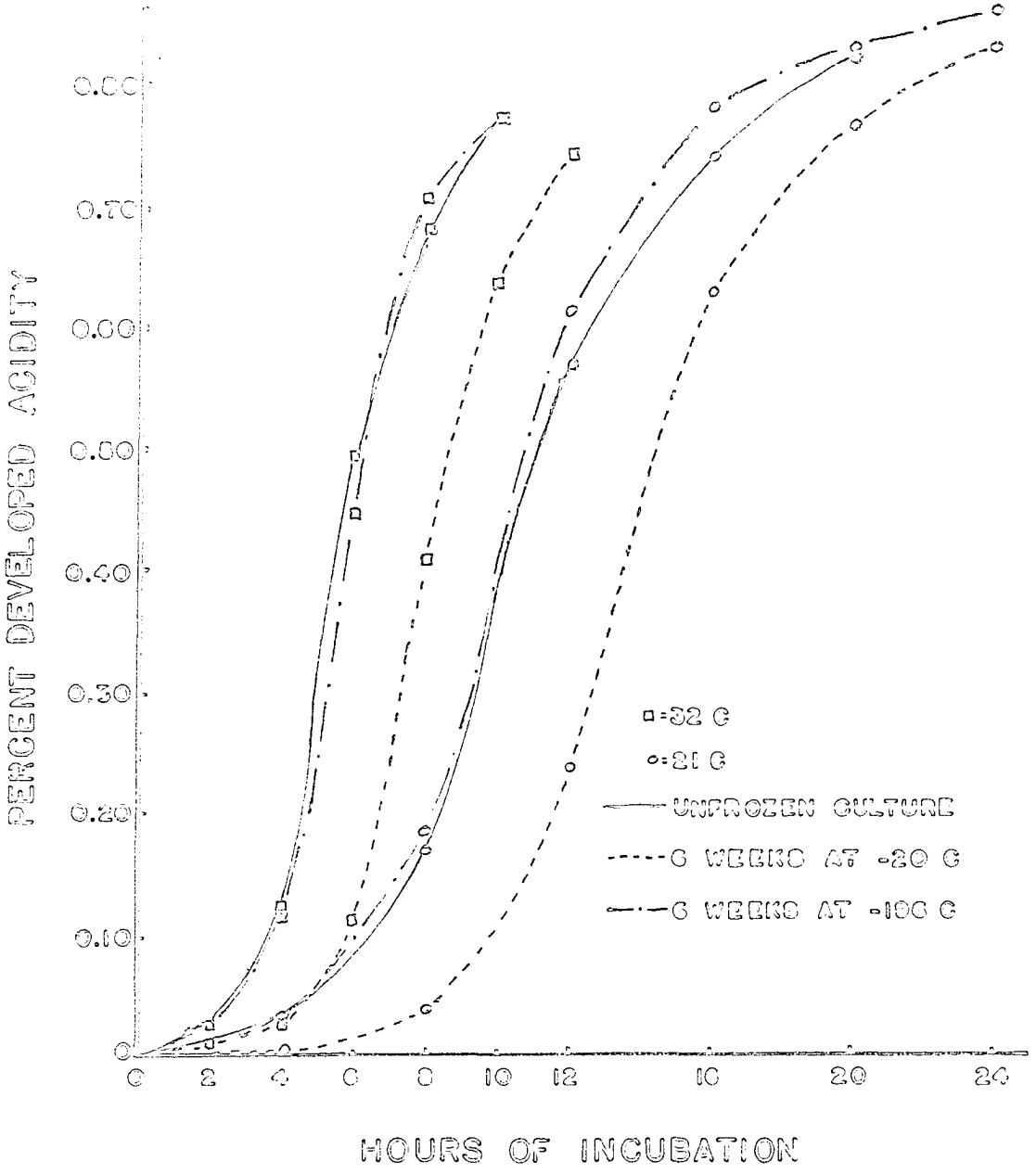

Figure 11. Effect of storage temperature upon growth of cultures at 21 and 32 C

Table 34. Effect of atmosphere and pH upon survival of lactic cultures stored for 4 weeks at -20 C[a]

Initial activity	Titratable acidity	Atmosphere	Activity upon thawing	Percent of initial activity
45.9	0.908	Air	7.9	18.8
		Vacuum	6.8	15.0
		CO_2	5.8	13.2
		N_2	8.6	22.5
		O_2	7.3	17.8
	0.16[b]	Air	18.7	42.6
		Vacuum	18.0	38.5
		CO_2	14.1	32.0
		N_2	19.6	44.1
		O_2	14.0	30.0

[a]Avg of cultures 573, FD-2, FS-103, 122, FL and Xi.

[b]Assumed value: the amount of Na_2CO_3 calculated to reduce the titratable acidity to this level was added to each culture.

in vats inoculated with culture stored at -20 C than in those inoculated with cultures held at -196 C. No activity of culture 60 was noted in vat 1B. A 2-hr ripening period was used in vat 2B instead of the 1-hr period used for all other vats. In comparisons using cultures 60 and FL, cultures stored at -196 C yielded higher milling acidities than the controls.

Table 35. Effect of frozen storage upon ability of a culture to function in Cheddar cheese manufacture

Culture	Vat no. and treatment of culture	Initial activity	Activity after 6 weeks	Titratable acidity of whey at milling
60	1A - Control; transferred 3 times weekly	54.5	56.2	0.475
	1B - Stored at -20 C	54.5	6.2	--[a]
	1C - Droplet frozen, stored at -196 C	54.5	53.5	0.665
FL	2A - Control; transferred 3 times weekly	68.0	58.0	0.450
	2B - Neutralized to 0.16% titratable acidity; 2.5% yeast extract added, stored at -20 C	68.0	15.8	0.505
	2C - Neutralized to 0.16% titratable acidity, 2.5% yeast extract added, droplet frozen and stored at -196 C	68.0	67.8	0.685
FD	3A - Control; transferred 3 times weekly	--[b]	69.0	0.490
	3B - Concentrated 35:1, stored at -20 C	56.2	36.2	0.380
	3C - Concentrated 70:1, droplet frozen and stored at -196 C	56.0	66.8	0.470

[a]No developed acidity in 3 hr; cheese was not made.

[b]Initial activity not determined.

DISCUSSION

Selection of the Activity Test

Activities of frozen cultures often are determined by inoculating milk with 1% of the frozen culture and measuring the titratable acidity after incubation at 21 C for 16 to 18 hr. From Figure 11 it can readily be seen that, for cultures stored at -20 C, this method of determining activity will result in higher apparent recoveries than incubation at 32 C for 6 hr as was done throughout this study. This accounts for the ability of frozen culture to be used as direct inocula for Cottage cheese manufacture, in which the time and temperature of ripening are similar to those in the former activity test. These same cultures could not be used as direct inocula for Cheddar cheese manufacture, where a higher temperature and shorter making time are involved. Some loss of activity of cultures through freezing damage would not be as objectionable in Cottage cheese manufacture as in Cheddar cheese manufacture since the longer incubation time in the former process would allow more multiplication of cells, resulting in greater total acid production. For this reason, 32 C for 6 hr was selected as the time and temperature of incubation for activity tests in this study. This activity test appears to be more sensitive to variations in techniques of preservation. Perhaps similar results could be obtained with incubation at 21 C for shorter periods of time, for example, 12 hr.

The shape of the curves in Figure 11 is sigmoidal, as it often occurs in graphic presentation of bioassays. Finney (18) pointed out that for such an assay to be valid, responses should be taken from the linear

part of the curve. Here, the linear range would fall in the middle, since the curves converge at both ends. Selecting incubation times and temperatures to fall in the linear range would lead to the inclusion of 32 C for 6 hr. After 16 hr at 21 C the slope of the curve is beginning to change, making this time less desirable.

An activity test was desired which would duplicate, as nearly as could be easily achieved in the laboratory, time and temperature conditions found in the manufacture of Cheddar cheese. From the data plotted in Figure 11, incubation at 21 C for 12 hr may give results similar to incubation at 32 C for 6 hr. Incubation for 12 hr was found inconvenient to work into a laboratory schedule. Since 32 C is within the range of temperatures used in Cheddar cheese manufacture, this temperature was selected, with the shorter incubation time of 6 hr easily fitting into a work schedule.

Rate of Freezing and Thawing

Cowley et al. (11) explained how freezing rates can be increased by the addition of insulating coats as shown in Figure 4. Coating materials used in this study were arbitrarily selected from the stock of similar materials readily available. Greater freezing rates have been attained by Luyet (44) using other substances. The primary interest in this investigation was to compare the effects of different freezing rates. Therefore, no attempt was made to use the coating material resulting in the fastest freezing rate attainable.

Data presented in Figure 5 and Table 2 related to cooling rates for droplet freezing are taken from theoretical data reported by Rinfret (66)

for water droplets 1 mm in diameter. Skimmilk was used as a freezing menstruum in this study and the droplets were generally larger than 1 mm in diameter. Therefore, actual freezing rates probably were slower than those reported by Rinfret.

Thawing rate curves plotted in Figure 6 are similar to those reported by Rinfret (66). The warming rate greatly decreases once the temperature reaches -20 C.

Initial studies of the effect of freezing and thawing rates upon lactic cultures were undertaken assuming no interaction between these two factors. The interaction shown in Table 6 accounts for the results shown in Tables 3, 4 and 5. This interaction could also have contributed to the conclusions drawn by Lamprech (39) and Moss and Speck (55) that freezing or thawing rates had little influence on survival. Lamprech expressed his data as percent of initial agar plate counts, while the data reported in Tables 3 to 6 are expressed as percent of initial activity. His data showed the same general trends as the data given in Table 6. Throughout the course of this study on lactic culture preservation, experimental errors were greater when agar plate counts were used than when activities were used as a basis for determining recovery. This means that a large difference in bacterial colony recovery rates would have to exist before it becomes significant in a statistical analysis. Possibly for this reason, freezing and thawing rates did not appear to affect significantly cultures of Leuconostoc sp. (Table 7).

Droplet freezing is the simplest method of achieving high freezing and thawing rates. This is especially true when large quantities of culture are frozen. Results presented in Tables 20, 32 and 35 and Figures

9 and 10 indicate that cultures survive droplet freezing as satisfactorily as any other method of freezing that was used.

If the data in Table 6 represent the true effect of freezing and thawing rates upon lactic cultures rather than from a chance in sampling, the reason for the efficiency of a fast freeze-fast thaw treatment remains to be determined. Mazur (52) felt that slow cooling allowed time for water to flow out of the cell, resulting in higher survival. Luyet (43) noted that even at high cooling rates, cells of S. lactis gave no signs of intracellular ice, indicating the ability of these cells to lose water rapidly. At higher freezing rates any ice present in cells would freeze in smaller crystals, which could result in less damage to the cells. Fast warming probably inhibits growth of existing intracellular crystals.

Atmosphere of Cultures During Growth

Growth under aeration or inclusion of additional CO_2 did not increase activity of frozen lactic cultures. Aerated cultures (Table 8) had lower activities upon thawing than did cultures grown under CO_2 or in normal atmosphere. Results shown in Table 9 suggest this may be due to the increased content of dissolved O_2 or CO_2. These two gases resulted in low survivals when used as the storage atmosphere (Table 34). The high viscosity of skimmilk cultures apparently reduced the rate of removal of dissolved gases, resulting in no significant difference between recoveries of evacuated and nonevacuated aerated cultures (Table 10).

Harrison and Cerroni (26) found that aeration improved the resistance of E. coli to freezing. They concluded that, through aeration, cells were

altered in permeability or concentration of intracellular metabolites and enzymes. Since E. coli is more aerobic than S. lactis or S. cremoris, it is not surprising to find aeration improving the resistance to freezing of E. coli. Since lactic cultures do not require large amounts of oxygen, little benefit would be expected from aeration. Lamprech (39) found high cell yields and rapid growth of lactic cultures with aeration, but attributed this to the agitation provided by aeration.

Growth Temperature

Results shown in Tables 11 to 14 indicate that highest recovery after freezing can be obtained by growing cultures at temperatures above 21 C. Cultures grown at 32 C had high percentage recoveries but because of low initial activities the advantage of growing cultures at this temperature is diminished. At higher growth temperatures, acid is produced more rapidly than at lower temperatures, causing the activity to decrease earlier. The decrease in activity is quite rapid, making it more difficult to arrest growth at the time of peak activity.

Generally, cultures of bacteria are grown at their optimum temperature for use in studies on freezing. Consequently, little work has been done on the effect of growth temperature upon resistance to freezing. Results given in Tables 11 to 14 indicate that the freezing resistance of lactic cultures increases as the optimum growth temperature is approached. This is what one would expect, recognizing the fact that growth at the optimum temperature for a given organism increases its resistance to effects of other physical factors such as high temperatures (38, 17, 88).

Commercial lactic cultures were used in this study. Since acid

production was used as an index of recovery, there was no measure of the survival of Leuconostoc sp. Altering the growth temperature to favor survival of one type of organism in a mixed-strain, multiple-type culture may reduce the resistance of another type of organism. It is quite possible that the initial cell concentration of Leuconostoc sp. would be lower when lactic cultures are grown above 21 C and that their percentage survival would be lower after freezing. If this is the case, methods employed by Lamprech (39) could be used. He grew cells of S. lactis or S. cremoris and Leuconostoc sp. separately in broth, centrifuged, resuspended in skimmilk and then froze them. The two organisms were then mixed in the desired proportions upon thawing. If flavor development in the culture is of no interest, growth of cultures at temperatures in the vicinity of 26 to 32 C should result in highest acid producing activity upon thawing.

pH of Cultures

The value of neutralizing the acidity of cultures before freezing has been mentioned often in the literature. Results of pH studies at -20 C, shown in Table 15 and Figure 7, support the findings of Lamprech (39) in that highest survival occurred at pH values of 6 and 7. The primary purpose of this investigation was to determine the effect of pH upon survival of cultures held at -196 C. Results plotted in Figure 7 show a decrease in survival as the pH is increased above 6. The curve has the same general shape as the one plotted for the -20 C data, although the differences in survival were not found to be significant. This shows that storage at -196 C decreases the effect of pH upon survival of cul-

tures. Little advantage is seen in neutralizing cultures to be stored at -196 C. This same conclusion is reached from the neutralization studies reported in Tables 17 and 31.

The work reported in Tables 16 and 17 was done to determine if the type of neutralizer had any effect on survival of frozen cultures. In the first comparison, shown in Table 16, $Ca(OH)_2$ and $CaCO_3$ appeared to be of no benefit in protecting frozen cultures. These substances are less soluble than the sodium neutralizers. Cultures may have been frozen before complete solution and neutralization occurred, as indicated by the pH values at freezing. Therefore, in the second comparison, Table 17, cultures were held 1 hr at 0 C before freezing. This holding temperature was selected to prevent further growth of the cultures. Apparently $CaCO_3$ dissolved to some degree, judging by the pH of cultures before freezing. Recoveries of cultures containing $Ca(OH)_2$ and $CaCO_3$ were higher than those shown in Table 16 and were higher than those of unneutralized cultures. However, in both comparisons, these neutralizers resulted in lower activities after frozen storage than did NaOH and Na_2CO_3. This may be due largely to the relative insolubility of the calcium compounds, resulting in incomplete neutralization. If sufficient time had been allowed for the calcium neutralizers to raise the pH to the same level as attained with the sodium neutralizers, probably no significant differences in recoveries would have occurred. No significant differences were noted in activities of cultures containing Na_2CO_3 or NaOH. Therefore, for rapid neutralization, either one of these should be used in preference to the calcium compounds.

Other studies in which cultures were neutralized (Tables 29, 31 and

34) were undertaken to see if neutralization would exert a synergistic action with other additives in protecting frozen cultures. The only significant interaction found was between the effects of neutralization and the additives listed in Table 31. Neutralization was more beneficial to cultures frozen with no additive or with egg white or egg albumin than to cultures frozen with yeast extract or 15 or 20% glycerol. The latter additives were more effective in protecting cultures during freezing than egg white or egg albumin. Assuming a maximum of 100% survival possible after frozen storage, a more effective additive would result in less additional protection from neutralization. This same reasoning would apply to the observation made previously concerning the lack of benefit of neutralizing cultures stored at -196 C.

Precooling Before Freezing

Hegarty and Weeks (28) observed death of E. coli cells upon cooling cultures from 37 C to 0 C. The work reported in Table 18 was done to determine if a similar cold-shock existed with lactic cultures and if the shock of cooling to freezing temperatures could be diminished by allowing a time of adjustment to temperatures barely above freezing. From the results given in Table 18, it appears there is no advantage in precooling lactic cultures before freezing. Prolonged holding at either 0 or 5 C resulted in decreased activity, presumably because of effects of pH and maturing of the cells. There apparently is no detectable "shock" in cooling cells rapidly from the incubation temperature to subzero temperatures.

Cell Concentration

It was found necessary to neutralize the developed acidity in broth cultures to be concentrated to attain activities comparable to those in milk cultures. This is probably due to the lack of buffer capacity in broth, allowing a more rapid drop in pH than would occur in milk. Data in Table 19 were obtained to determine the optimum amount of neutralizer and time of addition to cultures. The largest amount of Na_2CO_3 used, 0.55 g/100 ml, resulted in the highest activity and the highest recovery after freezing. There also appeared to be some benefit from several additions of Na_2CO_3. Continuous neutralization to provide a nearly constant pH would be ideal. The pH would be held at a desirable level for cell growth, allowing a larger population, since inhibition by end-products would be diminished or delayed. Since this was impractical during the investigations of freezing concentrated cultures, a total of 0.50 g Na_2CO_3/100 ml culture was added in two equal portions after 11 and 15 hr incubation. This procedure resulted in acceptable culture activities.

Considerable variation in recoveries of droplet-frozen concentrated cultures is evident in Table 20. Difficulty was encountered from time to time in obtaining uniform resuspension of cell paste after centrifuging. This would not be as great a problem when working with larger quantities of culture, for a Waring blendor could be used to resuspend the cells (39). The data in Tables 19 to 22 also are more variable because of the small number of replications used.

The variation in activities must be kept in mind in interpreting the

data in Tables 21 and 22. There is an apparent decrease in recovery with increasing cell concentration in cultures stored at -20 C. To a lesser degree, the reverse is apparent in Table 21, with cultures held at -196 C. The data in Table 22 for cultures held at -196 C indicate that this apparent trend may only be due to sampling variation. This also may be true for the data from cultures held at -20 C, since the 25:1 concentration without additive in Table 22 had a slightly higher activity after 4 weeks than the corresponding 15:1 concentration. The results in Table 22 may come largely from the effects of the additive. (See discussion on additives.)

These data and those in Table 32, when compared with data in other tables obtained in separate experiments, indicate that concentrated cultures can successfully be frozen. The degree to which a culture is concentrated before freezing probably has little effect upon the ability of the culture to withstand freezing.

Age of Cells

Studies of the effect of cell age upon ability to withstand freezing were made using centrifuged resuspended broth cultures to eliminate the effect of acidity upon freezing damage to cells. To avoid the possibility of cell concentration influencing results, centrifuged cells were resuspended as nearly as possible to concentrations normally found in 16-hr cultures. Since cells were resuspended in skimmilk, optical density could not be used as a means of verifying that the desired concentration had been attained. In earlier trials, as shown in Tables 23 and 24, difficulty was experienced in resuspending cells of different ages to

the same concentration. Consequently, activities of the cultures before freezing varied considerably. The cell concentration may have influenced the recoveries of frozen cultures. Inspection of the data in Tables 23 to 26 reveals that, although cultures with a higher cell concentration had higher initial activities, rates of acid production per cell were greater in cultures with lower cell concentrations. This may have influenced activities of cultures upon thawing. The highest concentration of cells in the study reported in Table 24 occurred in 8-hr cultures. Highest percentages of initial activity were found in 8-hr cultures after 4 weeks at -20 C. Recoveries reported in Table 24 for 8-hr cultures appeared abnormally high and could have resulted partially from high cell concentrations.

Since most fluctuation in recovery rates occurred in younger cells, the experiment was repeated, with the results shown in Table 25. In this second trial, initial cell concentrations for each age group were more nearly equal than before. Recoveries were calculated on the basis of plate count, activity and activity per 10^8 cells to determine if the initial cell concentration influenced the activity of cultures after frozen storage. With both counts and activities as criteria for recovery, 8-hr cultures had the lowest recoveries of the age groups studied. However, 8-hr cultures showed greatest recoveries when activity per 10^8 cells was used as a basis for determining recovery. With higher initial cell concentrations, the high activity per cell in frozen 8-hr cultures could cause the results shown in Table 24.

Data in Tables 24 and 25 indicate that, of the ages of cells studied, cells older than 8 hr survived freezing better than 8-hr cells. Under

incubation at 21 C, cultures may be in their early logarithmic growth phase and would be more susceptible to effects of physical factors than cultures later in the growth phase. Higher recoveries of cultures younger than 8 hr is probably partially due to the older cells introduced in the original inoculum.

Additives

Sucrose seemed to be less effective than either glycerol or dimethyl sulfoxide in protecting lactic cultures against freezing. In one comparison (Table 10), cultures containing sucrose had lower activities after freezing than did cultures with no additive. Heller (30) found sucrose to be effective in protecting cultures of S. pyogenes and E. coli. This was attributed to the ability of the sucrose to penetrate the cells, since carbohydrates which were not utilized by the organisms were less effective in protecting the cells. This factor may have contributed to the low efficiency of sucrose in this investigation. According to Bergey's Manual of Determinative Bacteriology (7), S. pyogenes utilizes sucrose but S. lactis and S. cremoris rarely ferment sucrose. Possibly sucrose doesn't penetrate cells of lactic streptococci as well as other species of organisms, thereby reducing its effectiveness as a protective agent.

Dimethyl sulfoxide has shown promising results in studies with frozen animal tissue. In these studies, it was equivalent to or slightly less effective than glycerol in protecting frozen lactic cultures. However, its usefulness as an additive for lactic cultures is doubtful. The toxicity of dimethyl sulfoxide for humans has not been determined, and it is quite certain some would remain in the cheese or other products

manufactured using lactic cultures. This additive has a definite unpleasant taste and odor which carry through one or two transfers of the culture to which it has been added. Since this factor made the use of dimethyl sulfoxide impractical, no further studies of this additive were made.

Glycerol resulted in higher recoveries than sucrose or dimethyl sulfoxide. In these comparisons 10% additive was used. Heinemann (29) found higher recoveries using 20% glycerol. This prompted the use of 15 and 20% glycerol in the comparisons shown in Table 31. When these data are compared with the data in Tables 8, 9, 10, 27 and 28, it appears that 10% glycerol is too low a level for maximum protection. Little difference was noted in activities upon thawing of cultures containing 15 or 20% glycerol. The reason for this diminishing efficiency is probably connected with the protective mechanism of glycerol. This is still not known and is probably a combination of several factors. Glycerol is apparently able to bind water and prevent some of it from freezing, although it does not entirely prevent the formation of ice crystals, according to Smith et al. (76). It easily penetrates cells, thereby binding some intracellular water and inhibiting crystal formation and growth within the cells. The necessity for glycerol to penetrate the cell is questioned by several authors. It is doubtful if this is an important factor in the preservation of lactic cultures, for in these studies cultures were frozen within 5 min of the time of adding glycerol. This should not allow complete penetration before freezing. One theory which may have merit is that glycerol prevents intracellular freezing by preventing seeding by extracellular ice crystals (52). However, the results obtained

by Luyet (43), tend to contradict this theory, since he found no evidence of ice crystals within cells of S. lactis frozen without additive. It appears that no definite conclusion can be drawn. Additional investigation is needed before the action of glycerol in protecting cells against freezing is known.

A definite decrease in the activity of cultures was noted upon the addition of 15 or 20% glycerol (Table 31). Slight decreases in activity were observed with the addition of 10% glycerol, sucrose or dimethyl sulfoxide. This same reaction was observed by Richardson (64), who attributed it to an osmotic pressure difference created by reduction in glycerol content of a sample of culture when it was transferred into milk for an activity test. He reported that this osmotic shock was not evident after frozen storage. Percentage recoveries reported are based upon the activities of cultures immediately after the addition of protective agents. If the initial activities of cultures with no additive are taken as a basis for computing percentage recoveries, the survival rates listed in Tables 8, 9, 10 and 27 for sucrose, dimethyl sulfoxide and glycerol and in Tables 28 and 31 and Figures 8 and 9 for glycerol would be somewhat lower. Even with this decrease glycerol appears superior to the other additives, especially at the higher levels.

In a comparison of colloidal substances, Heller (30) found the use of peptone to result in highest recovery of dried cultures of S. pyogenes. The three colloidal substances used in this study were selected on the basis of ability to stimulate acid production. This was done primarily to reduce the number of materials examined; less stimulatory substances may well have protective effects equivalent to those studied. A secondary

reason for selecting these compounds was to determine if injured cells could be stimulated into acid production upon thawing.

All three substances proved superior to glycerol in protective effect as illustrated in Table 28 and Figure 8. The action of these substances probably is due to their hydrophilic property; water is bound, leaving less for crystallization. Heller (30) also suggested that this type of material may exert a protective colloid effect, shielding the cells from adverse environmental conditions. The reason for the lack of increased protective effect above a level of 5% additive may be the same as for the decreased effectiveness of glycerol at levels greater than 15%. The decreased protection of 10% yeast extract may also be due to toxicity at high levels and to experimental variation.

The stimulation of unfrozen cultures followed the same pattern as did the protective effects of these additives, as shown in Figure 8. Again, 10% yeast extract resulted in a decreased stimulation, either because of toxicity at high levels or the osmotic shock discussed in relation to glycerol.

Little advantage was found in adding yeast extract, N-Z Amine A or Edamin S to the skimmilk used for activity tests, except for cultures frozen without additives. There was enough additive in the inoculum for the activity tests of cultures containing these compounds to reduce the effectiveness of additional stimulant. This is only reasonable since bacterial cells have a definite limit of activity.

Serum or serum albumin have been utilized as protective additives for frozen cells (86, 71), but have never been studied to determine their efficiency in protecting lactic cultures. Egg white and egg

albumin were selected primarily because of their availability in relatively large quantities. Egg white and 1% egg albumin appeared to protect cells during frozen storage, according to the data in Table 30. However, no benefit was found from addition of these substances in a second comparison (Table 31). Some toxicity of the egg white was noted from the reduced initial activities in Tables 22, 30 and 31. This counteracted any protective effect so that activities after thawing were little different than activities of cultures stored with no additive.

The viscosity of the egg white and the egg albumin suspension made mixing with cultures difficult. Any uneven distribution of additive would contribute to variability of results. Lack of efficiency of these additives probably is partially due to their limited water-binding ability. The water included in the egg white would increase the total volume of potential ice. Hydrophilic properties of the egg albumin were probably decreased through some degree of denaturation during drying.

Casein was selected for the purpose of investigating a different type of protein than had previously been investigated. Casein is the major protein in milk and may contribute to the protective action of milk. The value of milk as a freezing menstruum is recognized and is illustrated by comparing the data in Tables 8 and 9 for broth cultures with the data in Table 10 for skimmilk cultures. The serum proteins, salts and lactose of milk also may offer protection to frozen cells. More pronounced effects of egg white, egg albumin and casein may have been noted if cultures had been frozen in broth. These additives apparently added little to the value of similar materials already present in milk.

Storage Temperature

Results of temperature comparisons leave little doubt as to the advantage of storing cultures at -196 C rather than at -20 C. At -196 C there is practically no opportunity for ice crystal growth. At the temperature of liquid N_2 biochemical activity within the cell is at a virtual standstill. Gas diffusion is practically eliminated since O_2 and CO_2 are no longer gases at -196 C.

Problems would be encountered in adapting these techniques to freezing large quantities of lactic starters, such as would be required in a cheese plant. Although this was not a matter of immediate concern during this study, such problems will have to be solved if these procedures are to have a practical application. A definite advantage of droplet freezing can be seen here, since this method of freezing could readily be adapted to a large-scale continuous process. Thawing and inoculation are accomplished simultaneously, simply by pouring the frozen droplets directly into the milk used for starter or cheese manufacture. Thawing is completed in a few seconds.

The data plotted in Figure 9 illustrate that lactic cultures can be stored at -196 C for extended periods of time with no loss in activity. One could anticipate high survival indefinitely, since no evidence of decreased activity was apparent after 28 weeks.

From Figure 9 and the tables in which results of storage studies at -196 C are presented, it is evident that cultures often were more active upon thawing than before freezing. This suggests a possible stimulatory effect of some phase of the ultra-low temperature freezing treatment.

More research is needed to explain this phenomenon. One observation that might be helpful in approaching the problem is that increases in activities were more pronounced in single-strain cultures and in less active multiple-type cultures. Single-strain cultures used in this study were less active than the mixed-strain, multiple-type cultures. This could have been simply due to strain differences or could have been partially due to the lack of symbiosis resulting from the absence of Leuconostoc sp.

Droplet-frozen cultures (Figure 9) were frozen in 0.2 ml quantities dispensed from a syringe calibrated in 0.2 ml quantities. Measurement errors could possibly account for some of the increase in activity shown in Figure 9. The position of the line representing droplet-frozen cultures containing 10% glycerol was influenced largely by survival rates of one culture (W2), which were abnormally high. Activities of droplet-frozen samples of culture W2 varied from 112 to 224% of the activity of the culture before freezing.

The high survival rates immediately after freezing, shown in Figure 10, were typical of results obtained using the plate count method of determining recovery. This apparently was due to breaking of chains and clumps upon freezing (30, 51). Microscopic examination of frozen cultures showed considerable shortening of chain length in cultures held at -20 C, but little change in appearance of cultures held at -196 C. This is probably the reason for the greater increases shown in Figure 10 for cultures held at -20 C. Cultures without added glycerol increased in count more than cultures with 10% added glycerol. These two observations suggest that part of the function of glycerol and the lower temperature in protecting cells during freezing is by limiting ice crystal formation.

Chain breakage is probably a result of extracellular ice crystals, but glycerol and temperature would also influence the formation of intracellular ice crystals.

Percentage recoveries based on plate count were more variable than percentage recoveries based on acid-producing activity. Lamprech (39) reported considerable variation in plate counts of cultures after frozen storage. This is one reason most recovery rates in this work were based on culture activity. The primary reason for using acid-producing ability as an index of survival is that the manufacturing processes as well as the quality of cultured dairy products are dependent upon the ability of a culture to produce acid at a desired and essentially predetermined rate.

The data in Table 33 were obtained to determine if recovery rates based on activity were the same as recovery rates based on plate count. Also, it was of interest to determine if cell injury occurred at -196 C as Moss and Speck (55) and Straka and Stokes (81) had reported for cultures stored at higher temperatures. Standard methods agar would correspond to their minimal medium, i.e. one on which unharmed cells will grow but injured cells will not. Eugon agar with added V-8 Cocktail Vegetable Juice would theoretically sustain growth of both injured and uninjured cells. Although not significant, the differences between counts obtained on Standard methods agar and Eugon agar corresponded favorably with the percentage of injured cells in cultures stored at -20 C, reported by Moss and Speck. The percentage of cells injured at -20 C, as determined by the difference in activity and plate count on Eugon agar also was comparable to the percentage reported by Moss and Speck. Because of the variability of the counts obtained, differences were not

significant. The data still may be interpreted to show some degree of cell injury, in the light of the results of Moss and Speck and Straka and Stokes. No injury was apparent in cultures stored at -196 C. Percentage recoveries based on plate counts were higher than those based on activity, partially because of experimental variation and partially because of the chain breakage mentioned previously. Any chain breakage would influence counts, even after extended storage at -196 C, because of the extremely low death rate.

Atmosphere of Storage

The higher recoveries of cultures stored under N_2 can be attributed to the relative inertness of this gas. Since CO_2 and O_2 are involved in biochemical processes in the cell, their presence would allow respiration and cell activity to continue, resulting in aging and death of the cells. Since air is predominantly N_2 with lesser amounts of O_2 and CO_2, recoveries of cultures stored under air might be expected to be intermediate between survivals of cultures stored under the pure gases. This is what was found, with recoveries slightly less than those obtained after storage under N_2.

The advantages of both low temperature and storage under N_2 could be realized by storage in liquid N_2 or even in the vapor phase above liquid N_2.

Manufacture of Cheddar Cheese Using Frozen Culture

The most practical activity test for a culture is the manufacture of cheese or some other cultured product. More vats of cheese would have to

be made than were used in this study before a preservative technique could be considered as "best" or even satisfactory. The objective here was not to determine the best treatment, but to see if Cheddar cheese could be manufactured with culture frozen under some of the conditions found favorable for survival in the laboratory.

The data in Table 35 indicate that neutralization or the use of an additive are essential if Cheddar cheese is to be made using culture stored at -20 C. The concentrated culture stored at -20 C yielded a milling acidity slightly lower than desirable. Probably if this had not been a neutralized culture, it would not have been sufficiently active to use for cheese manufacture.

The milling acidity of cheese made using culture FL stored at -20 C was higher than cheese made with the same culture transferred 3 times weekly for two reasons. The first is that 2 hr elapsed between the addition of starter and the addition of rennet to the milk, instead of the usual 1-hr ripening period. This was done to verify that the culture was sufficiently active to be used for cheese manufacture. The second is the decreased activity of the control. The activity shown in Table 35 was slightly lower than the activity at the beginning of the 6-week period. However, this activity test may be misleading for there was evidence of decreased culture activity during the cheese-making process. On the same day, this culture after being carried separately in two laboratories showed marked decrease in activity by requiring 24 hr to coagulate milk held at 21 C. Whether this was due to bacteriophage or to chance, it emphasizes one advantage of culture preservation. Cultures of known high activity in storage are a form of insurance against culture failure.

In two of the cheese-making comparisons, milling acidities were higher with cheese made from culture stored at -196 C than with cheese made from unfrozen cultures. In the third instance, the milling acidities were virtually equal. These higher milling acidities correspond to the higher activities discussed earlier in connection with the effect of storage temperature.

All cheeses were judged for body and flavor defects. The cheese made in the first trial was judged after holding for 7 months. Cheeses from the second and third trials were judged at 4 and 2 months, respectively. Scores of cheese made from cultures held at -20 and -196 C were equivalent to scores of cheese made from fresh unfrozen culture. This indicates that none of the treatments applied to the cultures was detrimental to the quality of the cheese.

SUMMARY

Conditions influencing survival of frozen single-strain and mixed-strain, multiple-type lactic cultures were studied. Culture activity before freezing and after thawing was determined by inoculating 20-ml quantities of 11% solids reconstituted skimmilk with 0.2 ml culture and determining the titratable acidity after incubation for 6 hr at 32 C.

Studies of rates of freezing and thawing of lactic cultures indicated fast freezing followed by fast thawing resulted in highest survival. Therefore, in subsequent studies cultures were frozen by immersing in liquid N_2, 16 x 125 mm screw-capped test tubes containing 3 ml culture. Tubes were placed in a 40 C water bath for thawing. Freezing of cultures in droplet form, by directing a stream of culture onto the surface of liquid N_2 with a syringe, was found advantageous because of the high freezing and thawing rates attainable and the relative ease of freezing and thawing large quantities of culture.

No significant difference was noted in survival rates of cultures frozen after having been grown under normal atmosphere, aeration or 10% CO_2.

Cultures grown at 26 to 32 C were found to have significantly higher activities after 4 weeks at -20 C than cultures grown at 15 or 21 C.

Cells younger than 8 hr appeared more susceptible to freezing damage than older cells. Resistance to freezing increased with increasing age of cultures up to 16 hr, which was near the maximum age studied.

No advantage was found in precooling cultures before freezing.

Neutralization of cultures to pH 6 or 7 was found to result in high

survival of cultures held at -20 C. Neutralization had no significant effect on survival of cultures stored at -196 C.

Yeast extract or glycerol appeared superior to sucrose, dimethyl sulfoxide, casein, egg white and egg albumin in protecting cells against freezing damage at -20 C. Edamin S was slightly less effective than yeast extract in protective action; N-Z Amine A was less effective than yeast extract at low levels. Additives had little effect on survival of cultures stored at -196 C.

Storage at -20 C for 4 weeks under an atmosphere of N_2 or air resulted in higher activities than storage under O_2, CO_2 or vacuum.

The most significant factor in this study found to influence the survival of lactic cultures was the temperature of storage. The use of additives, pH of cultures, growth temperature and age of culture were found to have little effect upon survival of cultures held at -196 C. Cultures showed no loss of activity after periods of storage up to 28 weeks at -196 C. In several instances there was a definite, but unexplained, increase in culture activity after storage at -196 C.

Results of experiments in which Cheddar cheese was made using droplet-frozen culture stored in liquid N_2 indicate the potential usefulness of this method. Droplet freezing conceivably could be adapted to a large-scale, continuous operation. Rapid freezing and thawing rates attained and the low storage temperature are virtual guarantees of high culture activity after storage. Milk for bulk starter or cheese manufacture can be inoculated directly with the frozen culture, with no loss of time due to the thawing operation. This method would also be advantageous from the standpoint of time and labor saved in transfer of cultures.

Results obtained in this study indicate cultures can be held at -196 C with no other preservative treatment. For cultures to be stored in the vicinity of -20 C, neutralization and the addition of 15% glycerol or 1 to 5% of colloidal substance such as yeast extract will be beneficial. There still remain, however, several unsolved problems connected with the preservation of lactic cultures. Primary among these is a problem common to preservation of all microbiological materials: the determination of the cause of death at sub-zero temperatures. This must be known to determine the best possible method of preserving lactic cultures. A second question, related to the first, is the mechanism of action of additives. A third factor which should be investigated is the apparent stimulation of lactic cultures at -196 C. To answer this question would be a major step toward discovering the exact mechanism of death and injury of bacterial cells during frozen storage.

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APPENDIX

Table 36. Error mean squares for analyses of data obtained in this study

Table in which results are reported	Error degrees of freedom	Error mean square
3	83	36.78
4	47[a] (94)	43.45 (33.71)
5	35	71.78
6	49	40.79
7	58	357.89
8	121	149.76[b] (684.77)
9	47	96.31 (478.74)
10	165	189.18 (295.14)
12	24	55.22[c] (17.14)
13	75	153.72 (47.04)
14	9	46.38 (14.71)
15	14	40.76 (7.34)

[a]Upper no. of degrees of freedom and upper mean square are for cultures frozen to -20 C; those in parentheses are for cultures frozen to -196 C.

[b]Upper mean square is for percent of initial activity; mean square in parentheses is for percent of initial plate count.

[c]Upper mean square is for percent of initial activity; mean square in parentheses is for final activity.

Table 36. (Continued)

Table in which results are reported	Error degrees of freedom	Error mean square
16	11	13.36 (5.58)
17	16	102.50 (37.13)
18	192	212.61
23	49	233.55
24	28	50.26 (15.20)
25	22	193.47 (89.66)
26	30	89.93 (14.28)
27	33	164.14
28	42	19.16
29	15	126.89
30	24	122.10
31	46	99.81 (11.47)
33	68	331.56
34	45	77.43
Figure 9	258	323.74