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Preservation of starter cultures in liquid nitrogen

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PRESERVATION OF STARTER CULTURES IN LIQUID NITROGEN

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INTRODUCTION

The manufacture of cheese and fermented dairy products requires large quantities of high quality starter culture. As a source of starter culture, dairy plants commonly receive lyophilized culture from one of several commercial suppliers. Lyophilized cultures need to be transferred prior to use by the dairy plant to reactivate the microorganisms. These cultures are then maintained by frequent transfer with periods of refrigeration between transfer. A culture program such as this requires considerable time, expense, and experienced personnel. Also, difficulties may frequently occur, such as loss or non-uniformity of acid-producing ability, commonly referred to as "activity"; loss of flavor production; contamination by foreign microorganisms or bacteriophage; the use of mother-culture milk containing growth inhibitors; strain dominance; or other less serious defects.

Freezing has been studied as an alternative to frequent culture transfer. Considerable work has been done concerning the freezing of cultures at various deep-freeze temperatures. However, these frozen cultures still must be transferred in the dairy plant before use, and cultures of this type have limited storage life.

A recent development in the preservation of microorganisms

has involved the use of liquid nitrogen (-196 C) as the coolant. Recent investigations have indicated the applicability of liquid-nitrogen freezing for cheese and other fermented-milk cultures.

The ideal method of culture preservation would involve maximum retention of culture activity and viability, and would require minimum or no handling in the dairy plant. This investigation was planned with these objectives in mind. Growth conditions before freezing were studied to insure retention of maximum activity and viability after frozen storage. Culture characteristics before and after frozen storage were studied to determine effects of freezing and frozen storage upon the usefulness of cultures in making fermented-milk products. Concentration of culture, together with liquid-nitrogen freezing, was investigated as a means of reducing the volume of culture required to make cheese. It was hoped that this study would help to solve some of the problems common to handling cultures and increase the knowledge of low-temperature preservation of starter microorganisms.

REVIEW OF LITERATURE

A great amount of work appears in the literature concerning preservation of microorganisms by drying and freeze-drying. Excellent reviews on these methods of preservation are found in the literature (28, 40, 91).

As this investigation involves only preservation by freezing, other methods of preservation will not be reviewed.

Many factors influence the recovery of microorganisms after frozen storage. Especially concerned are the following factors: (i) preparation of cells before freezing, (ii) nature of the freezing menstruum, (iii) conditions of freezing and thawing, and (iv) conditions of frozen storage.

Preparation of the Cells before Freezing

Culture incubation conditions

Foster (27) considered that physiological age of lactic cultures before freezing was an important factor influencing survival, and recommended that cultures be frozen when in the late log or early stationary phase of growth. In their investigations, Cowman and Speck (19) grew lactic cultures at 22 C until the late log growth phase was reached before freezing at -196 C.

Hegarty and Weeks (41) studied the sensitivity of Escherichia coli to cold shock and reported that cells from

the lag or stationary growth phase were quite resistant to rapid temperature drops, as only 8% of the mature cells displayed sensitivity to cold. Immediately after the first multiplication, the number of sensitive cells increased rapidly throughout the log phase. Woodburn and Strong (123) also reported that bacterial cells tended to be especially sensitive to freezing during the log phase of growth.

Swartling and Lindgren (112) found that the most active lactic cultures were those frozen immediately after inoculation, and cultures allowed to ripen before freezing were the least satisfactory. However, Nilsson and Wass (82) froze cells of Lactobacillus bulgaricus at -18 to -22 C immediately after inoculation into milk, and found that after 6 weeks of frozen storage, activity was slightly weakened.

Simmons and Graham (99, 100) froze samples of culture at various times during incubation at 21.1 C; after thawing, incubation was resumed for the interval necessary to give a total incubation time of 16 hr. They found that incubation beyond 8 hr before freezing resulted in a less active culture after frozen storage. Varying the initial inoculum from 0.5 to 10% did not influence final activity after storage for 2 weeks at -28.9 C. When cultures were frozen after a 1% inoculation into sterile skimmilk and stored up to 6 months at 28.9 C and thawed, activity and flavor characteristics were comparable to fresh cultures. In contrast, Baumann (6)

found that minimum viability and activity occurred after frozen storage at -20 C with cultures grown at 21 C for 8 hr before freezing. Increasing recoveries occurred with advancing culture age from 10 to 16 hr. In another experiment, Baumann (6) froze cultures at -20 C and -196 C after 6 and 16 hr of incubation at 21 C. At both freezing temperatures, older cells had higher survival rates.

Cardwell and Martin (11) grew cultures for 12, 16, and 20 hr at 21.1 C before freezing, and found that after 30 days' storage at -17.8 C, cultures incubated 12 hr before freezing produced slightly less acid than when incubated 16 or 20 hr before freezing. Similarly, Lamprech and Foster (58) froze suspensions of Streptococcus lactis at -20 C after incubation for 10, 12, 15, 18, 21, and 24 hr at 25 C. Cells frozen at various times up to 18 hr were more active than those frozen at 21 or 24 hr, and maintained higher activity during storage at -20 C. Richardson and Calbert (92) froze cultures of S. lactis after growth for 12 hr at 32 C and found no loss of activity after storage at -28 C for 3 and 6 months.

Thus, contradictory relationships between culture age and the optimum time for freezing occur frequently in the literature. Precise and detailed information on the relationship between the physiological age of cultures before freezing and the viability and activity of cultures after

storage in liquid nitrogen is especially lacking.

In a study comparing 15 and 21 C incubation for obtaining maximum culture activity, Sellars, Eakle, and Sanders (97) found that 15 C was preferable to 21 C. Baumann and Reinbold (7), however, found no significant difference in recovery of activity with cultures grown at 15 or 21 C after frozen storage at -196 C for 4 weeks. The maximum culture activity after frozen storage was obtained with cultures grown at 26 C for 10 hr.

Neutralization before freezing

The pH of the menstruum in which cells are frozen is often believed to play a significant role in the survival of the organisms. Straka and Stokes (111) froze cultures of E. coli and Pseudomonas fluorescens suspended in 0.5% beef extract at pH values from 5 to 8 and stored them for 5 days at -18 C. Death was greatest at pH 5 and 8 and death was least at pH 6 and 7. Similar results have been reported for the same organisms at the same pH values at various freezing temperatures with storage up to 15 days (4).

Lamprech (57) froze suspensions of S. lactis and a Leuconostoc culture in skimmilk, with and without added glycerol, at pH 5, 6, and 7 at -20 C. Activity of cultures frozen with glycerol and stored up to 1 year decreased most rapidly at pH 5; pH 7 was the most favorable level for

freezing. With 20% added glycerol, culture activity after frozen storage was not influenced by pH. Baumann (6) found that neutralization of cultures to pH 6 or 7 resulted in greater survival of cultures stored at -20 C. Neutralization was more beneficial to cultures frozen with no additives than to cultures frozen with yeast extract or 15 or 20% glycerol. However, neutralization showed no significant effect upon survival of cultures stored at -196 C. Duggan, Anderson, and Elliker (26) neutralized cultures of Lactobacillus acidophilus to pH 7 before freezing.

Anderson (2) ripened lactic cultures to 0.22, 0.25, 0.65, 0.85, and 0.98% acidity before freezing at -13.3 C for 2, 6, and 12 months. After frozen storage, the cultures were used for making Cottage cheese. It was found that the cultures produced satisfactory cheese whether frozen shortly after incubation or highly ripened. The author recommended that cultures be frozen at their maximum activity and used within 12 months. Contrary to these findings, Johns (48) reported that lactic cultures neutralized to 0.16% acidity before freezing were more active than unneutralized cultures after frozen storage up to 14 weeks. Although there was a gradual decrease in bacterial numbers and activity during storage, it was found that satisfactory bulk starters could be prepared if the count did not drop below 500,000/ml.

Initial cell concentration

The question of the effect of initial cell concentration upon freezing survival is far from settled (9). Major, McDougal, and Harrison (67) studied the effect of initial cell concentration upon survival of different species of bacteria after freezing in broth at -22 C. They found that species of Salmonella, Pseudomonas, and Escherichia manifested a percentage survival which varied in proportion to the initial bacterial numbers. On the other hand, species of Lactobacillus, Microbacterium, Chromobacterium, and Bacillus showed survival which was constant and independent of initial numbers. However, Postgate and Hunter (85), using Aerobacter aerogenes as the test organism, found no population effect with freezing. Cells at a concentration of 10 mg of dry cells/ml survived -20 C storage equally well as concentrations of 1 mg/ml.

After storage of Ps. fluorescens in beef broth for 5 days at -18 C, the extent of death was independent of cell concentration within the range of 1×10^5 to 8×10^6 cells/ml (111). Record and Taylor (89) froze E. coli in phosphate buffer at -78 C and reported a 40% kill after thawing regardless of the original concentration of cells from 10^4 to 10^{10} mg/ml.

Harrison (36) studied the survival of bacteria after repeated freezing and thawing and found that after one

freezing of L. fermenti at -22 C, the survival was constant and independent of initial cell concentration. However, the survival which resulted after a series of repeated freezings and thawings varied in proportion to the initial cell concentration. Duggan et al. (26) froze concentrates of L. acidophilus and found no detectable storage death, whereas dilution of the concentrate by 10- or 100-fold resulted in increased mortality on freezing and storage.

Baumann (6) concentrated lactic cultures by centrifugation from broth, resuspended the cells to various concentrations in skimmilk, and froze the suspensions at -20 and -196 C. The lower concentrations of cells survived storage at -20 C better than did higher concentrations. The reverse was true with cells stored at -196 C. However, additional experiments with storage at -196 C indicated that the results may have been due to sampling variation and it was concluded that the degree of cell concentration had little effect upon the ability of cultures to withstand freezing.

Lamprech (57) prepared concentrates of lactic cultures containing over 50 x 10^9 cells/ml. Concentrates were frozen and stored at least 5 months at -20 C without excessive loss of viability or activity.

Nature of the Freezing Menstruum

The nature of the suspending medium has a profound effect upon the lethality of microorganisms during freezing and thawing and during frozen storage. Borgstrom (9) explained that the nature of the environment was more important in freezing than is generally realized.

As long ago as 1913, Keith (51) demonstrated the importance of changing environmental conditions to increase resistance of bacteria against freezing. The addition of milk or glycerol to the freezing medium was superior to water for the recovery of E. coli after frozen storage at -20 C. When E. coli was frozen in pure and diluted milk, the death rate after freezing increased with dilution of the milk; the highest numbers surviving in the undiluted milk. Further experiments by Hilliard, Torossian, and Stone (44) in 1915, and Hilliard and Davis (43), in 1918, showed similar results with E. coli and Bacillus subtilis in cream and milk media. The improved preservation was attributed to the physical protection offered by the colloidal and solid matter in suspension.

The most widely used cryoprotective agents for biological materials are glycerol, followed by dimethyl sulfoxide (10). Excellent reviews are available concerning the use of numerous cryoprotective compounds for microorganisms and other living cells (23, 103); spermatozoa (84, 103); living cells and

tissues (80, 102), mammalian organs (103), blood cells (25, 103) and enzymes (12, 115).

Numerous investigations also have been conducted concerning the adequacy of various compounds for the protection of lactic cultures against freezing effects. Milk is the most widely used substrate for propagation of lactic cultures, and cultures are frequently suspended in milk when frozen. This is quite fortunate since milk is a commonly used protective agent for bacteria against freezing damage.

Lamprech (57) froze cultures in skimmilk containing 11% solids, distilled water, and 1% peptone water. Twenty percent glycerol additions also were used for comparison. Of the media tested, skimmilk allowed greatest survival. Anderson (2) froze active cheese cultures in skimmilk with 9% solids. Cowman and Speck (17, 19) and Moss and Speck (77) froze lactic streptococci in skimmilk containing 10% solids. Cardwell and Martin (11) froze buttermilk cultures in skimmilk containing 9, 14, and 18% solids. The lower percentage of solids offered slightly less freezing protection than the higher percentages. Simmons and Graham (99) recommended that cultures be frozen in skimmilk with 10% solids.

Rudnick, Bucy, and Glenn (93) froze lactic cultures in 10 to 16% solids skimmilk with added $CaCO_3$ to neutralize

developed acidity. At various intervals, samples were removed from storage at -28.9 C and activity tests and plate counts were determined. After about 100 days of storage, little difference between treatments was found. After 190 days of storage, cultures grown and frozen in skimmilk containing 16% solids were most active. Cultures in the 10% medium were least active after frozen storage.

Heinemann (42) studied the activity of frozen cheese cultures to which 0, 10, and 20% glycerol had been added before freezing. After storage, culture activity was measured as titratable acidity after a 16-hr incubation at 21 C. Cultures containing 20% glycerol were found to be active up to 2 months of storage at 1.7 C, and up to 6 months at -15 and -28.9 C. Culture activity without glycerol was appreciably lower at all temperatures of storage. Richardson (91) studied the addition of glycerol to lactic cultures before freezing at -52 C in a dry-ice alcohol bath with storage at -23 to -28 C. Glycerol addition at the levels of 10 or 20% gave no beneficial effect after 6 or 12 weeks of frozen storage.

Woodburn and Strong (123) froze a Streptococcus culture in the presence of 4% solutions of rice flour, corn syrup, egg white, and sodium alginate. After frozen storage at -11, -21, and -30 C for 1, 4, and 10 weeks, all additives offered marked protection to the suspended cells over phosphate

buffer alone.

Gibson, Landerkin, and Morse (31) added various compounds to S. lactis, Streptococcus cremoris, and Streptococcus diacetilactis with freezing and storage at -23.3 and -196 C. The cultures were frozen and stored in skimmilk with 10% solids; in apple juice containing 10% skimmilk solids; and in skimmilk containing 10% glycerol, 10% dimethyl sulfoxide, 0.5 and 2.0% malic acid, 0.5 and 2.0% acetamide, and 0.5 and 2.0% succinimide. With cultures frozen and stored in liquid nitrogen, none of the additives gave any improvement over the skimmilk control; some additives gave poorer results. When S. lactis or S. cremoris was frozen and stored at -23.3 C, all additives except 2% succinimide improved the percentage survival. When S. diacetilactis was stored at -23.3 C, no additives improved the percentage survival. Similar results have been reported by Baumann (6). He studied the effects of various additives upon the survival of lactic cultures frozen at -20 and -196 C. Yeast extract and glycerol were superior to sucrose, dimethyl sulfoxide, casein, egg white, and egg albumin in protecting cells against cellular damage at -20 C. However, additives had little effect on survival of cultures stored at -196 C. His results showed that cultures could be stored at -196 C with no other preservative treatment.

Conditions of Freezing and Thawing

The amount of cellular damage from freezing and thawing may be influenced by the rate and temperature of freezing and thawing, the type of organism involved, and by certain environmental conditions. It is often difficult to distinguish between freezing rate and freezing temperature, since the most efficient way of accelerating the freezing rate is to use a lower temperature for freezing (9).

Mammalian spermatozoa, red blood cells, ovarian cells, and a variety of tissues survive freezing better if cooled slowly and thawed rapidly (103). For many living cells, Luyet and Gehenio (62) believed that fast freezing and fast thawing were necessary to rapidly pass through the temperature range in which crystallization of ice occurs. Hilliard and Davis (43) found no critical temperature below freezing where the germicidal effect of freezing was greatly hastened. Intermittant freezing of bacteria was more destructive than continuous freezing.

Meryman (73) explained that thawing can affect biological materials by exposure to high electrolyte concentrations and by growth of ice crystals by recrystallization prior to actual melting. Recrystallization may take place during storage at temperatures as low as -70 C (74). Rapid rates of thawing help to diminish the exposure to high electrolyte

concentrations (70) and prevent recrystallization (74). Chilson, Costello, and Kaplan (12) reported that rapid freezing and rapid thawing did not allow cellular protein to be exposed to critical pH and salt concentration long enough for inactivation to occur. However, with the inclusion of glycerol in the medium, the rate of thawing was of little significance (73).

Haines (35) observed that rapid freezing killed microorganisms at constant proportions depending upon the organism studied. For example, one freezing killed 89% of Saccharomyces cerevisiae cells, but only 5% of Staphylococcus aureus. Mazur, Rhian, and Mahlandt (71) found that when gelatin-saline suspensions of Pasteurella tularensis were cooled to -15 or -75 C, recovery was dependent on the rate of cooling and the minimum temperature attained. Recovery was significantly greater after slow cooling (74.7%) then after rapid cooling (45.4%). The proportion of viable cells could be increased by freezing slowly to, and thawing rapidly from, any given temperature.

Mazur (69) studied the effect of slow and rapid attainment of a temperature of -30 C upon the survival of S. cerevisiae suspended in distilled water. Less than 0.01% survived rapid freezing, whereas up to 50% survived slow freezing. Nei (81) studied the effect of slow (1 C/min) and rapid (about 10 C/sec) freezing rates upon the survival of

yeast cells suspended in distilled water. The temperature finally attained was below -40 C. Survival was much lower with rapid freezing than with slow freezing; 15 and 80%, respectively. Smith (103) reported results of freezing several different genera of yeasts under various freezing conditions. Only a small proportion of cells was viable in broth cultures which had been cooled rapidly to -79 or -190 C. Survival was improved by slow cooling to the same temperatures. However, with 15% glycerol in the broth, a large proportion of cells survived rapid or slow cooling to -79 or -190 C. With several yeasts, the proportion of survivors was higher in 15% glycerol cultures which had been cooled rapidly than in broth cultures which had been cooled slowly to -79 C. Remarkable difference in the resistance to freezing and thawing of organisms of closely related strains, species and genera, was demonstrated. For example, survival of different strains of S. cerevisiae cooled slowly to -79 C varied from 25 to 90%.

Lamprech (57) and Lamprech and Foster (58) studied the effects of rate of freezing and thawing upon cultures of S. lactis suspended in skimmilk, with and without added glycerol. Samples were frozen rapidly by immersion in alcohol at -20 C or slowly by placing in a freezer at -20 C. Samples of each treatment were thawed rapidly in a water bath at 30 C for 5 min or slowly in a water bath at 4 C for 2 hr.

With the rates and media studied, no differences in recovery of viable cells were found. Cardwell and Martin (11) reported no significant difference between the activity of cultures frozen slowly in a freezer at -17.8 C or rapidly in front of a blower at -28.9 C.

Richardson (91) investigated the effect of fast and slow freezing and added glycerol upon the activity of S. lactis cultures after frozen storage at -23 to -28 C. Slow-frozen samples were prepared by freezing gradually at the storage temperature over a period of 1.5 to 2 hr. Fast-frozen samples were frozen in a dry-ice alcohol bath. After 6 and 12 weeks of frozen storage, glycerol addition provided no advantage to the fast-frozen samples. However, glycerol addition to the slow-frozen samples showed a definite advantage over the fast-frozen cultures. Ten percent glycerol was more beneficial than was 20%. Fast-frozen and slow-frozen cultures, without added glycerol, showed equal activity after frozen storage. Moss and Speck (77) studied the influence of rate of freezing to -20 C upon injury and death of S. lactis cells suspended in buffered water and in skimmilk with 10% solids. Fast freezing was conducted at approximately 1 C/sec, and slow freezing at about 0.7 C/min. Only small differences in survival were found in cultures frozen either slowly or rapidly when suspended in skimmilk. When cultures were frozen in buffered water, however, maximum

survival occurred with certain strains when frozen rapidly, whereas other strains required slow freezing for similar results.

Rapid thawing is necessary for recovery of the greatest number of bacteria (56, 120). Turner and Brayton (116) reported that slow thawing of spirochetes from -78 to 0 C over a 2 to 6 hr period killed most of the organisms. However, greater survival was reported when cells were thawed rapidly in 30 sec.

Smith (103) reported that slow thawing of yeast cells suspended in distilled water was more destructive than rapid thawing from -79 or -190 C. Mazur (69) found that the mortality of yeast cells after exposure to -30 C in distilled water was 50 to 100 times greater with slow (1 C/min) than with rapid thawing (500 to 1,000 C/min).

Cardwell and Martin (11) found that the method of thawing was the most important factor influencing activity of frozen lactic cultures. They noted that fast thawing at 43.3 C for 1 hr greatly lowered culture activity over slow thawing at 4.4 to 6.7 C for 8 hr. However, when frozen culture was thawed by direct inoculations into starter milk, activity was comparable to the activity of slow-thawed culture. Decreased culture activity with thawing at 43.3 C was probably due to the temperature used, as temperatures over 38.9 C may seriously impair acid development of lactic

cultures (90).

Reports of ultrarapid freezing, as provided by freezing in the range of -196 C, have appeared in the literature for certain microorganisms. Clark (15) reported successful quick-freezing of Serratia marcescens and E. coli in liquid nitrogen whereas Vibrio comma, slow-frozen and cooled to -196 C, showed 90% loss of viability after thawing.

Early success of freezing microorganisms (63, 64, 65) to extremely low temperatures was reported in 1900. Ten bacterial cultures and two yeasts frozen on agar or in broth with liquid air at -182 to -190 C and about -252 C in liquid hydrogen, after frozen storage for 10 hr to 7 days showed no impairment in structure or vitality.

Doebbler and Rinfret (24) studied the effects of ultrarapid freezing and indicated the usefulness of droplet freezing for preservation of several microorganisms. Freezing was accomplished by spraying cell suspensions or mycelial particles onto a moving surface of liquid nitrogen. Thawing was conducted by stirring the frozen suspensions into 0.15 M NaCl at 37 C. The freezing and thawing rates were estimated to be several hundred degrees per sec. Viable cell counts before freezing and after thawing showed that E. coli and Staph. aureus were essentially uninjured. Azotobacter vinelandii, Aspergillus niger, and S. cerevisiae were markedly injured or killed; showing 28, 22, and 42% survival,

respectively.

Postgate and Hunter (85) found almost no difference in viability after freezing and thawing A. aerogenes irrespective of the freezing rate - slow at -20 C (about 20 min, rapid at -20 C (about 3 min), or ultrarapid at -196 C (less than 1 sec). All freezing was conducted in 10% glycerol suspensions. However, when freezing slowly at -79 C in phosphate buffer, survival was less than 1%, whereas 36% survival was obtained by ultrarapid freezing in liquid nitrogen. In a later paper, the same workers (86) reported that ultrarapid freezing of A. aerogenes in liquid nitrogen resulted in 50% survival when suspended in distilled water and almost complete survival in 10% glycerol. Van Eseltine et al. (118) reported that freezing in liquid air resulted in such rapid freezing that the bacterial counts of peas or corn were unchanged by the freezing process.

Successful reports have also been made for ultrarapid freezing of lactic cultures. Gibson et al. (31) reported that ultrarapid freezing and thawing of lactic streptococci gave greatest recovery of viable cells. Cultures were frozen slowly at -23.3 C and rapidly in liquid nitrogen. Baumann (6) and Baumann and Reinbold (7) froze and thawed lactic cultures at rates varying from slow (0.2 C/min) to ultra-rapid (130.7 C/min). Slow freeze-slow thaw or fast freeze-

fast thaw combinations resulted in greater survival than slow freeze-fast thaw or fast freeze-slow thaw combinations. Fast freezing followed by fast thawing was the best method to preserve maximum viability and activity.

Yurchenco, Piepoli, and Yurchenco (124) were able to maintain Streptococcus cultures up to 2 years without loss of viability after rapid freezing with storage at -76 C, followed by rapid thawing.

Sokolski, Stapert, and Ferrer (106) compared the growth response of Lactobacillus leichmannii after ultrarapid freezing in liquid nitrogen and slow freezing at the rate of 1 C/min to -40 C, with subsequent storage at -196 C. Thawing was conducted rapidly in a water bath at 40 C. After thawing, the nitrogen-frozen cultures produced a growth response comparable to unfrozen cells. Slowly frozen cells gave a delayed growth response.

Frozen Storage

Death of microorganisms during frozen storage is dependent upon the organism involved, time and temperature of frozen storage, suspending medium, and concentration of cells. Microorganisms are generally more resistant to freezing than animal or highly developed plant cells (81). Yeasts and molds are especially sensitive to freezing in the vegetative state; whereas spores of bacteria, yeasts, and

molds generally show low susceptibility to low temperatures and freezing (9).

As early as 1887, Prudden (87) reported that resistance of bacteria to freezing varied with different species. Staphylococci and streptococci are regarded by most workers to be more resistant to the effects of freezing (70).

Gibson, Landerkin, and Morse (30) studied the survival of 16 different strains of lactic streptococci during frozen storage at -17.8 and -23.3 C. At both storage temperatures, there were differences between the percentages of surviving strains, and in the rate of decrease with time. On the other hand, Simmons and Graham (99) found no appreciable difference in activity of four different cultures after frozen storage up to 6 months at -28.9 C.

Marked difference between the resistance of different organisms to freezing have been reported by many other investigators (4, 8, 34, 35, 38, 46, 59, 62, 67, 83).

Weiser and Osterud (121) presented evidence that death of bacteria by freezing involved a "rapid acting" or "immediate" death, and a "storage" death, which was a function of time and temperature, taking place at a reduced rate after the freezing period. Using E. coli as the test organism, the authors reported that immediate death was independent of the freezing temperature, and was uniform over the range from -5 to -196 C. Studies by Haines (35) and

Turner (115) also differentiated between immediate and storage death with the freezing of various microorganisms.

Major et al. (67) froze and stored 11 different species of bacteria for various periods of time at -22 C. After frozen storage, surviving bacteria versus storage interval was graphically plotted. They found that, initially, the negative slope of the survival curves was steep, representing rapid destruction of cells. However, with the passage of each successive time interval, the slope decreased, showing relatively slower rates of death. After a few days or weeks (depending on the species), the slope became negligible. Similar reports have been made about species of Salmonella stored at -25.5 C (34); strains of E. coli and Ps. fluorescens stored at -18 or -30 C (4); Achromobacter species stored at -28 C (53); Staph. aureus and Streptococcus faecalis stored at -11 or -30 C (123); and Pseudomonas aeruginosa stored at -1, -2, -3, or -5 C (35).

Variable success of extended storage of frozen lactic cultures has been frequently reported in the literature. Simmons and Graham (99) conducted a study to determine the length of time frozen cultures could be stored at -28.9 C without serious loss of activity. Six cultures were prepared by inoculating sterile skimmilk with 1% of a buttermilk culture. After freezing, one culture was removed from storage each month and was tested for activity. All frozen cultures

were slightly less active than fresh cultures. However, there appeared to be no progressive decrease in activity with increased storage time.

Richardson and Calbert (92) studied the activity of S. lactis after frozen storage at -28 C and found that culture activity decreased progressively as storage time increased. The criterion for activity was acid production after 12 hr of incubation at 32 C. Before freezing, activity was 0.44, and after 3 and 6 months of storage, activity was 0.31 and 0.25, respectively. Rudnick, Bucy, and Glenn (93) studied the preservation of viability and activity of cheese and buttermilk cultures after frozen storage at -28.9 C. Numbers of bacteria decreased rapidly as storage was prolonged to 190 days. Activity decreased less rapidly than bacterial numbers.

Rudnick and Glenn (94) studied the use of cultures frozen and stored at -27.8 C for Cottage cheese making. Storage time varied from 3 to 150 days. They reported that the activity of frozen cultures during cheesemaking tended to decrease slightly as the age of the frozen culture increased. Antila and Peltola (3) reported that starter cultures frozen and stored at -12 C retained initial flavor- and acid-producing capacities for about 1 month of frozen storage. Stoyanov (110) froze and stored yogurt cultures at -15 C and found that activity was substantially

decreased after 10 days of storage. After frozen storage, coagulation required 3 to 6 hr as compared to the normal coagulation time of 2 to 2.5 hr. However, Cardwell and Martin (11) reported that activity of cultures frozen at -28.9 C for 4 or 30 days of storage was as good as the activity of cultures transferred daily.

The success of freezing most microorganisms is largely dependent upon the freezing temperature; the lower the temperature, the greater the survival and maintenance of activity. As early as 1918, Hilliard and Davis (43) studied the germicidal action of various freezing temperatures on bacteria and reported that storage at 0 to -20 C was more destructive than the temperature below -20 C.

Howard (46) demonstrated progressively better survival of bacteria at lower storage temperatures. With freezing at -10 C, cultures of Neisseria gonorrhoeae remained viable for only 3 weeks, whereas cultures of the same organism at -40 C remained viable at least 3 months. Haines (35) observed that prolonged storage of various bacteria stored from -1 to -20 C exhibited the most rapid death rate near the higher temperature. Arpai (4) found that the percentage of killed cells of Ps. fluorescens and E. coli decreased as the temperature of freezing and storage was lowered from -7 to -18 C.

Woodburn and Strong (123) froze Salmonella,

Staphylococcus, and Streptococcus species at -11, -21, and -30 C for periods up to 30 weeks. With one exception, the greatest number of cells survived each time period at the lowest temperature and the least survived at the highest temperature. Gibson et al. (30) froze and stored lactic cultures at -17.8 and -23.3 C, and found that for the majority of strains studied, the survival rate was greater at the lower temperature. Duggan et al. (26) froze cultures of L. acidophilus at -10, -20, and -60 C and found that greater survival was obtained with storage at -60 C.

Mazur et al. (71) reported that the survival of Pasteurella tularensis frozen slowly in sucrose solution was lower at -45 C than at any temperature studied, and was higher at -75 C than at -60 C. Turner and Brayton (116) and Turner and Fleming (117) found that Treponema pallidum and relapsing fever spirochetes survived better at -78 C than at higher temperatures. Weiser and Osterud (121) studied the mortality of E. coli following freezing at -15, -20, -30, -50, -78, and -195 C. They found no significant difference in reduction of bacterial numbers frozen at temperatures of -30 C and below. However, with increasing temperatures above -30 C, the counts were progressively reduced. Storage death at -195 C either did not occur, or was too slow to be detected.

Recently, an increasing number of investigations have

dealt with ultralow freezing and storage of microorganisms. Meryman (73) reported that freezing and storage of living cells at -196 C has distinct advantages over preservation at higher temperatures as the ultralow freezing minimizes crystal growth and biochemical changes. He attributed the deleterious effects of frozen storage of cells primarily to the biochemical process of dehydration denaturation. He further explained that if a specimen survives the freezing process, further decreases of temperature result in a logarithmically decreasing rate of cellular denaturation, presumably reaching a standstill in the vicinity of or below -100 C.

Baumann (6) found that the most significant factor influencing survival of lactic cultures was the temperature of storage. Cultures showed no loss of activity or viability after storage for 28 weeks at -196 C, whereas the same cultures stored at -20 C retained less than 20% of their initial activity or viability. Studies by Gibson et al. (31) also showed that the most significant factor influencing the survival of lactic streptococci was the freezing and storage treatment. Cultures of S. lactis, S. cremoris, and S. diacetilactis were frozen and stored at -23.3 C and in liquid nitrogen. Cultures frozen and stored in liquid nitrogen up to 24 weeks, with or without additives, gave the greatest recovery of viable cells.

Studies by Cowman and Speck (17, 19) indicated that -196 C would be the temperature of choice for the preservation of maximum culture activity and viability for extended periods of time. Viability and activity of cultures was markedly reduced after storage up to 60 days at -20 C. When storage was at -196 C, viability and activity of the cultures remained at a high level during the 60-day storage period. After storage for over 3 years in liquid nitrogen, no measurable changes in the activity of lactic cultures were detected (49).

Mazur (70) reported that with storage of cells at -196 C, it was highly probable that no loss of viability and no physiological or genetic changes would occur for decades. Meryman (73) considered that storage of cells in liquid nitrogen could be considered as essentially indefinite. Sneath (105), in discussing the longevity of microorganisms, reasoned that at low temperatures, around absolute zero, not only would essential and labile metabolites be preserved, but the structural breakdown of proteins and nucleic acids would be exceedingly slow. He concluded that life at low temperatures could probably be preserved over a million years.

Mechanism of Freezing Death

The mechanism of death that occurs during frozen storage of bacteria is unknown (45). Much disagreement exists in the literature concerning the causes of death at low temperature. Luyet and Gehenio (62), in a review of the mechanisms of injury and death at low temperatures, discussed the following probable causes: precipitation of some of the components of living structure, impairment in the functions of elimination and accumulation of toxic products, impairment in the function of osmosis and permeability, impairment in the function of controlling protoplasmic water solutions and resulting dehydration, changes in the velocities of interrelated chemical reactions, changes in the adsorptive properties of some protoplasmic constituents, and solidification of protoplasmic fats. Burns (10) added the following chemical changes as possible causes of death: toxic formation of carbonyl compounds, toxic radical formation, and damage caused by changing sulfhydryl groups on protein molecules to disulfide bonds which denature protein. It appears most probable that death by freezing involves factors which have been proposed in several theories, and that the influence of the various factors may vary with different cells, especially cells which vary widely in structure and function (121).

Freezing Injury and Stimulation

Straka and Stokes (111) believed that early stages of frozen storage of bacteria involved nonlethal physical or metabolic injury, and as injury became progressively more extensive, the cell eventually dies. These authors demonstrated metabolic injury in cultures of E. coli and three species of Pseudomonas by a change of nutritional requirements after freezing. After frozen storage, cultures were plated on a minimal medium and on a complex medium rich in amino acids, peptides, vitamins, purines, pyrimidines, and other nutrients. Before freezing, cells grew equally well on either minimal or maximal media. It was thought that metabolic injury impaired the ability of bacteria to synthesize essential cellular components, therefore they would not be able to grow on minimal media after freezing. It was found that the freezing process injured few cells and that frozen storage was primarily responsible for cellular injury.

Other workers also have similarly demonstrated a metabolic injury to bacteria that results in increased nutrient requirements after frozen storage. Nakamura and Dawson (79) showed injury in cultures of Shigella sonnei after frozen storage at -20 C in saline, nutrient broth, or milk. Metabolic injury increased with an increase in the time of

frozen storage. Similar freezing-injury has been demonstrated with cells of Ps. fluorescens and E. coli (4), E. coli (78), A. aerogenes and E. coli (66), and A. aerogenes (86). MacLeod, Smith, and Gelinas (66) postulated that frozen storage damaged the cytoplasmic membrane of a portion of the cells rendering them more penetrable by toxic metal ions. The enriched medium would permit growth of injured cells by chelating the toxic ions.

Freezing injury also has been studied in lactic streptococci. Using a maximal and minimal agar medium, Moss and Speck (76, 77) found that cells of S. lactis were injured after freezing in such a way that their nutritional requirements were increased so that growth would not occur on the minimal medium. Injury and death were more pronounced when cells were frozen in distilled water than in skimmilk. Greatest injury occurred during early stages of frozen storage and decreased with time. Using the same type of media, Gibson et al. (30) studied metabolic injury of 16 single-strain cultures of S. lactis, S. cremoris, and S. diacetilactis after frozen storage. Cell damage, as indicated by failure of organisms to grow on minimal agar medium, was demonstrated with only two cultures. Baumann (6) found that 12 single-strain cultures of S. lactis and S. cremoris when stored at -20 C showed injury as reported by Moss and Speck (77). However, when cells were stored at

-196 C, no injury was found.

The term "metabolically injured" implies that the enriched or maximal medium supplied biochemical constituents that the cells have become incapable of synthesizing because of damage (70). Attempts have been made to isolate these constituents. Moss and Speck (78) found that the active components for growth of injured E. coli cells were five closely related peptides isolated from Trypticase, a pancreatic digest of casein. Trypticase also was found responsible for recovery of injured cells of Pseudomonas and Escherichia (111). Cysteine was found to be the effective component isolated from more complex materials for cells of E. coli and A. aerogenes (66). Enzymatic digests of casein also have been found responsible for recovery of injured cells of S. lactis (77).

Numerous supplements have been added to lactic cultures to increase their acid-producing ability or improve growth. These nutrients include amino acids or peptides (20, 96); corn steep liquor (50, 52); fat globule membrane (32); hydrolyzed lactalbumin (54); hypoxanthine, inosine, or adenine (21, 22, 55); inorganic phosphate (60) and phosphate buffer (98); malic acid or apple juice (31); pancreas extract (22, 107, 108, 122); peptones (29); protein hydrolysate, cysteine, glutathione, or thioglycolate (33); and yeast extract or glutamic acid (50).

Other than increasing a culture's activity by adding supplements, freezing itself has been reported to cause an increase in growth rate or activity. It has been observed that certain cultures, especially slow acid-producing, single-strain cultures of lactic streptococci, possessed greater activity after frozen storage in liquid nitrogen than before freezing (6). This increase in acid-producing activity of a culture after frozen storage has been referred to as "stimulation". Such a phenomenon has been most frequently noticed after 1 month of frozen storage. The mechanism of this phenomenon was not understood. Sometimes frozen-thawed cells show a higher enzymatic activity than do unfrozen controls, and the explanation usually given is that the substrate can more easily permeate damaged cell membranes and reach the site of the enzyme (70).

Other cases of apparent stimulation have appeared in the literature. Hartsell (39) found that after storage for 21 days at -9 C, the generation time of thawed cultures of E. coli had decreased from 36.4 to 22.4 min. Tanguay (113) noted that S. faecalis grew at a greater rate at the end of one year's storage than the original culture. Arpai (5) found that cultures of E. coli showed reduction of generation time after frozen storage at -4 and -30 C. This stimulation of growth was explained on the basis of selection of the more rapidly growing cells, assuming that they belonged to the

more cryoresistant fraction of the bacterial population. Squires and Hartsell (109) found that the generation time of E. coli could be decreased by freezing, depending on the freezing medium and length of frozen storage. Stimulation was attributed to stimulatory materials accumulated during storage.

EXPERIMENTAL METHODS

Acquisition and Identification of Cultures

Thirty-nine single-strain cultures of lactic streptococci and 14 multiple-type, mixed-strain cultures were used in the study. One single-strain culture of Streptococcus lactis, three cultures of Streptococcus diacetilactis, and ten cultures of Streptococcus cremoris, were of New Zealand and Australian origin.[1] One culture of S. lactis was obtained from the A.T.C.C.[2], and the remaining 24 isolates were obtained by plating and picking colonies from two cultures of S. cremoris, received from New Zealand and Australia. All multiple-type, mixed-strain cultures were obtained from the culture collection of the Food Products Analysis Laboratory at Iowa State University.

The species identity of each single-strain culture was established by the criteria given by Sandine, Elliker, and Hays (95). Methylene-blue milk was made according to the method of Clark (16). Arginine-hydrolysis medium was made according to the method of Skerman (101). Trypticase soy broth[3], adjusted to the desired salt level or pH, was

[1]J. Czulak, Commonwealth Scientific and Industrial Research Organization, Highett, Victoria, Australia.

[2]American Type Culture Collection, Rockville, Md.

[3]Baltimore Biological Laboratory Div., B-D Laboratories, Inc., Baltimore, Md.

used for salt and pH tolerance tests. Cultures were tested after incubation for 1 day at 32 C and periodically up to 7 days, if necessary.

Routine Analysis and Handling of Cultures

Culture propagation

Cultures were routinely propagated in sterile Matrix[1] medium, reconstituted with distilled water to 11% solids. The medium was inoculated at the rate of 1% and incubated for 16 hr at 21 C. Cultures were transferred at least three times weekly and between transfers were stored in a refrigerator at approximately 2 C. At least three propagations were made on successive days prior to use.

Cultures also were grown in Matrix before use in freezing and related studies. Since no other milk powder was used, reconstituted Matrix will hereafter be referred to as "skimmilk." Skimmilk was steamed for 1 hr and immediately cooled in flowing tap water to the desired incubation temperature, usually 21 C.

Some experiments required the use of whey medium which was prepared by the isoelectric precipitation of skimmilk followed by steaming for 30 min to facilitate whey removal from the curd. After cooling to room temperature, the whey was filtered through several layers of cheese cloth, and the

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

pH was adjusted to 6.8-7.0. To obtain bacterial counts in whey medium that were comparable to counts in skimmilk, it was found necessary to add 0.25% yeast extract[1] to the whey.

Enumeration and activity determination

Total counts of cultures were determined as outlined in Standard Methods for the Examination of Dairy Products (1). Duplicate plates were poured with Eugonagar[2], and incubated for 7 days at 21 C.

Acid-producing activity was determined by inoculation of 20-ml quantities of skimmilk which had been dispensed into 20 x 125 mm screw-capped tubes, steamed 30 min and immediately cooled in flowing tap water. The skimmilk was inoculated with 0.2 ml culture and incubated in a thermostatically controlled water bath for 6 hr at 32 C. After incubation, an 18-g sample was titrated to the phenolphthalein end point with 0.1 N NaOH, using 10 drops of a 2% phenolphthalein solution as indicator. To facilitate titrating culture samples to the same end point, a reference color stick[3], magnetic stirrer[4], and color-corrected fluorescent lamp[5] were used for

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

[2]Baltimore Biological Laboratory Div., B-D Laboratories, Inc., Baltimore, Md.

[3]Kimble Glass Co., Toledo, Ohio.

[4]E. H. Sargent and Co., Chicago, Ill.

[5]Luxo Lamp Corp., Port Chester, N.Y.

all titrations. The titratable acidity of an uninoculated control was subtracted from that of the culture to obtain values for developed acidity, calculated as lactic acid. Culture activity was then reported as 100 times developed acidity. All activity tests were determined either in duplicate or triplicate. All activity-test medium was prepared from a single lot of skimmilk powder which was stored in a refrigerator at approximately 2 C.

An attempt also was made to relate acid-producing activity to population, and to compare individual cultures on the basis of activity per cell. The total counts obtained from replicate plates of the same culture did not vary significantly one from another whether the culture was fresh or had been frozen. Also microscopic smears did not show clumping. Therefore, to facilitate comparisons when taking viable population into account, the apparent values for acid production per clump or cell were calculated by dividing the acid-producing activity by the total counts. The quotient was expressed as [activity per cell] x 10^{-8}.

Freezing and thawing of cultures

Cultures were quick-frozen in 20 x 125 mm screw-cap tubes, stoppered with styrofoam plugs, by immersion in liquid nitrogen. The samples were stored in the same coolant in a LN-35 or LN-180 liquid-nitrogen container.[1]

[1]Linde Company, Div. of Union Carbide Corp., New York, N.Y.

Cultures were thawed from the frozen state by 1-min agitation in a 40 C water bath. One minute was found to be the minimum time required to obtain complete dissolution of ice in the culture tube. Samples for plate counts or activity tests were taken immediately after thawing.

Proteolysis determination

Proteinase activity was measured by detection of liberated tyrosine and tryptophan according to the method of Hull (47). Before testing for proteolysis, cultures were grown for 6 hr at 32 C or for 12 hr at 21 C. Five-gram aliquots of culture were removed and after the addition of 1 ml of distilled water to the aliquot, the protein was precipitated by adding 10 ml of 0.72 N trichloroacetic acid. After standing at room temperature for 10 min, the mixture was filtered and a 5-ml portion of the trichloroacetic acid filtrate was transferred to a 50-ml Erlenmeyer flask. Ten milliliters of the sodium-carbonate reagent were added, mixed, and a 3-ml volume of Folin-Ciocalteau phenol reagent[1] was added to the contents of the flask and allowed to stand for 5 min for maximum color development. The intensity of the blue color was measured at 650 mμ in a Coleman Model 11 spectrophotometer. The values obtained were converted into their tyrosine equivalent by reference to a standard tyrosine curve. Proteolysis in the milk medium due to growth of the

[1]Fisher Scientific Co., Fair Lawn, N.J.

culture was determined by subtracting the value obtained for the skimmilk control.

Rate of Freezing Cultures

Rate of freezing of cultures placed in a freezing cabinet at -20 C, or in a liquid-nitrogen refrigerator, was determined by inserting a copper-constantan thermocouple in 3 ml of culture in a 20 x 125 mm screw-cap tube. The temperature change was recorded continuously on a Electronik 16 Multipoint Recorder[1].

Two rates of freezing were used for frozen storage at -20 C: "fast" and "slow". Fast freezing was conducted by immersion of the culture tubes in liquid nitrogen for 30 sec and then placing them in the -20 C freezer. Slow freezing was conducted by placing the tubes directly into the freezing cabinet at -20 C.

Frozen Storage of Cultures

Preliminary tests were planned to determine the maintenance of culture activity and total counts after frozen storage at -196 C. Six multiple-type, mixed-strain cultures were grown and frozen at -196 C as previously described. After 1 day and after 2 and 4 weeks of frozen storage, samples of culture were thawed. Activity and total counts were

[1]Honeywell Inc., Philadelphia, Pa.

determined before freezing and after storage. Three replications of the experiment were made to obtain average values.

Physiological Age of Culture in Relation to Activity and Total Count

Studies were made to determine the relationships of temperature and time of culture incubation to total counts and activity of cultures grown in skimmilk. Four multiple-type, mixed-strain cultures were incubated at 15 and 21 C. Total counts were determined on samples incubated at 15 C, immediately after inoculation, and after 8, 14, 18, 20, 22, and 24 hr of incubation. Culture activity was determined at 18 hr and thereafter at times corresponding to the sampling times for total counts with additional measurements at 28 and 32 hr. Total counts on cultures grown at 21 C were determined immediately after inoculation, and after 8, 12, 14, 16, 18, and 24 hr. During 21 C incubation, activity tests were determined at 12 hr and thereafter at times corresponding to the sampling times for total counts.

A further study was made to determine the effect of freezing and storage at -196 C upon maintenance of viability, acid producing ability, and proteinase activity of cultures grown for various periods of time at 21 C before freezing. While some workers determine proteinase activity of cultures with growth at 21 C (13), and others conduct the test with growth at 32 C (122); it was felt desirable to perform the

tests using both temperatures for comparison. During incubation at 21 C, total counts, activity tests, and proteolysis tests were determined after 12, 18, 24, 30, and 36 hr. During the sampling times, portions of the cultures were quick-frozen and stored in liquid nitrogen. Duplicate tubes of cultures were thawed after 1 month of frozen storage. Then, total counts, activity and proteolysis were determined. Four multiple-type, mixed-strain and four single-strain cultures were used for this study.

Cultures Grown in Whey Medium

Trials were conducted to develop a suitable medium from which bacterial cells could be easily separated and concentrated. Various agents that prevent the coagulation of casein, as urea and citrate, were added to the skimmilk medium. Concentrations of citrate or urea required to prevent coagulation of skimmilk were found to be highly inhibitory for adequate culture growth.

In view of the desirable characteristics of whey medium in comparison to milk (clarity and the absence of interfering effect of colloidal protein in preparing cell concentrates), it was considered desirable to compare the growth and activity of cultures in skimmilk and whey media. First, a study was made to determine the time of incubation required at 21 C to obtain maximum culture activity and cell

numbers. Four multiple-type, mixed-strain cultures were used. Three days prior to the study, the cultures were transferred daily in skimmilk; on the fourth transfer, the cultures were inoculated into whey medium using a 1% inoculum. At 12, 14, 16, 18, and 20 hr of incubation, total counts and activity tests were determined.

A further study was made to determine if single-strain cultures would maintain their activity after being maintained in skimmilk, transferred to whey, and back to skimmilk again. Ten single-strain cultures were used. At the time of each transfer from one to the other medium, total counts and activity tests were determined.

Effect of Frozen Storage upon Activity, Viability, and Proteolysis of Cultures

Two fast acid-producing and two slow acid-producing, single-strain cultures of S. cremoris were grown and frozen in skimmilk and whey media at -196 C. Before freezing and after 1, 3, and 6 months of frozen storage, activity tests, total counts, and proteolysis determinations were made.

The effect of subsequent transfer after frozen storage also was studied. After these additional transfers, activity tests, total counts, and proteolysis determinations again were made.

Daily Comparison of Unfrozen Culture to Frozen Culture after Extended Storage at -196 C

Two slow acid-producing and two fast acid-producing, single-strain cultures of S. cremoris were stored for 6 months at -196 C. Activity tests were made on each culture before freezing, immediately after thawing, and after daily 16-hr transfers for a 2-week period. Also, activities were determined daily on the same four, unfrozen cultures. Total counts were determined on the cultures before freezing, immediately after frozen storage, after the first 16-hr transfer, and after the 2-week period of daily transfers. Culture proteolysis was ascertained before freezing, immediately after thawing, and after the 2-week period.

Effect of Frozen Storage upon Cellular Integrity of Cultures

The effect of liquid-nitrogen freezing upon the cellular integrity of the lactic streptococci employed was studied by use of maximal-minimal media. Two fast acid-producing and two slow acid-producing cultures of S. cremoris were used. The maximal medium used was Eugonagar. Minimal media were developed by progressively lowering the concentrations of Trypticase[1] and Phytone[1] from 100% of that used in the Eugonagar formula to 12.5%, giving five different minimal

[1]Baltimore Biological Laboratory Div., B-D Laboratories, Inc., Baltimore, Md.

media. The proportions of the other components used in the minimal media were unaltered as contained in Eugonagar: sodium chloride, 0.4%; sodium sulfite, 0.02%; l-cystine, 0.07%; dextrose, 0.55%; and agar, 1.5%. Preliminary experiments showed that the media containing 50% initial protein concentration or less were inadequate to support bacterial growth of several cultures in comparison to Eugonagar before freezing. Therefore in subsequent experiments, the lower protein concentrations were only used when growth was equivalent to that of Eugonagar. The pH of the media was adjusted to 7.0 before sterilization. The various protein concentrations of the experimental media were as follows:

Agar medium	Trypticase	Phytone	% of control
Control (Eugonagar)	1.50	0.50	100.0
B	1.13	0.38	75.0
C	0.75	0.25	50.0
D	0.56	0.19	37.5
E	0.38	0.13	25.0

The cultures were held frozen at -196 C and at -20 C for 1 month. Slow and fast freezing rates were used for cultures stored at -20 C. Before freezing, and after storage, the cultures were plated using Eugonagar and the four minimal media.

Selection of Cultures Stimulated by Frozen Storage in Liquid Nitrogen

To further investigate the phenomenon of stimulation as observed by Baumann (6), it was necessary to first select individual cultures that showed stimulation by frozen storage at -196 C. Ten single-strain and seven multiple-type, mixed-strain cultures were employed for study. To obtain a more reliable figure for culture activity before and after frozen storage, activity tests were conducted before freezing and on the two preceding transfers, immediately after frozen storage for 1 month and on the next two succeeding transfers. The three values for activity tests before freezing were averaged, and the three activity-test values after frozen storage were averaged for each culture.

Using information gained from the preceding study, two cultures were selected for further investigation. It also was found that to more clearly demonstrate stimulation, the activity should be calculated on a per-cell basis. Appropriate dilutions of these cultures were made and plated with Eugonagar. After incubation, 100 bacterial colonies were picked from each culture into tubes of sterile milk. The cultures were grouped on the basis of coagulation time in milk at 32 C. With daily transfers and elimination of apparently similar cultures, all but 24 were eliminated after

5 days of daily transfer. Seventeen of the cultures were capable of coagulating skimmilk within 16 hr at 21 C, and were referred to as "fast"; the remaining seven cultures were incapable of coagulating milk in 24 hr, and were designated as "slow." Therefore, based on their activity in skimmilk, the slow cultures were transferred on a 24-hr schedule; whereas the fast cultures were maintained on a regular 16-hr basis. Before freezing, and after frozen storage for 1 month at -196 C, total counts and activities were determined on each culture. This experiment was conducted in triplicate with activity determinations made on triplicate samples.

Effect of Freezing upon Subsequent Carbohydrate Utilization of Cultures

A study was made to determine if freezing affected fermentation patterns of cultures after frozen storage. Four single-strain cultures of S. cremoris that were routinely propagated and the same cultures after frozen storage in liquid nitrogen were used. Fermentation of added lactose, glucose, or galactose was studied in Eugonbroth[1]. Ten percent solutions of the sugars were prepared and filter sterilized. Two-milliliter amounts of sterile sugar solution

[1]Baltimore Biological Laboratory Div., B-D Laboratories, Inc., Baltimore, Md.

were then added to tubes containing 18 ml of sterile Eugonbroth, giving a final concentration of 1% fermentable sugar. After tempering the tubes at 32 C, they were inoculated with 1% fresh or frozen culture, mixed, and incubated in a 32 C water bath. After a 6-hr incubation, the tubes were placed in an ice-water bath to stop growth and were titrated to the phenolphtalein end point using 0.1 N NaOH. Simultaneously, activity tests were run on the fresh and frozen cultures.

Effect of Freezing Cultures upon the Probable Release of Biologically Stimulatory Substances

An investigation was made to determine if freezing of culture may produce leakage of biologically active material into the culture medium. Cell-free supernatants were prepared from Culture I after routine propagation and from the same culture after frozen storage in liquid nitrogen. The cultures were centrifuged and the whey separated and filtered to remove the suspended curd particles. A cell-free supernatant was then obtained by sterilization of the whey using a Selas filter. After heat-treatment of the skimmilk, the filtrates from fresh and frozen culture were added in varying amounts from 0 to 25% by replacing a portion of distilled water used to reconstitute the skimmilk medium. Resulting mixtures were inoculated with Culture I. After a 6-hr period of incubation at 32 C, 18-g samples were taken for activity tests.

Effect of Freezing upon Bacterial Growth Curves

Using three fast and three slow acid-producing cultures of S. cremoris, the growth curves at 21 C were studied in skimmilk. Portions of the inoculum, from which the growth-curve cultures were inoculated, were frozen in liquid nitrogen for 2 and 4 weeks. After frozen storage, cultures were thawed and growth curves redetermined. At 2-hr intervals, culture growth was determined by total plate counts. Two trials of the study were conducted.

Effect of Frozen Storage upon β-Galactosidase Activity of a Culture

Assay of β-galactosidase of S. lactis was conducted according to the method of Citti, Sandine, and Elliker (13). Culture 7962 was propagated daily in a broth with incubation for 12 hr at 32 C before the assay. The broth consisted of the following components: lactose, 10 g; Tryptone[1], 10 g; yeast extract[1], 5 g; gelatin[1], 2.5 g; sodium chloride, 4 g; sodium acetate, 1.5 g; ascorbic acid, 0.5 g; and distilled water to 1.0 liter. The pH of the broth was adjusted to 7.0 prior to autoclaving for 15 min at 121 C. Cultures were grown in the broth in 1-liter quantities for 12, 16, 20, and 24 hr at 32 C, after which they were chilled in ice water and centrifuged at 9,000 x g for 10 min in a

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

refrigerated Spinco Model L ultracentrifuge[1], operated at 1 C. The harvested cells were washed twice with cold 0.05 M potassium phosphate buffer at pH 7.0.

Washed cells were suspended in 20 ml of cold buffer. Several 3-ml quantities of each culture were frozen and stored in liquid nitrogen for examination after 1 month of frozen storage at -196 C.

For preparation of toluene-treated cells, 4-ml volumes of the whole-cell suspensions were treated with 0.2 ml of toluene-acetone (1:9) solution, and incubated at room temperature for 5 min with vigorous agitation by a Vortex-Genie[2] tube mixer.

The chromogenic substrate o-nitrophenol-β-D-galactopyranoside[3] (ONPG) was used to measure enzyme activity. A solution of 0.005 M ONPG was prepared in the pH 7.0 buffer.

A 1-ml volume of the whole-cell or toluene-treated cell suspension was incubated with 4.0 ml of ONPG solution for 15 min at 37 C. Color development was then stopped by adding

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[1]Beckman Instruments, Inc., Spinco Div., Palo Alto, Calif.

[2]Scientific Industries, Inc., Queens Village, N.Y.

[3]Calbiochem, Los Angeles, Calif.

5.0 ml of cold 0.5 M sodium carbonate to the reaction mixture. Cells were removed from the assay mixture by centrifugation at 9,000 x g for 12 min at 1 C. The absorbancy of appropriate dilutions of the supernatant liquid was measured at 420 mμ by a Beckman Model DU spectrophotometer.

The unit of enzyme activity was defined as the number of μmoles of o-nitrophenol liberated from ONPG per min of reaction time, and was calculated by multiplying absorbancy at 420 mμ by the molar extinction of 0.238 $\mu mole^{-1}\ cm^{-1}$ $[1/E_{cm}^{\mu M}]$ (72).

The β-galactosidase activities of fresh whole and toluene-treated cells were measured immediately after preparation. Similar analyses were made on cells frozen and stored in liquid nitrogen immediately after thawing. Activity tests and total counts were determined on the same culture after 16, 20, 24, and 28 hr of incubation at 21 C in skim-milk. Samples of these cultures were frozen in liquid nitrogen, and, after 1 month of frozen storage, activity tests and total counts were again determined. All activity tests were determined in duplicate or triplicate.

Concentration and Freezing of Cheese Culture

A commercial culture was incubated in whey medium for 15 hr at 21 C, aseptically poured into sterile, 250-ml centrifuge flasks, and centrifuged at 2800 rpm for 15 min.

Preliminary trials showed that a centrifugation time of 15 min was the minimum time required to obtain maximum viability of concentrated culture with the equipment used. After concentration, the cellular concentrate was aseptically ladeled into pre-formed, heat-sealable aluminum bags[1]. The aluminum bags were prepared for use by chlorine sanitization followed by rinsing with sterile water before filling with culture. After filling and sealing the mouth, the bags of culture were frozen in liquid nitrogen and placed in the liquid-nitrogen container for further study. Total counts and activity tests were determined before concentration and after 1 month of storage at -196 C.

Manufacture of Cheese with Concentrated, Frozen Culture

Commercial cheese culture was grown at 21.1 C. After 12, 16, 20, and 24 hr of incubation, 10-fold concentrates were prepared by centrifugation. These concentrates were placed in sterile cans, sealed, and frozen in liquid nitrogen. After approximately 2 months of storage at -196 C, the concentrated cultures were used for making Cheddar cheese. Bulk cultures are commonly prepared to inoculate milk for making cheese. This is accomplished by transferring a culture into successively larger amounts of milk, usually requiring

[1]Buffalo Laboratories, Inc., Buffalo, N.Y.

two or three daily propagations. By using a concentrated culture, several or all transfers needed to prepare the bulk culture may be eliminated. Inoculating a concentrated culture directly into milk for cheese making is commonly referred to as "direct set" inoculation.

Each vat of experimental cheese was made from 240 lb of heat-treated (62.8 C for 17 sec) milk. To compare rates of acid production between vats, a conventional make procedure was used, with 4.5 hr elapsing from addition of rennet until milling (90). The experiment was conducted three times. Culture for each of the three trials was prepared at the same time, using the same culture in three different fermentation batches. The same culture, unconcentrated, served as a control for two 5000-lb vats; and concentrated culture was used for the third control.

During the manufacture of cheese, total counts were determined on the milk immediately before inoculation, after thorough mixing of milk and culture, and after 1 hr ripening. Titratable acidity and pH determinations were conducted at intervals during the make procedure, and pH readings also were made on the curd at the time of milling and the following morning after the cheese was made.

RESULTS

Identification of Cultures

The results of taxonomic tests on single-strain cultures used in these studies is presented in Table 1.

Rate of Freezing Cultures

Freezing rates used are shown in Figures 1 and 2. Table 2 shows the maximum and average cooling rates depicted in Figures 1 and 2.

Frozen Storage of Cultures

Maintenance of culture viability and activity after frozen storage at -196 C for 4 weeks is shown in Table 3. Values for maintenance of total count, activity, and activity/cell after 1 day and 2 and 4 weeks are presented in Figures 3, 4, and 5, respectively. Total counts of cultures decreased slightly after 1 month of storage at -196 C; no loss of culture activity occurred after frozen storage. Final activity/cell of all cultures was greater than before freezing.

Ranges and averages for total counts and activity tests of cultures grown for various periods of time at 15 and 21 C are presented in Table 4. Average values are shown in Figures 6 through 9. Cultures grown at 15 C had a maximum total count at about 20 hr of incubation (Figure 6) and a maximum acid-

Table 1. Classification of single-strain cultures of Streptococcus species

Culture no.	Growth in 0.3% methylene blue	Growth in 4.0% NaCl	Growth at pH 9.2	Growth at 40 C	Arginine hydrolysis	Production of aroma	Species
1	-	-	-	-	-	-	cremoris
2	-	-	-	-	-	-	cremoris
3	-	-	-	-	-	-	cremoris
4	-	-	-	-	-	-	cremoris
5	-	-	-	+	-	-	cremoris
6	-	-	-	-	-	-	cremoris
7	-	-	-	-	-	-	cremoris
8	+	-	-	+	-	-	cremoris[a]
9	-	-	-	-	-	-	cremoris
10	+	-	-	-	-	-	cremoris
11	-	-	-	-	-	-	cremoris
12	-	-	-	-	-	-	cremoris
13	-	-	-	-	-	-	cremoris
14	-	-	-	-	-	-	cremoris
15	-	-	-	-	-	-	cremoris

[a]Cultures 8 and E8 deviated in characteristics from the typical S. cremoris, suggesting classification as variants of S. cremoris.

Table 1 (Continued)

Culture no.	Growth in 0.3% methylene blue	Growth in 4.0% NaCl	Growth at pH 9.2	Growth at 40 C	Arginine hydrolysis	Production of aroma	Species
16	-	+	-	-	-	-	cremoris
17	-	-	-	-	-	-	cremoris
18	-	-	-	-	-	-	cremoris
19	-	-	-	-	-	-	cremoris
20	-	-	-	-	-	-	cremoris
21	-	-	-	-	-	-	cremoris
22	-	+	-	-	-	-	cremoris
23	-	-	-	-	-	-	cremoris
24	-	-	-	-	-	-	cremoris
C 1	-	-	-	-	-	-	cremoris
C 3	-	-	-	-	-	-	cremoris
C 7	-	-	-	-	-	-	cremoris
C 10	+	+	+	+	+	-	lactis
C 11	-	+	-	-	-	-	cremoris
C 13	-	+	-	-	-	-	cremoris
ML	-	-	-	-	-	-	cremoris

Table 1 (Continued)

Culture no.	Growth in 0.3% methylene blue	Growth in 4.0% NaCl	Growth at pH 9.2 40 C		Arginine hydrolysis	Production of aroma	Species
E 8	+	+	-	-	-	-	cremoris[a]
EB 2	-	-	-	-	-	-	cremoris
EB 4	-	-	-	+	-	-	cremoris
HP	-	-	-	-	-	-	cremoris
DRC 1	+	+	+	-	-	+	diacetilactis
DRC 2	-	+	+	+	+	+	diacetilactis
DRC 3	+	+	+	+	+	+	diacetilactis
7962	+	+	+	+	+	-	lactis

Figure 1. Cooling rate obtained in a 20 x 125 mm screw-cap test tube containing 3 ml culture, placed at -20 C

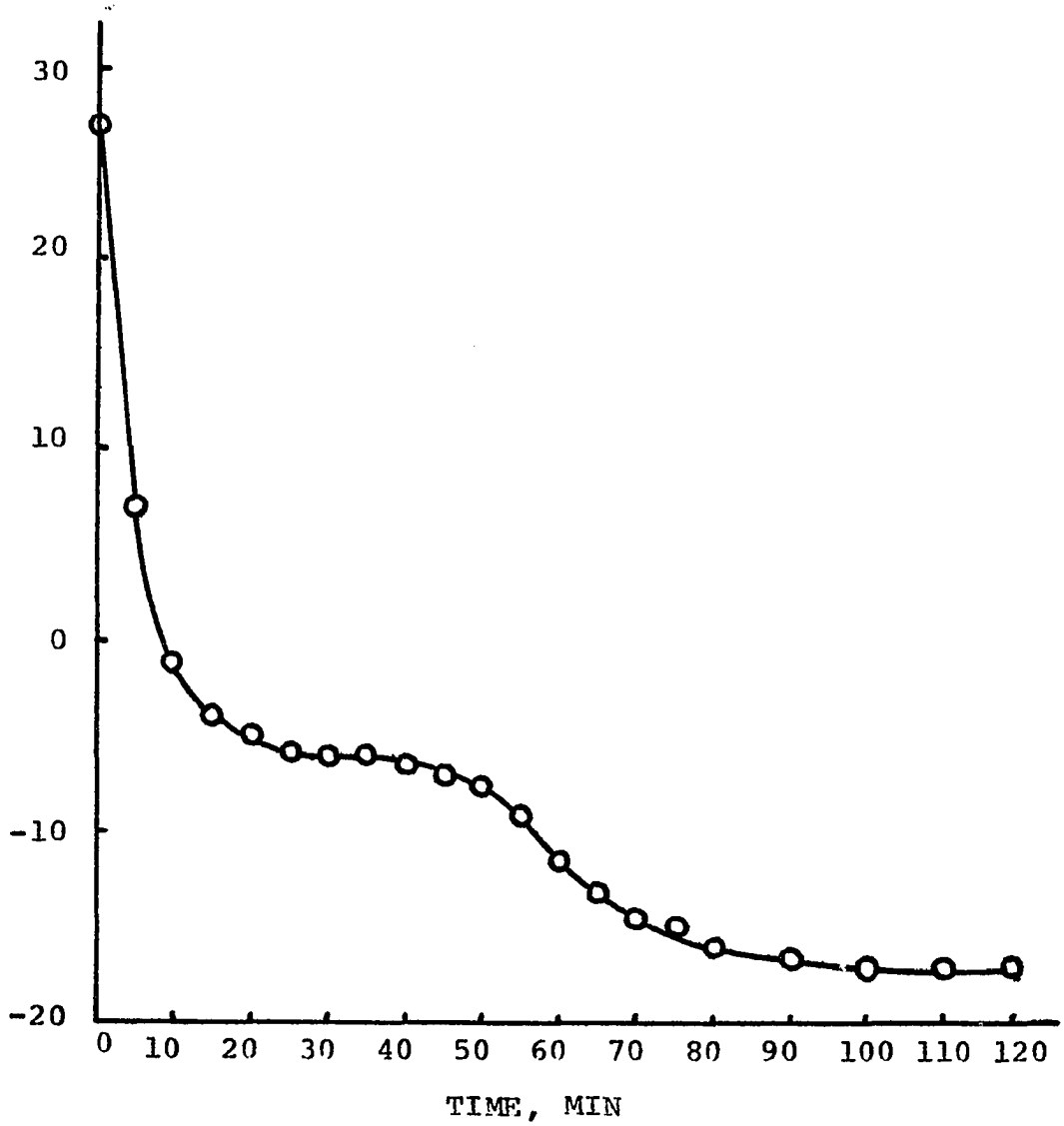

30
20
10
0
-10
-20
0 10 20 30 40 50 60 70 80 90 100 110 120
TIME, MIN

Figure 2. Cooling rate obtained in a 20 x 125 mm screw-cap test tube containing 3 ml culture, placed at -196 C

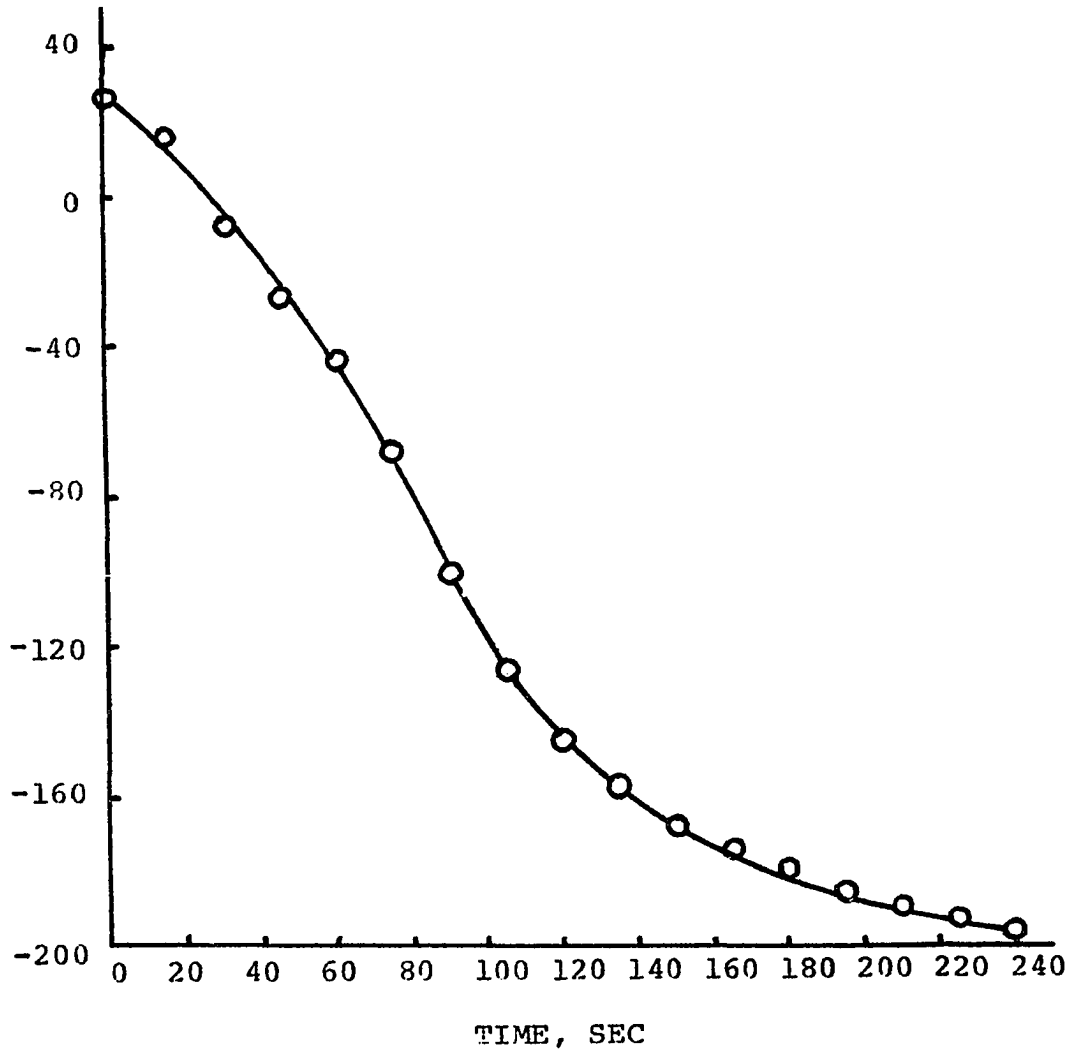

40
0
-40
-80
-120
-160
-200
0 20 40 60 80 100 120 140 160 180 200 220 240
TIME, SEC

Table 2. Maximum and average freezing rates of 3.0 ml culture in 20 x 125 mm screw-cap test tube

Freezing method	Maximum rate	Average rate	Range over which average computed
3 ml/tube at -20 C	5.0 C/min	0.17 C/min	0 to -17 C
3 ml/tube at -196 C	144.0 C/min	58.8 C/min	0 to -196 C

producing activity at 25 to 28 hr (Figure 7). Cultures grown at 21 C showed a maximum total count at about 14 hr (Figure 8), whereas the maximum acid-producing activity occurred at approximately 20 hr (Figure 9). Culture activity was nearly equal for cultures grown at either temperature.

The effect of freezing and storage at -196 C upon maintenance of culture activity of different aged cultures is presented in Figures 10 and 11. As shown in Figure 10 for the multiple-type, mixed-strain cultures, activity was well maintained for all aged cultures after storage at -196 C for 1 month with loss of approximately 10% of initial activity. After 18 to 24 hr, cultures possessed less activity than at successive sampling intervals. This was true before and after frozen storage.

Maintenance of activity of different aged single-strain cultures after frozen storage is shown in Figure 11. Retention of activity after frozen storage was comparable to the results shown in Figure 10. In general, activity of 18- to

Table 3. Effect of 4-week storage at -196 C upon total counts and activities of cultures

Culture	Before storage			After storage		
	Total count/ml x 10^7	Activity[a]	[Activity/cell] x 10^{-8}	Total count/ml x 10^7	Activity	[Activity/cell] x 10^{-8}
A	53	55.2	10.5	36	53.3	14.9
B	44	54.9	10.4	34	53.3	15.5
C	46	53.5	11.6	35	53.6	15.2
D	47	50.5	10.8	32	53.7	16.6
E	98	53.4	5.5	77	50.4	6.6
F	110	45.5	4.1	100	45.3	4.5

[a]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Figure 3. Effect of frozen storage at -196 C upon total counts of six commercial cultures

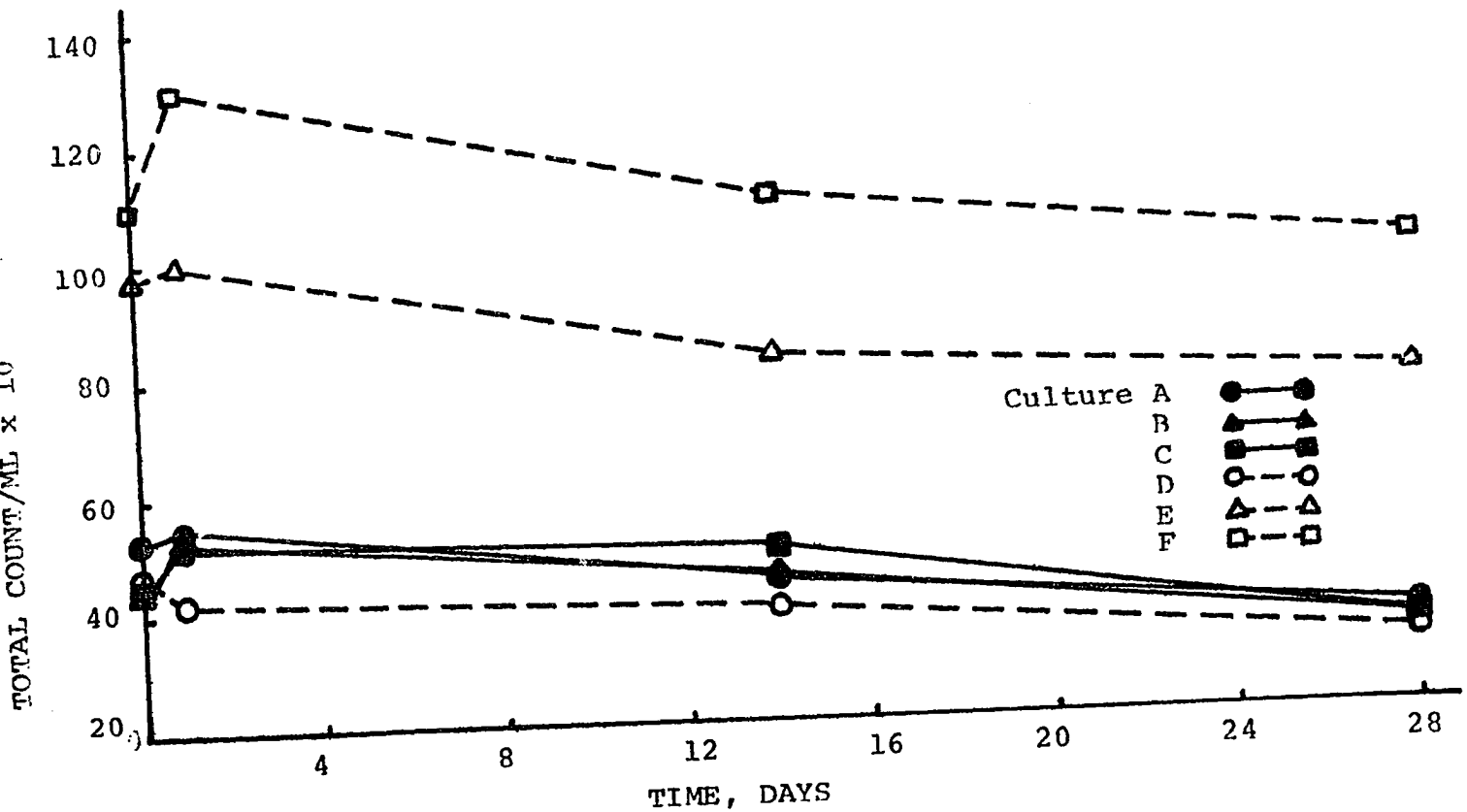

140
120
100
80
60
40
20
TOTAL COUNT/ML x 10^7
4
8
12
16
20
24
28
TIME, DAYS
Culture A
B
C
D
E
F

Figure 4. Effect of frozen storage at -196 C upon culture activity of six commercial cultures

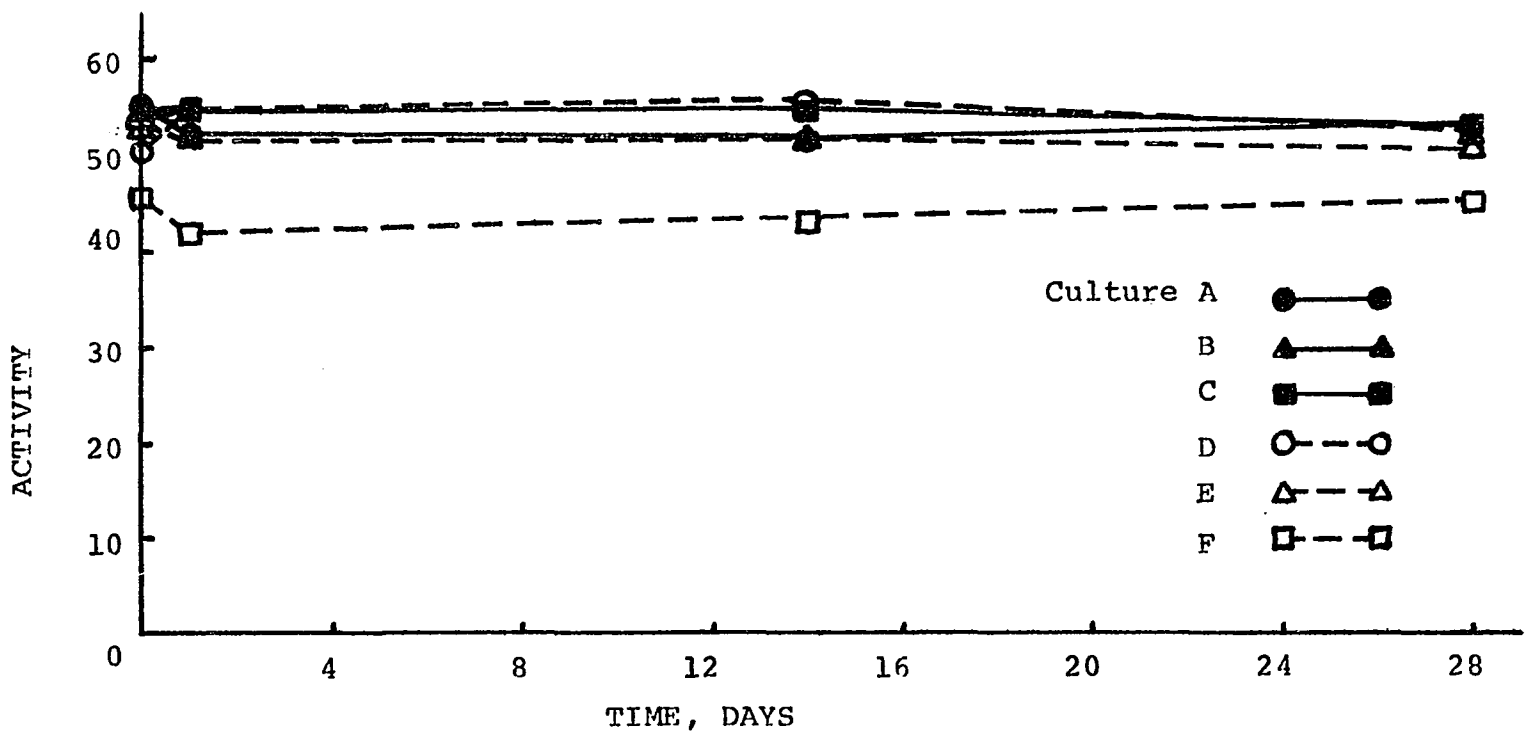

60
50
40
30
20
10
0
ACTIVITY
4
8
12
16
20
24
28
TIME, DAYS
Culture A
B
C
D
E
F

Figure 5. Effect of length of storage at -196 C upon culture activity of six commercial cultures calculated on a per cell basis

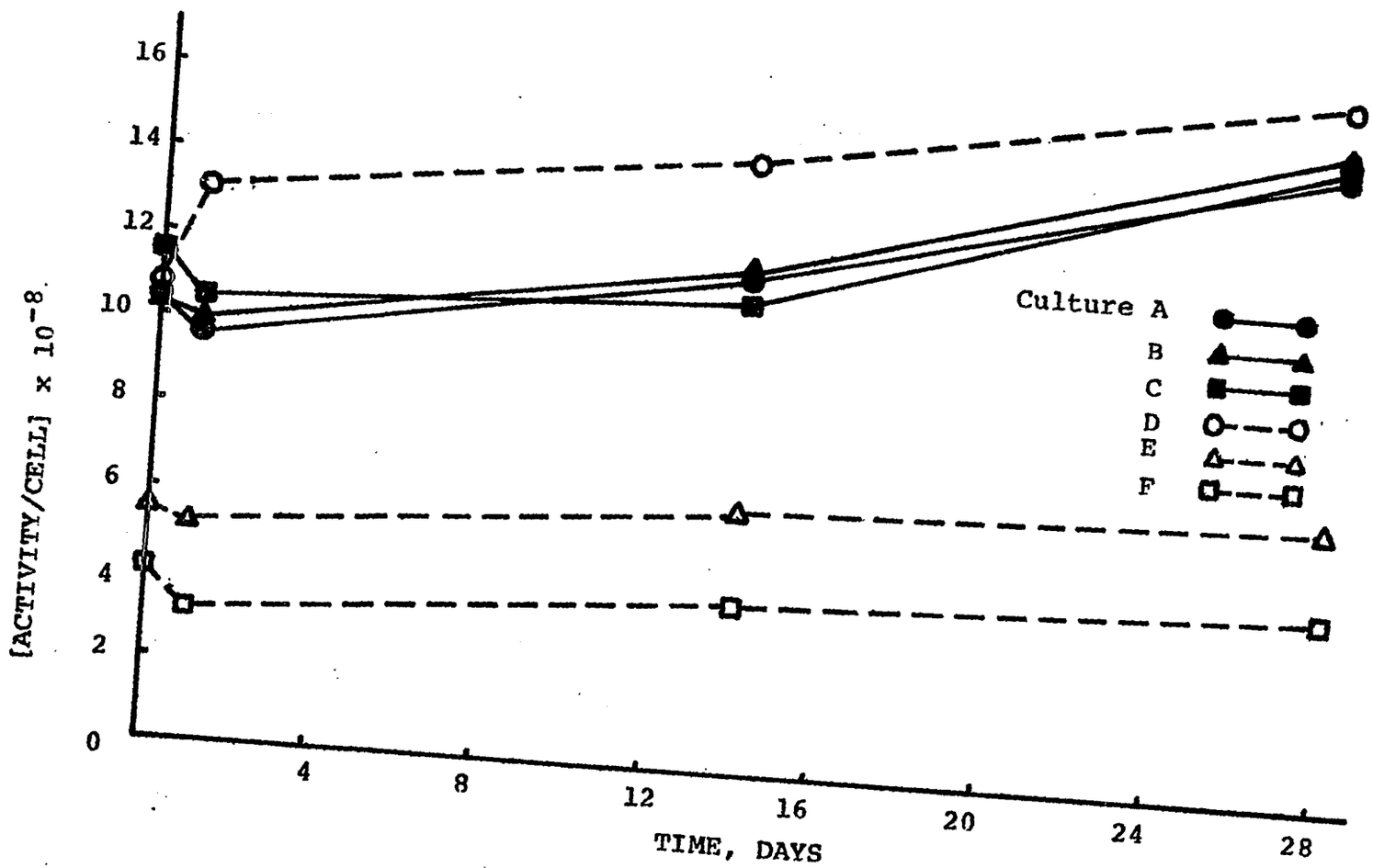

16
14
12
10
8
6
4
2
0
[ACTIVITY/CELL] x 10^{-8}
4
8
12
16
20
24
28
TIME, DAYS
Culture A
B
C
D
E
F

Table 4. Activities and total counts of cultures after various periods of incubation in skimmilk

Age of culture, hr	Incubation temperature	Total count/ml x 10^7			Activity[a]		
		Culture	Individual value	Avg.	Individual value		Avg.
0	15	A	0.58				
		B	0.35	0.43			
		C	0.37				
		D	0.40				
	21	A	0.58				
		B	0.35	0.43			
		C	0.37				
		D	0.40				
8	15	A	2.8				
		B	2.4	2.7			
		C	2.9				
		D	2.5				
	21	A	6.0				
		B	5.5	5.8			
		C	6.0				
		D	5.6				
12	21	A	25		51.0		
		B	19	28	46.0		48.5
		C	24		48.0		
		D	44		49.2		
14	15	A	7.0				
		B	6.0	5.9			
		C	4.9				
		D	5.8				
	21	A	37		51.0		
		B	28	33	52.0		51.9
		C	20		51.2		
		D	48		53.2		
16	21	A	20		55.2		
		B	24	24	54.2		54.7
		C	24		53.2		
		D	28		56.2		
18	15	A	96		44.2		
		B	130	120	41.0		37.2
		C	120		31.5		
		D	140		32.0		

[a]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Table 4 (Continued)

Age of culture, hr	Incubation temperature	Total count/ml x 10^7			Activity[a]	
		Culture	Individual value	Avg.	Individual value	Avg.
	21	A	20		56.8	
		B	23	21	57.0	57.1
		C	17		57.5	
		D	25		57.0	
20	15	A	180		53.5	
		B	120	130	51.2	45.2
		C	72		38.2	
		D	150		38.0	
	21	A	17		57.0	
		B	22	20	58.2	57.9
		C	17		59.2	
		D	23		57.2	
22	15	A	33		57.2	
		B	21	30	52.5	51.8
		C	36		48.5	
		D	31		48.8	
24	15	A	27		57.5	
		B	37	31	52.5	54.8
		C	23		55.8	
		D	38		53.2	
	21	A	19		54.2	
		B	20	18	56.0	55.7
		C	15		58.2	
		D	18		54.5	
28	15	A	28		57.8	
		B	28	30	55.8	56.2
		C	28		57.2	
		D	36		54.0	
32	15	A	46		59.5	
		B	42	43	51.2	52.6
		C	38		47.0	
		D	46		52.5	

Figure 6. Effect of incubation time upon total counts of four commercial cultures grown at 15 C

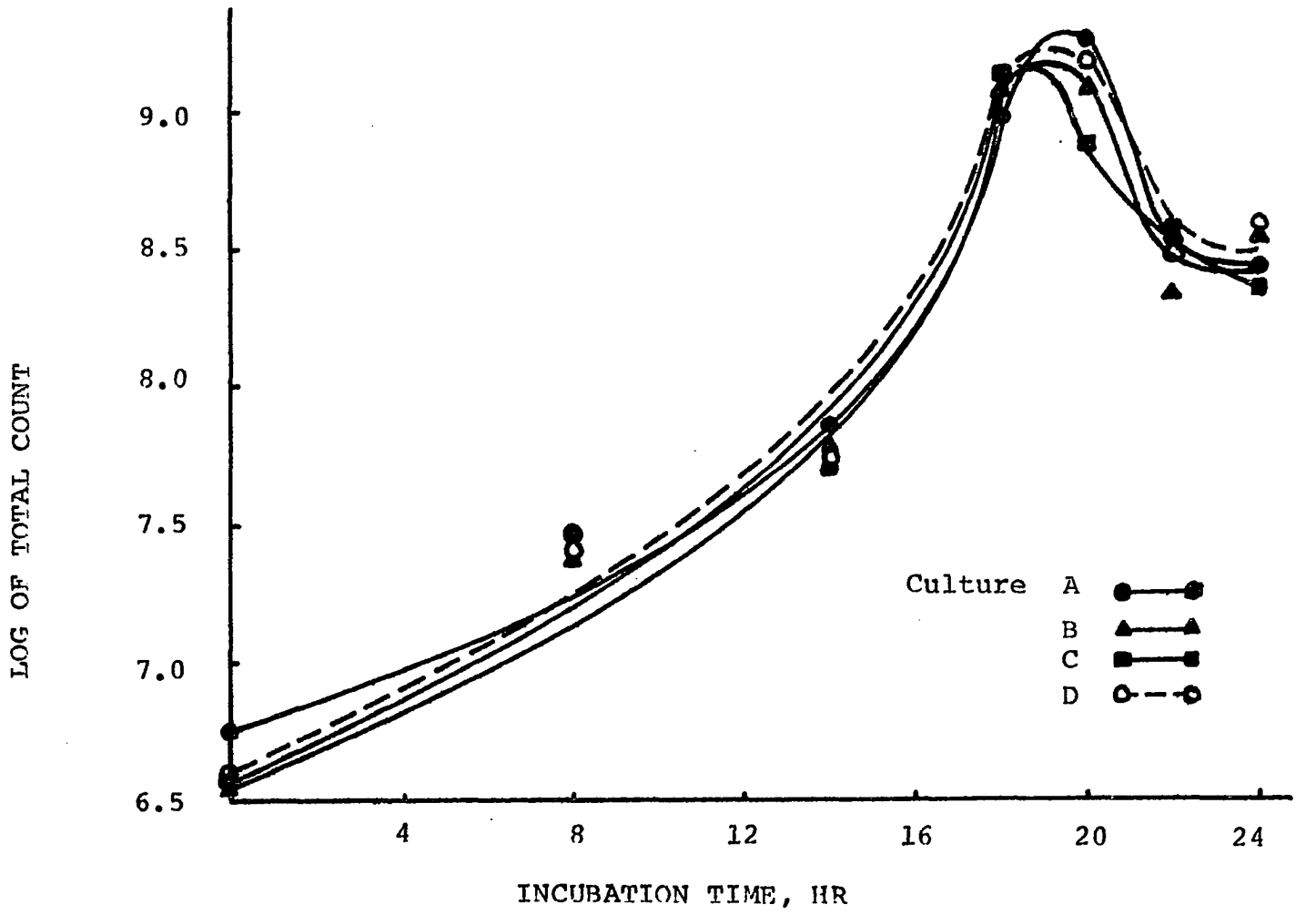

9.0
8.5
8.0
7.5
7.0
6.5
LOG OF TOTAL COUNT
4
8
12
16
20
24
INCUBATION TIME, HR
Culture
A
B
C
D

Figure 7. Effect of incubation time upon the activities of four commercial cultures grown at 15 C

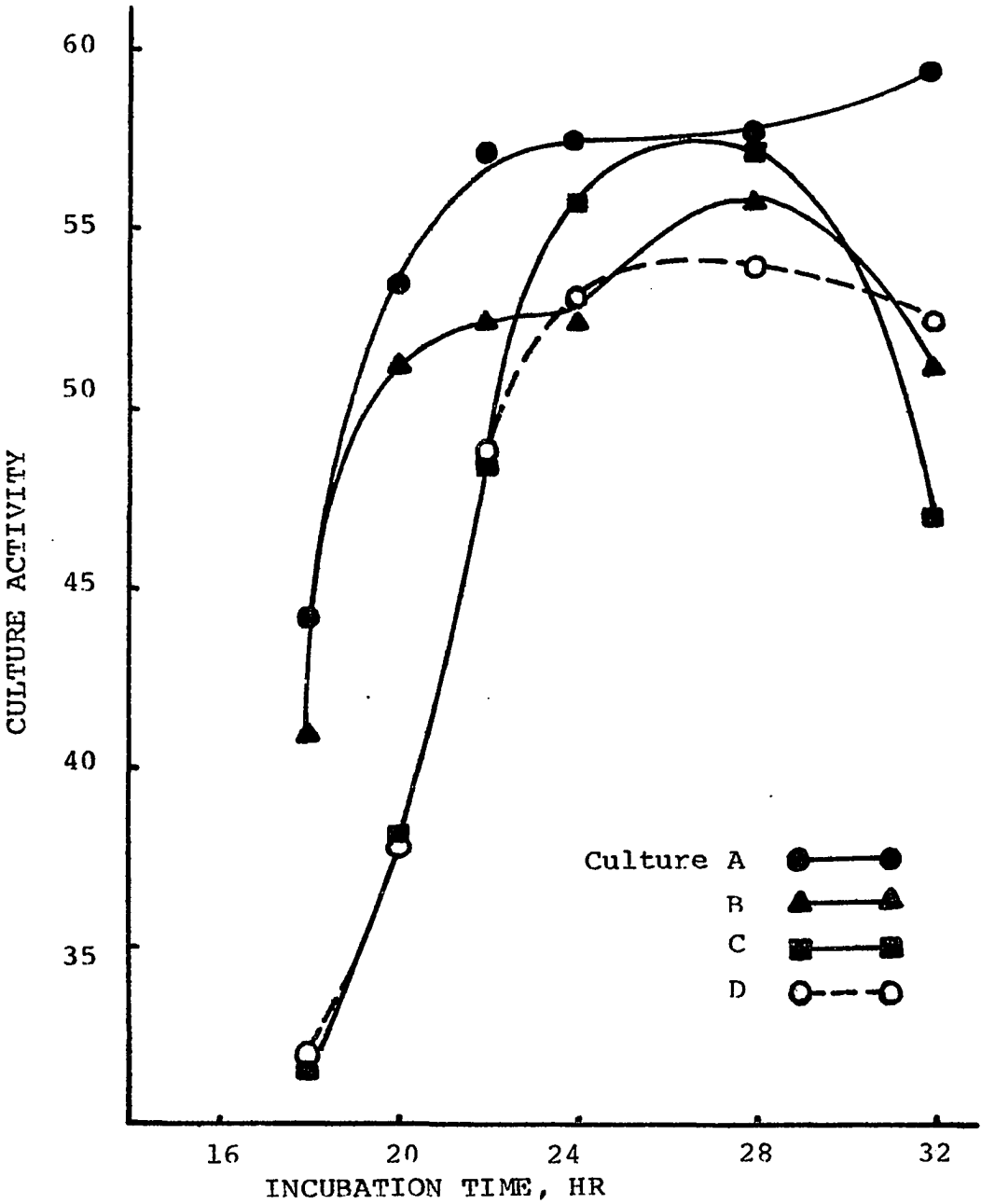

60
55
50
45
40
35
CULTURE ACTIVITY
16
20
24
28
32
INCUBATION TIME, HR
Culture A
B
C
D

Figure 8. Effect of incubation time upon total counts of four commercial cultures grown at 21 C

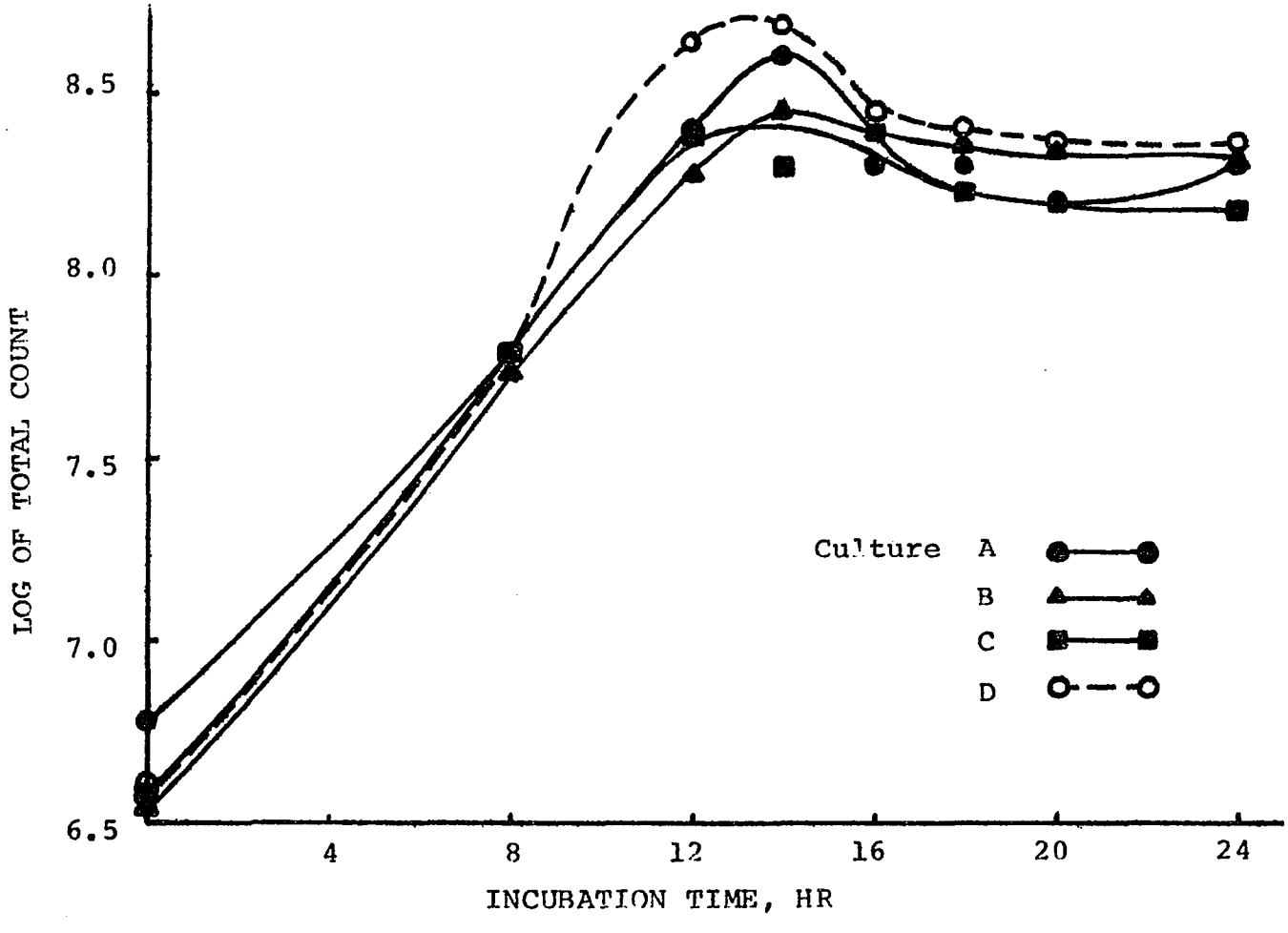

8.5
8.0
7.5
7.0
6.5
LOG OF TOTAL COUNT
4
8
12
16
20
24
INCUBATION TIME, HR
Culture A
B
C
D

Figure 9. Effect of incubation time upon the activities of four commercial cultures grown at 21 C

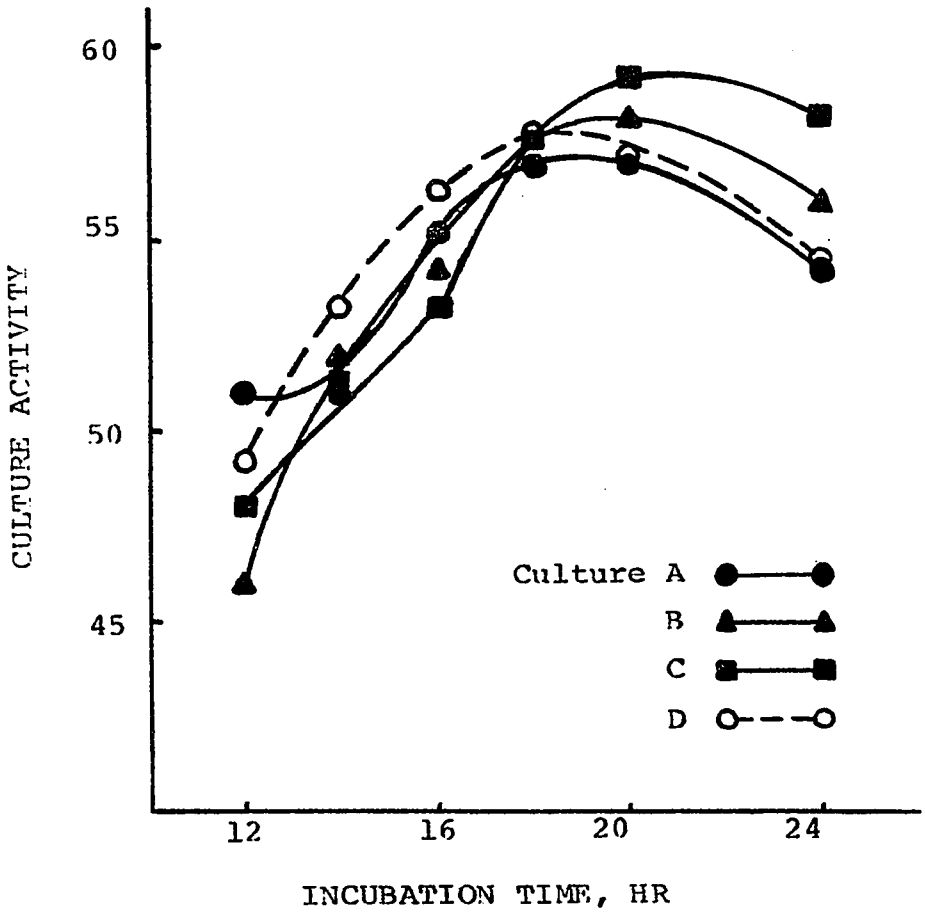

60
55
50
45
CULTURE ACTIVITY
12
16
20
24
INCUBATION TIME, HR
Culture A
B
C
D

Figure 10. Maintenance of culture activity of different aged commercial cultures after storage in liquid nitrogen for 1 month

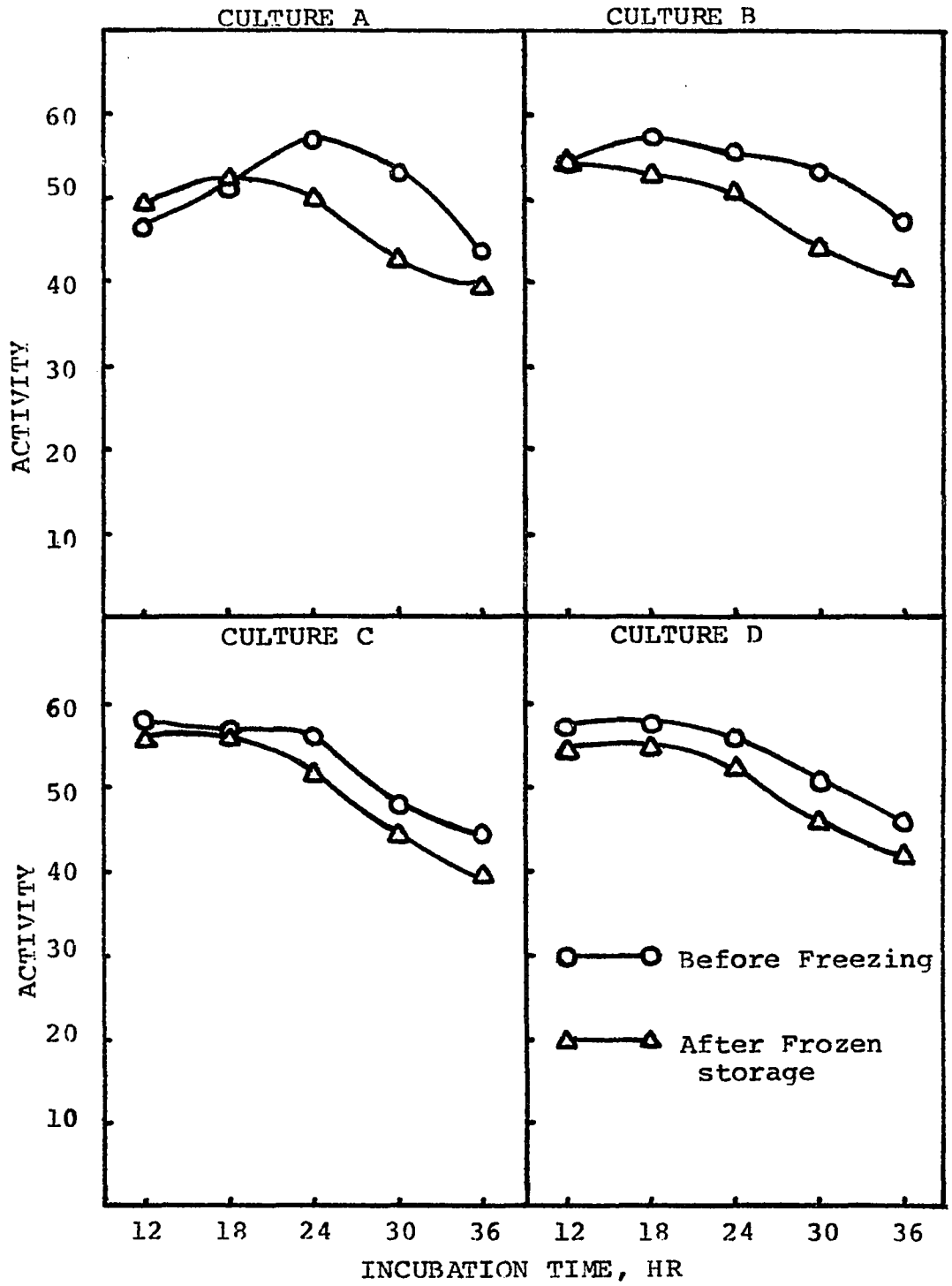
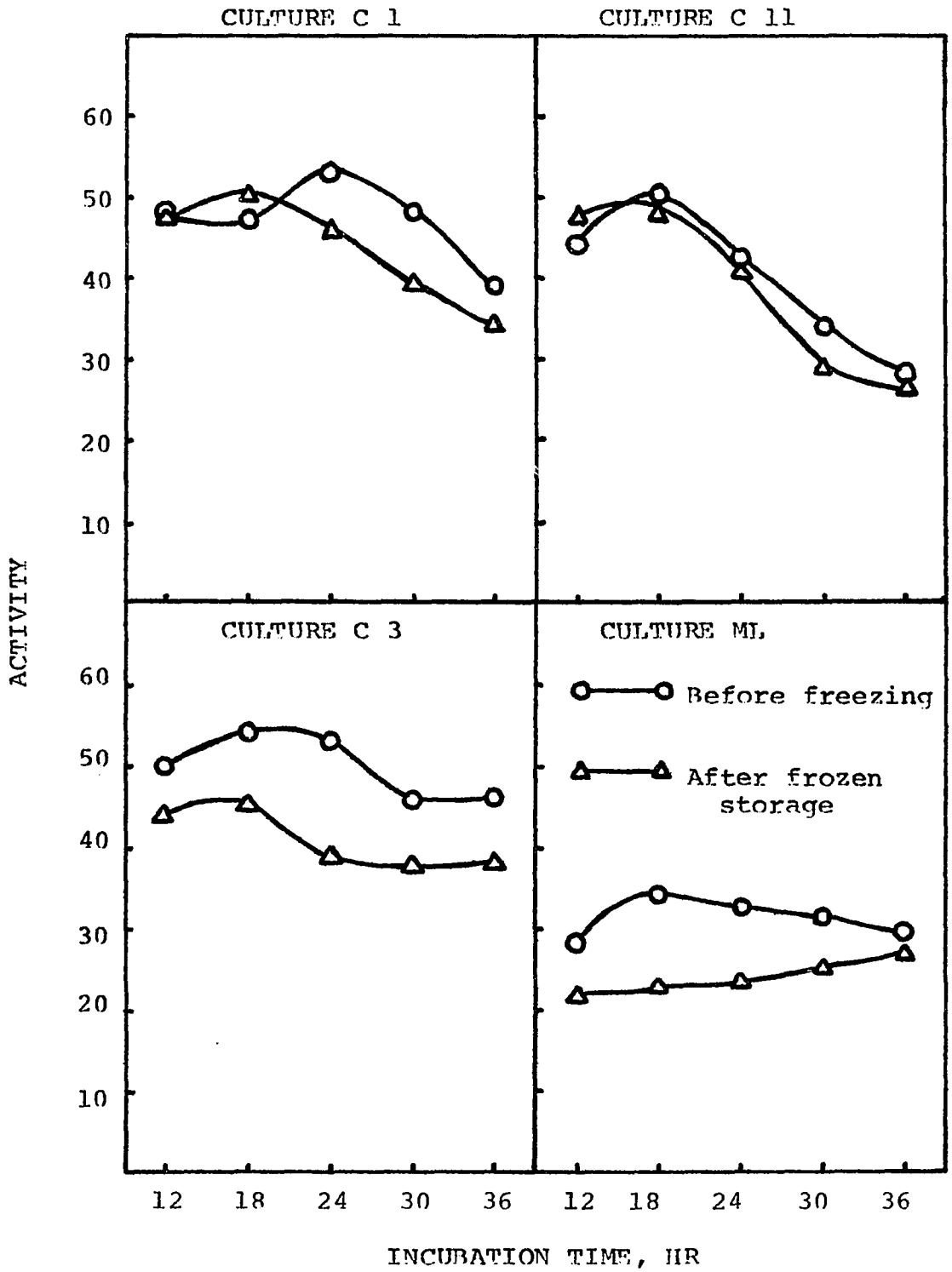

CULTURE A
CULTURE B
CULTURE C
CULTURE D
ACTIVITY
60
50
40
30
20
10
12
18
24
30
36
INCUBATION TIME, HR
Before Freezing
After Frozen storage

Figure 11. Maintenance of culture activity of different aged single-strain cultures after storage in liquid nitrogen for 1 month


CULTURE C 1
CULTURE C 11
CULTURE C 3
CULTURE ML
Before freezing
After frozen storage
ACTIVITY
60
50
40
30
20
10
12
18
24
30
36
INCUBATION TIME, HR

24-hr cells was greater than older cells before or after frozen storage.

Maintenance of culture activity after frozen storage expressed on a per-cell basis is presented in Figures 12 and 13. Activity/cell values for commercial and single-strain cultures at different ages plotted in Figures 12 and 13 exhibited greater variation than the corresponding activity values for the single-strain and commercial mixtures shown in Figures 10 and 11. For the commercial cultures (Figure 12), maintenance of activity after storage on a per-cell basis was poorer than that found for culture activity (Figure 10). In general, cells of younger commercial cultures possessed greater activity/cell before and after frozen storage than the older cells.

Retention of activity/cell of single-strain cultures after frozen storage (Figure 13) was greater than, and showed less variability than the commercial cultures. Except for Culture C 11, activity/cell remained at the same level, regardless of age. Activity/cell values for single-strain cultures were considerably less than for commercial cultures, as the total counts of the latter were lower. Further studies of the same commercial cultures showed high maintenance of culture activity and viability after storage at -196 C for 4 months.

Proteinase activity of different aged cultures before

Figure 12. Maintenance of activity/cell of different aged commercial cultures after storage in liquid nitrogen for 1 month

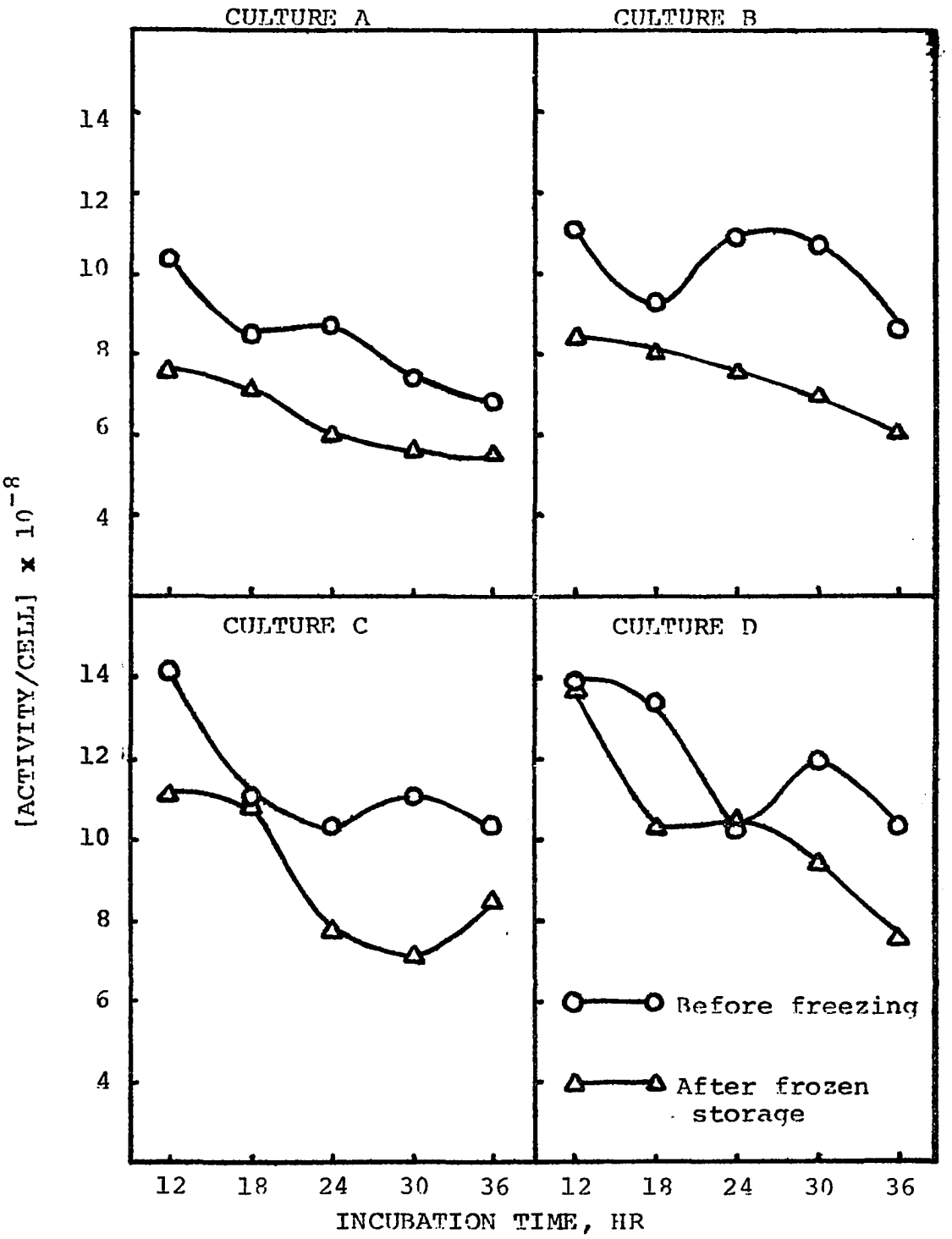
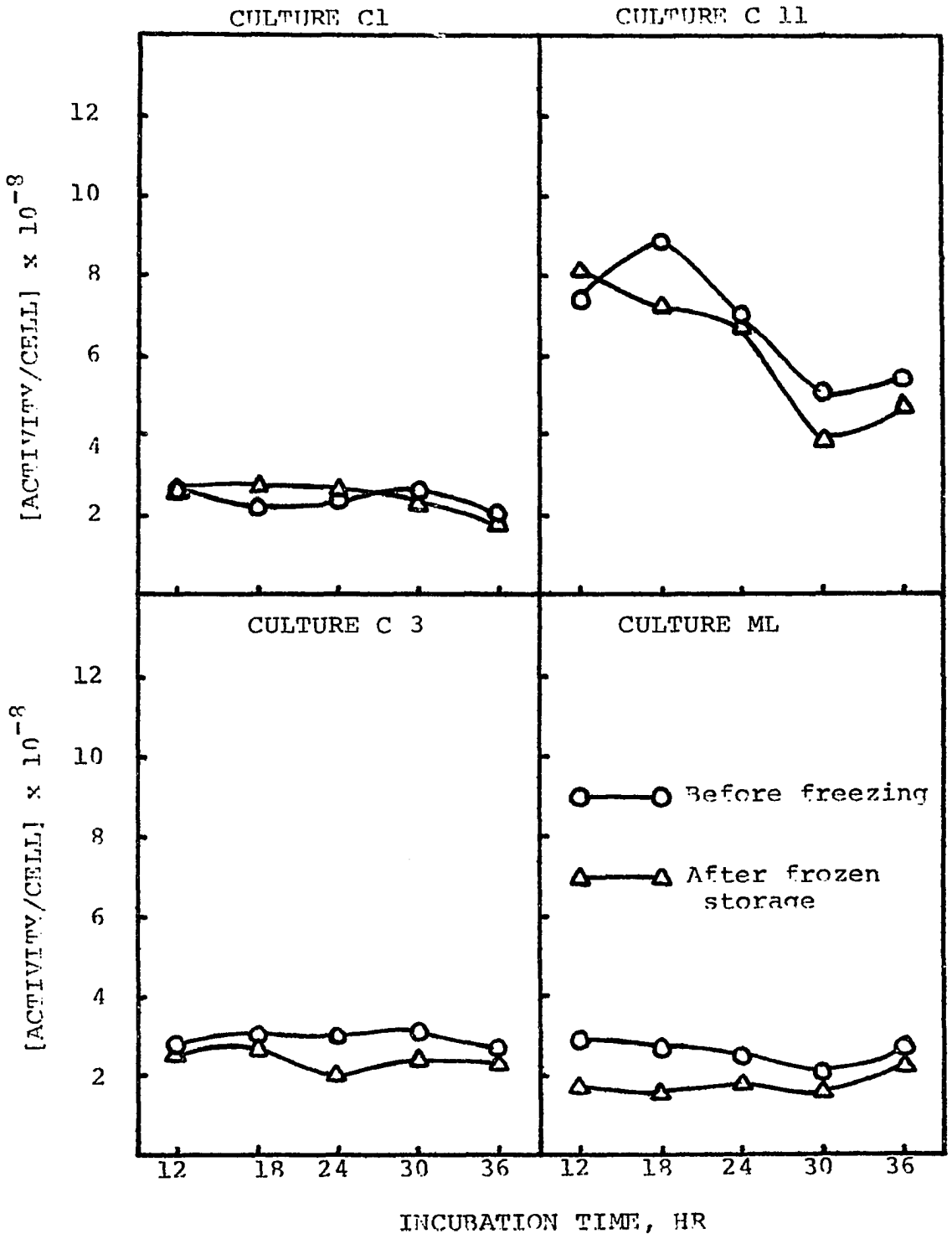

CULTURE A
CULTURE B
CULTURE C
CULTURE D
14
12
10
8
6
4
[ACTIVITY/CELL] x 10⁻⁸
12
18
24
30
36
INCUBATION TIME, HR
Before freezing
After frozen storage

Figure 13. Maintenance of activity/cell of different aged single-strain cultures after storage in liquid nitrogen for 1 month


CULTURE C1
CULTURE C 11
CULTURE C 3
CULTURE ML
[ACTIVITY/CELL] x 10^{-8}
12
10
8
6
4
2
12
18
24
30
36
INCUBATION TIME, HR
Before freezing
After frozen storage

and after frozen storage is shown in Table 5. Nearly all cultures possessed greatest proteinase activity at 12 hr of incubation. Decreases after 12 hr were not uniform. No consistent difference between proteinase activity of commercial and single-strain cultures was found. Fast acid-producing cultures (C 1 and C 11) generally possessed greater proteinase activity than did slow acid-producing cultures (C 3 and ML). For nearly all analyses, proteinase activity was markedly greater for cultures grown at 32 C for 6 hr than when grown at 21 C for 12 hr. With the proteinase test conducted at either 21 or 32 C, maximum retention of proteinase activity was found in 18- and 24-hr cultures.

Cultures Grown in Whey Medium

Effect of incubation time upon activity and total count of cultures grown in whey at 21 C is presented in Table 6. Total counts varied little between 12 and 20 hr of incubation; culture activity was slightly more variable, reaching a maximum at approximately 16 hr. Activity/cell calculations were highest from 16 to 20 hr.

Maintenance of culture activity upon transfer from skimmilk to whey and back to skimmilk medium is shown in Table 7. Culture activity or activity/cell was not diminished in the final skimmilk medium when transferred from whey. In several cases, culture activity in whey was less than when

Table 5. Maintenance of proteinase activity[a] of different aged cultures after frozen storage in liquid nitrogen for 1 month

Incubation time, hr	Culture	Before freezing		After frozen storage	
		21 C	32 C	21 C	32 C
12	A	3.37	5.20	2.88	4.86
	B	3.12	6.53	2.56	4.99
	C	4.00	7.92	3.12	5.68
	D	3.68	7.12	3.44	6.14
	C 1	6.34	7.12	5.71	6.43
	C 11	5.63	4.67	4.93	4.32
	C 3	4.32	4.50	3.92	3.97
	ML	1.73	3.84	1.36	3.60
18	A	3.12	5.44	2.21	4.27
	B	1.57	4.24	2.01	4.40
	C	2.16	5.06	2.50	5.44
	D	1.73	5.06	2.43	5.28
	C 1	4.67	5.12	4.40	5.52
	C 11	2.08	3.76	2.24	4.27
	C 3	1.86	3.68	2.08	4.00
	ML	2.17	3.12	2.08	3.20
24	A	3.33	4.86	2.30	4.58
	B	2.64	4.90	2.21	4.70
	C	2.21	4.93	2.80	5.28
	D	2.76	5.36	2.50	5.60
	C 1	3.12	6.59	3.01	6.66
	C 11	2.43	3.01	2.64	3.68
	C 3	2.64	3.52	2.80	3.52
	ML	1.79	2.21	2.43	3.04
30	A	1.52	4.74	2.16	4.58
	B	1.79	4.54	2.05	4.61
	C	2.50	6.34	2.50	5.36
	D	2.43	6.53	2.08	5.36

[a]Proteolysis in mg tyrosine per 100 g culture.

Table 5 (Continued)

Incubation time, hr	Culture	Before freezing 21 C	Before freezing 32 C	After frozen storage 21 C	After frozen storage 32 C
	C 1	4.48	5.52	3.76	6.14
	C 11	3.12	3.36	2.59	2.96
	C 3	2.72	3.20	2.16	3.28
	ML	2.72	3.60	2.30	2.41
36	A	2.30	5.20	2.05	4.61
	B	2.24	5.44	2.24	4.58
	C	2.96	6.11	2.69	4.70
	D	3.36	6.21	2.59	4.80
	C 1	4.00	6.40	3.68	5.60
	C 11	2.24	3.36	2.13	2.88
	C 3	2.96	4.80	2.56	4.00
	ML	2.56	3.76	2.16	3.12

Table 6. Activities and total counts of cultures after various periods of incubation in whey at 21 C

Age of culture, hr	Total count/ml x 10^7 Culture	Individual Value	Avg	Culture activity[a] Individual value	Avg	Average [activity/cell] x 10^{-8}
12	A	29		47.0		
	B	43	25	52.0	50.9	26.8
	C	11		53.3		
	D	17		51.5		
14	A	34		52.3		
	B	44	27	56.0	54.5	26.2
	C	13		56.5		
	D	16		53.3		
16	A	44		50.0		
	B	57	30	57.0	55.1	33.4
	C	11		56.5		
	D	10		56.8		

[a]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Table 6 (Continued)

Age of culture, hr	Total count/ml x 10^7 Culture	Individual value	Avg	Culture activity[a] Individual value	Avg	Average [activity/cell] x 10^{-8}
18	A	49	28	50.0	54.0	32.8
	B	41		55.8		
	C	11		55.3		
	D	10		55.0		
20	A	40	25	50.8	53.5	33.8
	B	40		55.3		
	C	10		54.5		
	D	10		53.5		

Table 7. Maintenance of culture activity upon transfer from skimmilk to whey and back to skimmilk

Culture[a]	Growth in skimmilk		Growth in whey		Growth in skimmilk	
	Culture activity[b]	[Activity/cell] x 10^{-8}	Culture activity	[Activity/cell] x 10^{-8}	Culture activity	[Activity/cell] x 10^{-8}
C 1	57.6	3.4	56.8	6.2	58.8	3.1
C 3	17.8	1.6	12.8	1.5	19.8	1.5
C 7	43.4	4.5	40.9	5.2	48.5	5.9
C 11	66.4	8.8	46.5	16.6	56.3	9.5
C 13	35.8	7.6	32.3	6.2	38.5	9.2
EB2	47.3	3.3	37.4	3.9	44.0	2.9
EB4	21.0	1.8	16.5	1.9	26.9	2.2
E8	49.2	2.6	44.5	5.0	47.8	2.7
HP	27.5	1.5	21.6	2.3	29.5	1.6
ML	16.3	1.5	12.3	1.6	19.5	1.5

[a]Cultures used in this study were all S. cremoris.

[b]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

grown in the initial skimmilk, but upon further transfer from whey to skimmilk again, final activity was comparable to initial values.

Effect of Frozen Storage upon Activity, Viability, and Proteolysis of Cultures

The effects of freezing and storage at -196 C upon culture activity, total count, activity/cell, and proteolysis of single-strain cultures are presented in Tables 8 and 9. Total count, activity, and activity/cell were maintained throughout the 6-month storage period. Maximum culture activity or activity/cell of stored cultures generally occurred after 1 month of frozen storage. Maintenance of activity during frozen storage was quite satisfactory for both the fast and slow acid-producing cultures. No distinct advantage was seen in growing cultures in one or the other medium.

Microscopic observations of cultures just prior to freezing and immediately after thawing showed no appreciable breakage of clumps due to freezing. However, for comparative purposes, calculations for activity/cell after frozen storage also were made on the basis of total counts before freezing. These values are shown in parentheses in Table 9.

Proteolytic activity was at a maximum before freezing and showed gradual decreases with frozen storage, especially after the first month. To determine whether the impairment

Table 8. Total counts and activities of milk and whey grown, fast(f) and slow(s), single-strain cultures after 1, 3, and 6 months frozen storage at -196 C

Medium	Culture	Total count[a]	Initial activity[b]	Storage period at -196 C					
				1 month		3 months		6 months	
				Total count	Culture activity	Total count	Culture activity	Total count	Culture activity
Whey	C 1-f	150	54.8	120	56.3	130	53.3	150	51.2
	C 11-f	16	41.8	17	52.9	28	51.5	19	51.3
	C 3-s	97	15.0	110	21.0	120	18.8	130	18.5
	ML-s	110	16.3	88	21.8	85	19.6	95	18.5
Milk	C 1-f	210	54.5	180	56.8	160	55.6	190	48.8
	C 11-f	28	54.0	25	54.5	32	51.2	32	49.5
	C 3-s	110	19.8	120	23.0	120	21.5	130	24.3
	ML-s	120	20.8	130	22.0	120	19.9	110	22.0

[a]Total count/ml x 10^7.

[b]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Table 9. Activity per cell and proteolysis of milk and whey grown, fast(f) and slow(s), single-strain cultures before freezing and after 1, 3, and 6 months at -196 C

Medium	Culture	[Activity per cell] x 10^{-8}				Proteolysis[a]			
		Before freezing	1-month storage	3-months storage	6-months storage	Before freezing	1-month storage	3-months storage	6-months storage
Whey	C 1-f	3.7	4.7 (3.8)[b]	4.1 (3.6)	3.4 (3.4)	9.12	8.48	6.78	6.08
	C 11-f	26.1	31.1 (33.1)	18.4 (32.2)	27.0 (32.0)	7.68	6.72	3.84	3.26
	C 3-s	1.6	1.9 (2.2)	1.6 (1.9)	1.4 (1.9)	7.52	7.20	2.82	2.34
	ML-s	1.5	2.5 (2.2)	2.3 (1.8)	2.0 (1.7)	6.72	6.62	2.62	2.72
Milk	C 1-f	2.6	3.2 (2.7)	3.5 (2.7)	2.6 (2.3)	8.80	8.32	7.30	5.76
	C 11-f	19.3	21.8 (19.5)	16.0 (18.3)	15.5 (17.7)	7.52	6.98	4.00	4.00
	C 3-s	1.8	1.9 (2.1)	1.8 (2.0)	1.9 (2.2)	7.04	6.88	2.94	2.88
	ML-s	1.7	1.7 (1.8)	1.7 (1.7)	2.0 (1.8)	6.88	6.88	2.40	2.72

[a]Proteolysis in mg tyrosine per 100 g culture.

[b]Figures in parentheses calculated on basis of initial total counts before freezing.

in proteolytic ability was temporary, the cultures after thawing were transferred into skimmilk and after 16 hr of incubation, proteolytic determinations again were made (Table 10). In some cases after the 16-hr transfer, the proteolytic activities of the cultures had risen considerably from the time of thawing, indicating that the diminished proteolytic activity was restored upon further transfer.

The effect of subculture upon activity and activity/cell of cultures after frozen storage is shown in Table 11. In nearly all cases, activity values were slightly increased after the subculture. Activity/cell values were, however, more variable than activity values. This probably is because of greater variability in total counts.

Daily Comparison of Unfrozen Culture to Frozen Culture after Extended Storage at -196 C

Maintenance of culture viability and activity of four single-strain cultures after frozen storage at -196 C for 6 months is presented in Table 12. Cultures were transferred daily with total-count and activity determinations after each period of incubation. As shown in the table, total-count values remained consistent after frozen storage and after subculturing daily for 2 weeks. Culture activity was maintained at comparable levels, except for Culture C 3,

Table 10. Proteolysis of milk and whey grown, fast(f) and slow(s), single-strain cultures after 1, 3, and 6 months at -196 C[a]

Medium	Culture	Proteolysis[b]			
		Before freezing	1 month storage	3 months storage	6 months storage
Whey	C 1-f	9.12	8.74	8.35	6.30
	C 11-f	7.68	7.04	4.99	3.94
	C 3-s	7.52	7.52	3.58	4.32
	ML-s	6.72	6.88	3.48	3.58
Milk	C 1-f	8.80	8.80	7.48	5.82
	C 11-f	7.52	6.88	5.12	4.32
	C 3-s	7.04	7.78	4.32	4.70
	ML-s	6.88	6.88	3.78	4.32

[a]Determinations made after one subculture following the respective storage treatments.

[b]Proteolysis in mg. tyrosine per 100 g culture, determined after 6 hr. at 32 C.

Table 11. Effect of 16-hr subculture upon recovery values of cultures after 1, 3, and 6 months at -196 C

Medium	Culture	Initial activity[a]	Subculture after storage for 1 month		Subculture after storage for 3 months		Subculture after storage for 6 months	
			Culture activity	[Activity/cell] x 10^{-8}	Culture activity	[Activity/cell] x 10^{-8}	Culture activity	[Activity/cell] x 10^{-8}
Whey	C 1-f	54.8	57.8	10.9	57.9	3.1	52.5	2.3
	C 11-f	41.8	53.8	17.3	57.0	13.9	52.4	12.2
	C 3-s	15.0	20.5	1.9	26.5	2.2	24.4	1.9
	ML-s	16.3	21.0	3.0	23.5	2.2	29.8	2.1
Milk	C 1-f	54.5	56.8	3.3	56.5	3.1	48.8	2.6
	C 11-f	54.0	55.3	13.8	56.8	11.1	52.3	10.5
	C 3-s	19.8	19.4	1.5	23.8	1.8	33.3	2.8
	ML-s	20.8	21.8	2.3	23.7	2.2	29.0	2.1

[a]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Table 12. Culture viability and activity before and after storage at -196 C for 6 months and after daily subculture for 2 weeks

Culture	Before storage		After storage		After subculture	
	Total count x 10^7	Culture activity[a]	Total count x 10^7	Culture activity	Total count x 10^7	Culture activity
C 1	210	54.5	160	52.8	190	53.0
C 11	28	54.0	33	51.4	46	51.0
C 3	110	19.8	110	17.8	130	37.1
ML	120	20.8	120	18.0	120	23.7

[a]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

which displayed a remarkable increase in activity during the period of daily transfer.

When the results of Table 12 were compared to those of the same four cultures that had not been frozen (Table 13), no marked differences were found, except for unfrozen Culture ML, which increased considerably in activity during subculturing. Culture C 3 that had not been frozen increased in activity as did the frozen culture. Therefore, any increase of culture activity was not attributed to the freezing treatment.

Table 13. Culture viability and activity before and after daily subculture of unfrozen cultures for 2 weeks

Culture	Before subculture		After subculture	
	Total count x 10^7	Culture activity[a]	Total count x 10^7	Culture activity
C 1	200	55.9	210	55.0
C 11	52	60.6	55	52.5
C 3	100	23.7	110	36.0
ML	120	23.2	120	31.8

[a]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Effect of Frozen Storage upon Cellular Integrity of Cultures

The recovery of "injured" and "normal" cells after frozen storage for 1 month at -20 and -196 C is presented in Table 14.

Damaged or injured cells and killed cells were defined as follows: (i) injured cells were those which grew on the maximal agar (Eugonagar) but not on the minimal agar after freezing, and (ii) dead cells were those which failed to grow on the maximal agar after frozen storage (111).

As shown in the table, injury and death of cells by slow-freezing were more severe than fast-freezing and subsequent storage at -20 C. Death and injury of cells stored at -196 C were in all cases less severe than cells stored at -20 C. With storage at -20 C, slow acid-producing cells appeared equally susceptible to freezing death and injury as fast acid producers.

With storage at -196 C, it appears that fast acid-producing cells were more resistant to freezing death than were slow acid-producing cells These results indicate that all single-strain cultures may not be equally susceptible to freezing injury and death.

Table 14. Percent cellular injury and death of single-strain cultures after frozen storage for 1 month at -20 and -196 C

Culture	Minimal medium	Storage at -20 C				Storage at -196 C	
		Slow frozen[a]		Fast frozen[b]			
		Injury	Death	Injury	Death	Injury	Death
		%					
11	B[c]	14	77	0	40	9	17
	C[d]	19	82	8	45	6	21
13	B	4	74	6	37	9	19
14	B	54	62	7	18	24	0
18	B	12	67	26	17	4	0

[a]Slow freezing was conducted by placing culture tubes in freezer at -20 C.

[b]Fast freezing was conducted by immersion of culture tubes in liquid nitrogen for 30 sec and then placing them in the -20 C freezer.

[c]Minimal medium B contained 75% of the protein concentration found in Eugonagar.

[d]Minimal medium C contained 50% of the protein concentration found in Eugonagar.

Selection of Cultures Stimulated by Liquid-Nitrogen Freezing

Twenty-four, single-strain cultures of S. cremoris were examined for "stimulation" by freezing and frozen storage in liquid nitrogen for 1 month. Criteria used to indicate stimulation were activity and activity/cell. The observations are presented in Tables 15 and 16, respectively.

Cultures 7 through 13 were slow acid-producing; the remaining were fast acid-producing strains. In general, culture activity remained at comparative levels after frozen storage. Slight increases or decreases occurred randomly among different cultures and within different trials of the same culture.

The activity/cell calculations presented in Table 16 were calculated from the data for activity values shown in Table 15 and the corresponding total counts. As shown in Table 16, activity/cell values were more variable than were activity values for the same cultures, due to greater variability of total counts. In general, culture activity was maintained after storage in liquid nitrogen for 1 month, whether calculated on the basis of culture activity or activity/cell. Using either method of expressing activity, none of the fast or slow acid-producing varieties studied demonstrated stimulation.

Table 15. Activity tests of 24 single-strain cultures before and after storage at -196 C for 1 month

Culture	Trial 1		Trial 2		Trial 3	
	Initial activity[a]	Activity after 1 mo.	Initial activity	Activity after 1 mo.	Initial activity	Activity after 1 mo.
1	48.1	50.3	46.1	51.5	48.0	46.8
2	46.6	49.2	51.6	52.7	48.7	48.6
3	50.7	50.0	51.2	51.8	50.4	50.8
4	51.5	50.7	51.7	51.8	51.4	52.0
5	50.5	49.8	51.6	53.0	50.7	52.8
6	49.6	49.5	52.7	53.0	49.7	49.4
7	6.4	6.2	6.7	7.5	6.6	6.2
8	6.3	6.2	6.9	7.4	6.6	6.4
9	6.0	5.9	6.8	6.8	6.1	5.9
10	6.7	6.5	6.7	7.3	6.7	6.6
11	5.4	5.4	5.8	6.2	5.9	6.2
12	6.2	6.2	6.6	7.0	6.1	6.6

[a]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Table 15 (Continued)

Culture	Trial 1		Trial 2		Trial 3	
	Initial activity[a]	Activity after 1 mo.	Initial activity	Activity after 1 mo.	Initial activity	Activity after 1 mo.
13	5.2	5.6	5.6	6.3	5.4	5.8
14	49.9	46.4	51.7	52.4	51.2	51.6
15	47.3	42.6	52.4	53.0	56.6	56.5
16	45.5	40.0	42.6	40.5	43.2	51.8
17	49.9	44.1	54.0	54.1	53.7	54.6
18	52.0	47.4	52.4	52.0	52.8	52.9
19	45.5	40.7	37.3	37.9	39.5	38.7
20	44.3	40.3	36.8	37.7	37.9	37.9
21	46.6	41.4	41.0	41.5	43.7	44.1
22	41.6	36.3	36.7	35.2	38.4	38.6
23	40.6	36.9	34.2	34.6	38.3	37.9
24	46.6	42.7	39.2	35.7	42.2	42.9

Table 16. Activity/cell calculations for 24 single-strain cultures before and after storage at -196 C for 1 month

Culture	[Activity/cell] x 10^{-8}					
	Trial 1		Trial 2		Trial 3	
	Initial	After 1 month	Initial	After 1 month	Initial	After 1 month
1	9.1	9.7	10.5	12.9	11.4	10.6
2	4.7	5.6	6.4	6.9	5.4	5.2
3	12.4	11.1	20.5	21.6	11.2	12.1
4	5.9	5.8	12.3	11.8	6.9	6.8
5	7.5	6.1	10.8	10.6	9.1	8.3
6	14.2	12.6	27.7	27.9	19.9	17.0
7	1.7	1.1	1.2	1.2	0.9	1.0
8	1.3	1.1	1.5	1.8	1.4	1.4
9	2.5	1.8	1.7	1.5	1.6	1.4
10	2.2	1.7	1.5	1.5	1.7	1.6
11	1.9	1.3	1.4	1.2	1.4	1.4
12	2.5	1.9	2.4	2.8	2.4	2.3
13	1.9	1.3	1.7	1.9	1.9	2.0
14	15.6	12.5	21.5	18.7	22.2	23.5
15	4.8	4.3	6.3	6.0	6.0	5.8

Table 16 (Continued)

Culture	[Activity/cell] x 10^{-8}					
	Trial 1		Trial 2		Trial 3	
	Initial	After 1 month	Initial	After 1 month	Initial	After 1 month
16	2.9	2.5	3.0	2.4	3.1	3.5
17	4.5	4.0	12.0	9.5	6.3	6.6
18	8.3	7.4	23.8	26.0	12.0	11.0
19	3.3	4.2	2.3	2.4	3.0	2.6
20	3.4	2.9	3.7	3.4	3.5	3.4
21	3.5	3.0	3.4	3.2	3.1	2.8
22	2.8	2.6	2.5	2.7	2.4	2.4
23	2.5	2.2	2.3	2.3	2.6	2.7
24	3.9	3.6	2.0	2.2	3.0	3.1

Effect of Freezing upon Carbohydrate Utilization of Cultures

Fermentation of lactose, glucose, and galactose solutions by fresh cultures and those stored in liquid nitrogen is presented in Table 17. As shown in the table, there was no difference in the acid production from various sugars between the frozen and unfrozen culture; only a slight difference was noted for culture C 1. These data indicate that liquid-nitrogen freezing causes no appreciable change in carbohydrate utilization or specificity. Culture activity tests determined before and after frozen storage showed no stimulation of acid production.

Effect of Frozen Storage upon β-Galactosidase Activity of Culture 7962

β-Galactosidase and culture activity tests before and after storage for 1 month at -196 C are shown in Tables 18 and 19, respectively. β-Galactosidase activity was unaffected by frozen storage at any of the culture ages studied for whole cells.

Culture activity or activity/cell decreased slightly after frozen storage for 16- or 20-hr cells, and increased slightly for 24- or 28-hr cells. Total counts remained nearly constant during frozen storage.

Table 17. Effect of frozen storage upon sugar fermentation by lactic cultures

Culture	Substrate					
	Before freezing			After frozen storage		
	Lactose	Glucose	Galactose	Lactose	Glucose	Galactose
	ml 0.1 N NaOH[a]					
C 1	3.6	3.8	3.3	3.9	3.9	3.8
C 11	3.7	3.6	2.5	3.6	3.7	2.4
C 3	3.6	3.8	2.2	3.5	3.7	2.2
ML	1.8	1.7	1.8	1.7	2.2	1.8

[a]Sugar fermentation expressed as ml 0.1 N NaOH required to neutralize developed acidity after 6 hr incubation at 32° C.

Table 18. Effect of storage upon β-Galactosidase activity of Culture 7962 at -196 C for 1 month

Age of cells, hr	Enzyme Activity[a]			
	Initial activity		Activity after frozen storage	
	Whole cells	Toluene-treated cells	Whole cells	Toluene-treated cells
6	0.066	0.249	0.079	0.294
12	0.030	0.198	0.028	0.233
18	0.012	0.308	0.016	0.369
24	0.013	0.301	0.018	0.268

[a]β-Galactosidase activity reported as μmoles of o-nitrophenol liberated from o-nitrophenol-B-D-galacto-pyranoside per min of reaction time.

Table 19. Effect of storage upon total count and activity of Culture 7962 at -196 C for 1 month

Age of cells, hr	Initial total count/ml x 10^7	Initial activity[a]	Initial [activity/cell] x 10^{-8}	Viability or activity after frozen storage: Total count/ml x 10^7	Activity	[Activity/cell] x 10^{-8}
16	97	2.9	0.30	100	2.7	0.27
20	120	3.6	0.30	130	3.2	0.24
24	150	2.8	0.18	160	3.6	0.23
28	160	3.0	0.18	160	3.8	0.24

[a]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Effect of Freezing Cultures upon the Probable Release of Biologically Stimulatory Substances

Cell-free filtrates from both fresh and frozen culture were added in varying concentrations to the skimmilk growth medium. The results of activity tests determined using the skimmilk fortified with cell-free filtrates are presented in Table 20.

As shown in Table 20, culture activity was stimulated to the same levels using filtrate from either fresh or frozen cells. This indicates that stimulation may not be due to a leakage of stimulatory cellular materials during the freezing process.

Table 20. Effect of cell-free filtrate[a] from fresh and frozen cultures upon culture activity

% added filtrate	Culture activity[b] using	
	Filtrate from unfrozen culture	Filtrate from frozen culture
0	51.0	51.1
1	52.0	52.5
5	58.5	58.7
10	59.0	58.9
25	59.1	59.7

[a]Commercial cheese Culture I was used.

[b]Activity expressed as [per cent developed acidity] x 100 after incubation for 6 hr at 32 C using 1% inoculum.

Effect of Freezing upon Bacterial Growth Curves

The effect of freezing and storage at -196 C upon the subsequent growth responses of single-strain Cultures 10, 11, 13, 14, 17, and 18 are shown in Figures 14, 15, 16, 17, 18, and 19, respectively. The first three cultures were slow acid-producers; the remaining three were fast acid-producers.

Frozen storage for 2 or 4 weeks did not appear to cause any changes in the lag phase, growth rate during the log phase, or maximum populations attained. Growth response after frozen storage was equally good for slow acid-producing or fast acid-producing cultures.

Concentration and Freezing of Cheese Culture

The effect of concentration and frozen storage upon total count and and activity of culture is shown in Table 21. Centrifugation of culture grown in whey medium produced an approximate 11-fold increase in cell concentration/ml. Activity/cell after concentration was about 16% less than before centrifugation, but maintained uniformly after frozen storage at -196 C for 1 month.

Figure 14. Growth curve of Culture 10 before and after storage at -196 C

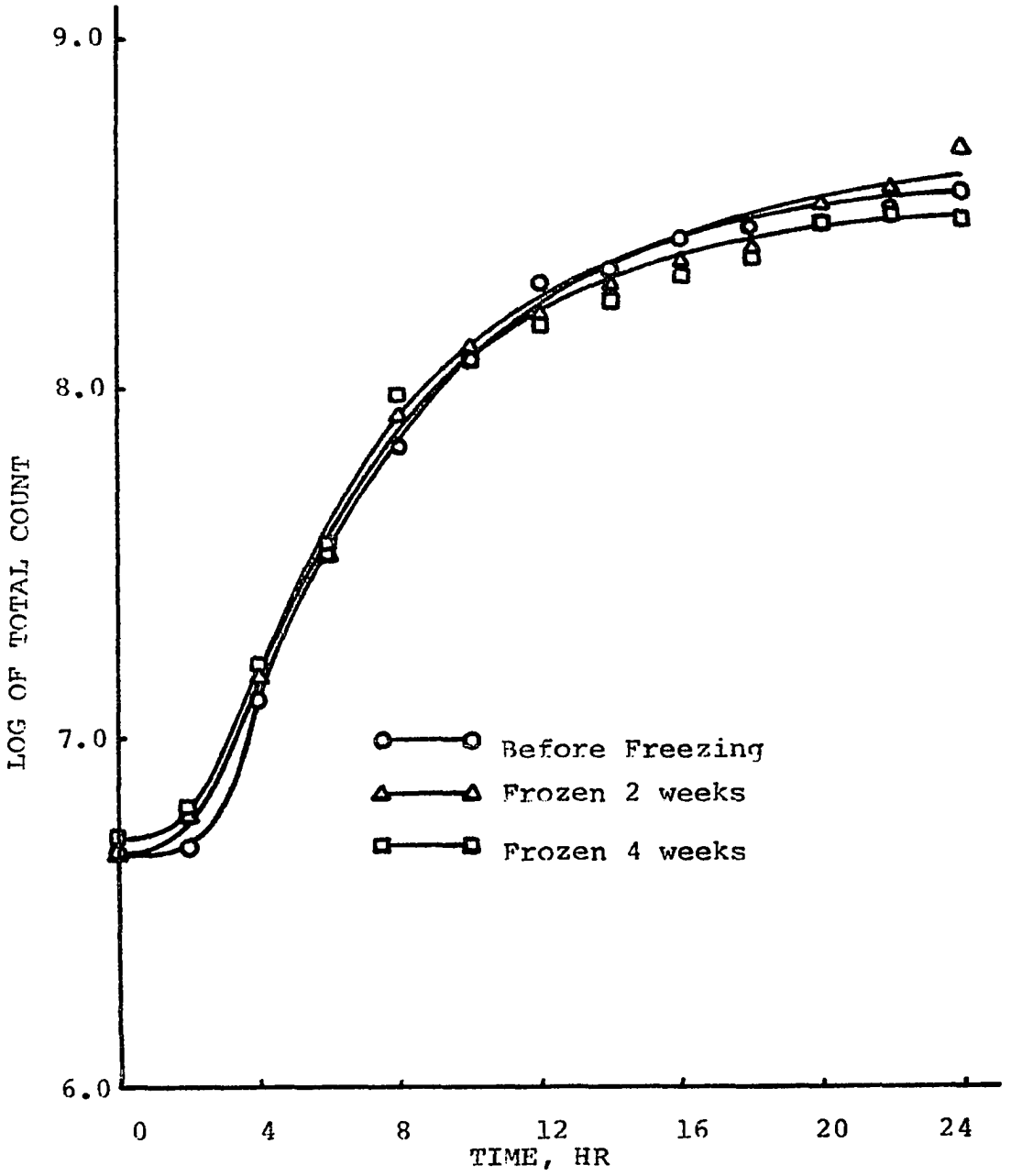

9.0
8.0
7.0
6.0
LOG OF TOTAL COUNT
0
4
8
12
16
20
24
TIME, HR
Before Freezing
Frozen 2 weeks
Frozen 4 weeks

Figure 15. Growth curve of Culture 11 before and after storage at -196 C

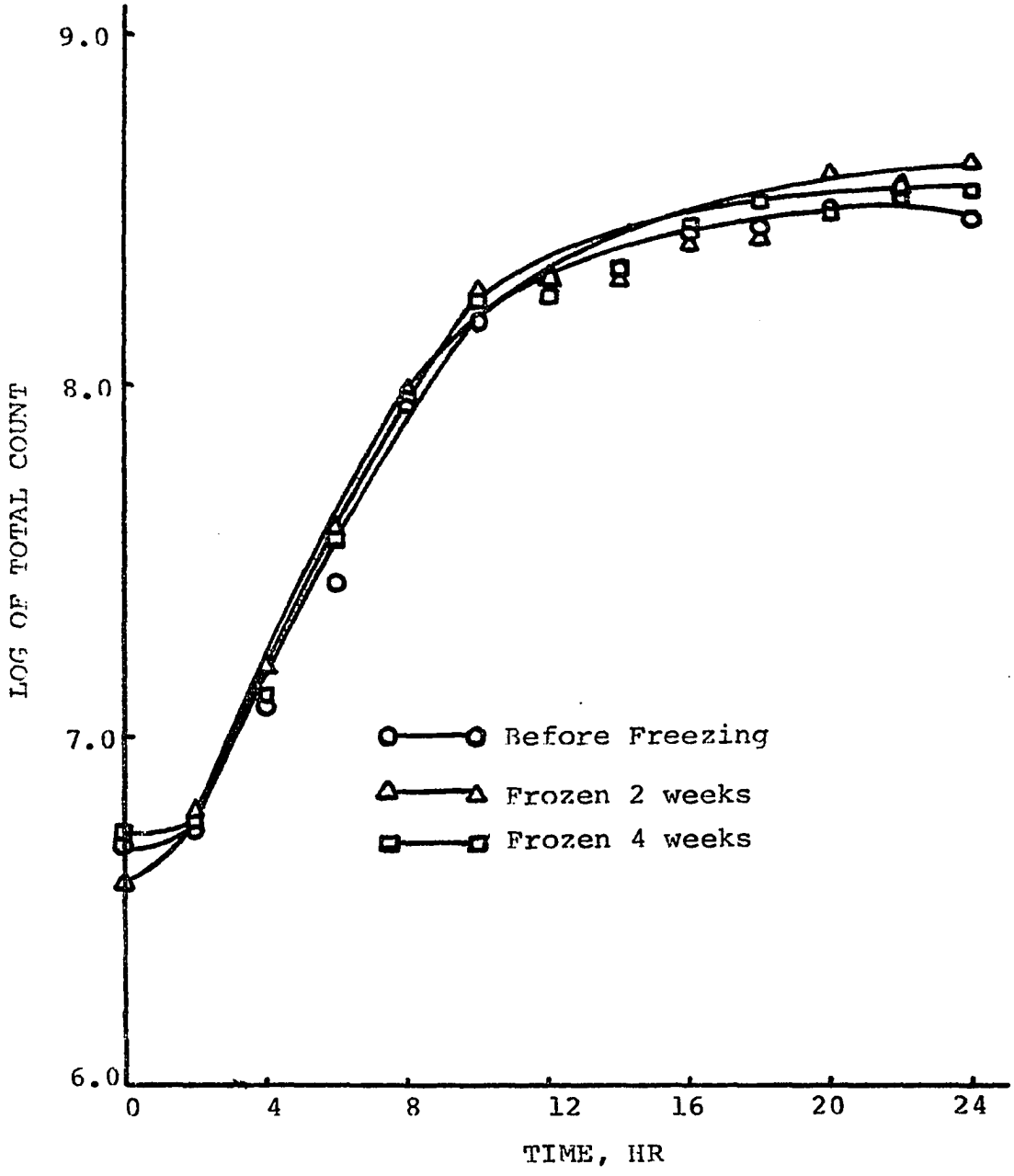

9.0
8.0
7.0
6.0
LOG OF TOTAL COUNT
0
4
8
12
16
20
24
TIME, HR
Before Freezing
Frozen 2 weeks
Frozen 4 weeks

Figure 16. Growth curve of Culture 13 before and after storage at -196 C

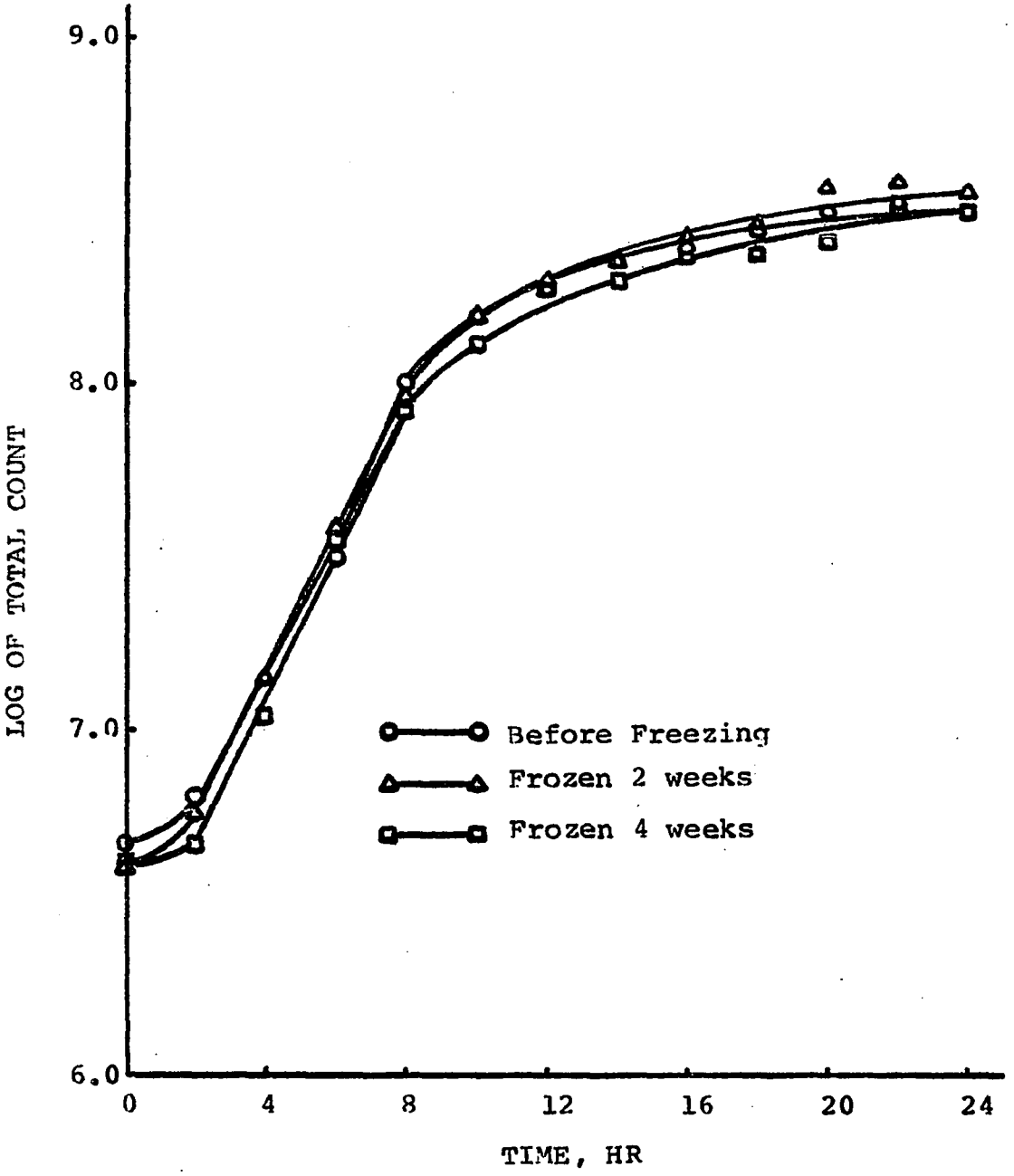

9.0
8.0
7.0
6.0
LOG OF TOTAL COUNT
0
4
8
12
16
20
24
TIME, HR
Before Freezing
Frozen 2 weeks
Frozen 4 weeks

Figure 17. Growth curve of Culture 14 before and after storage at -196 C

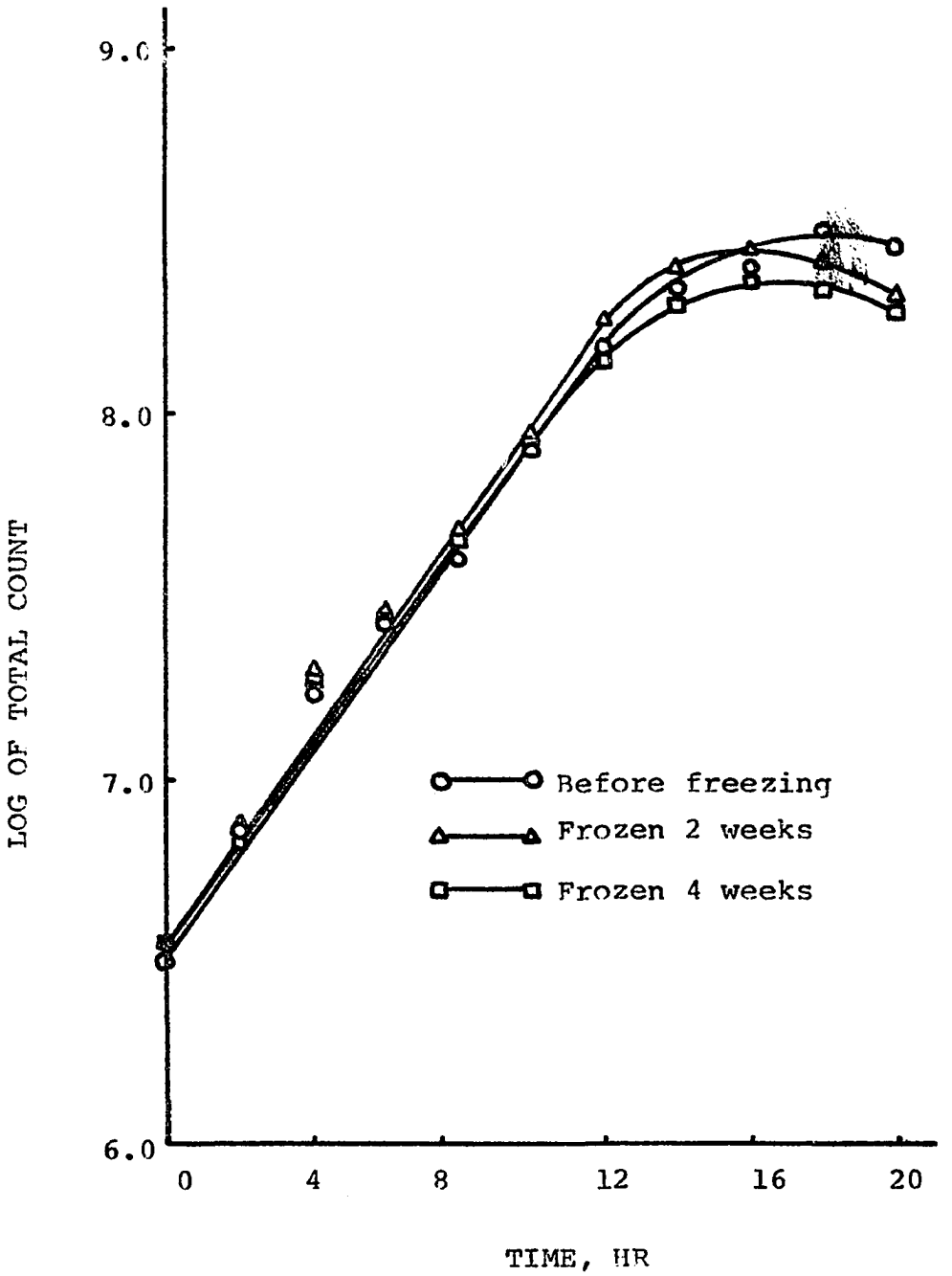

9.0
8.0
7.0
6.0
LOG OF TOTAL COUNT
0
4
8
12
16
20
TIME, HR
Before freezing
Frozen 2 weeks
Frozen 4 weeks

Figure 18. Growth curve of culture 17 before and after storage at -196 C

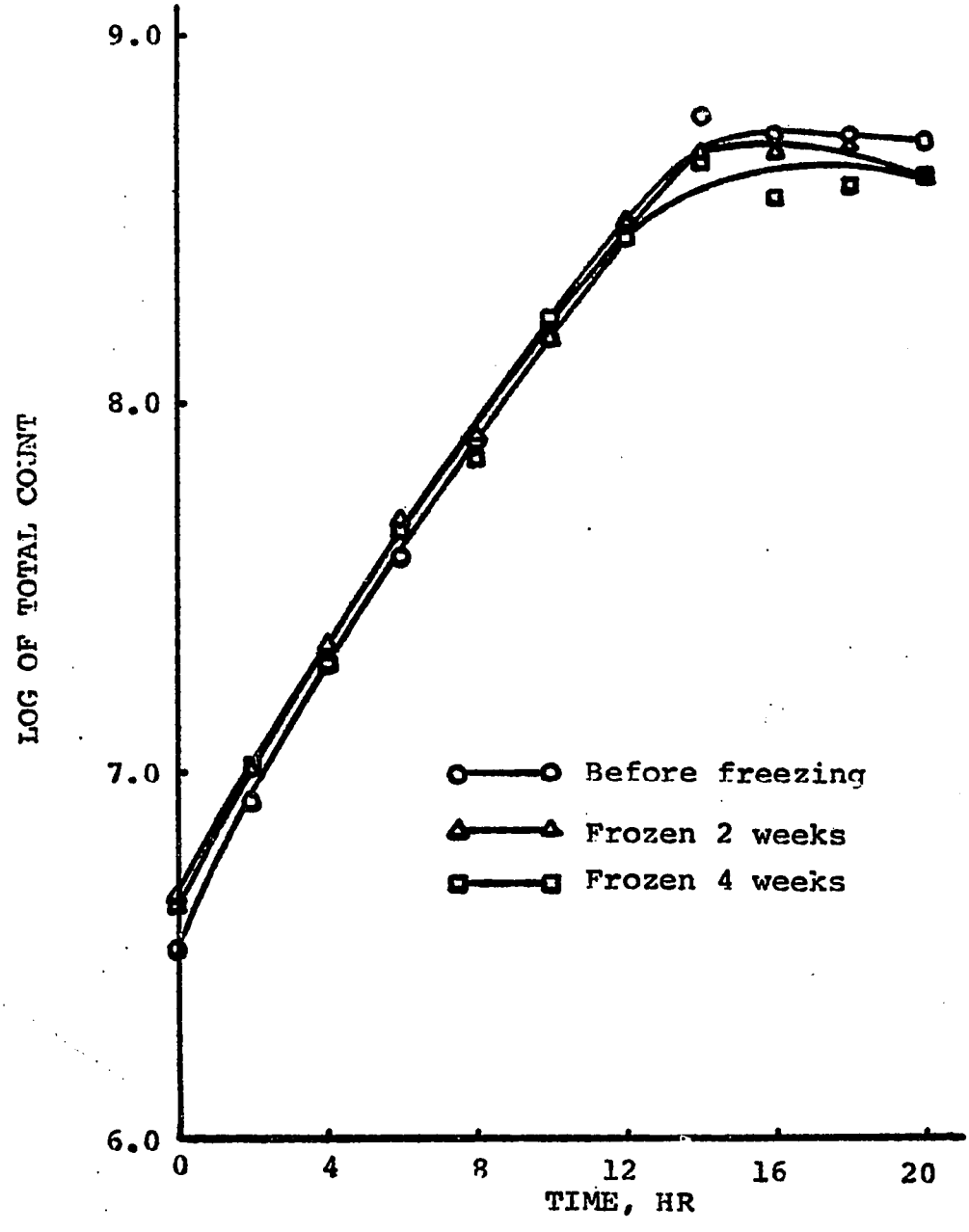

9.0
8.0
7.0
6.0
LOG OF TOTAL COUNT
0
4
8
12
16
20
TIME, HR
Before freezing
Frozen 2 weeks
Frozen 4 weeks

Figure 19. Growth curve of Culture 18 before and after storage at -196 C

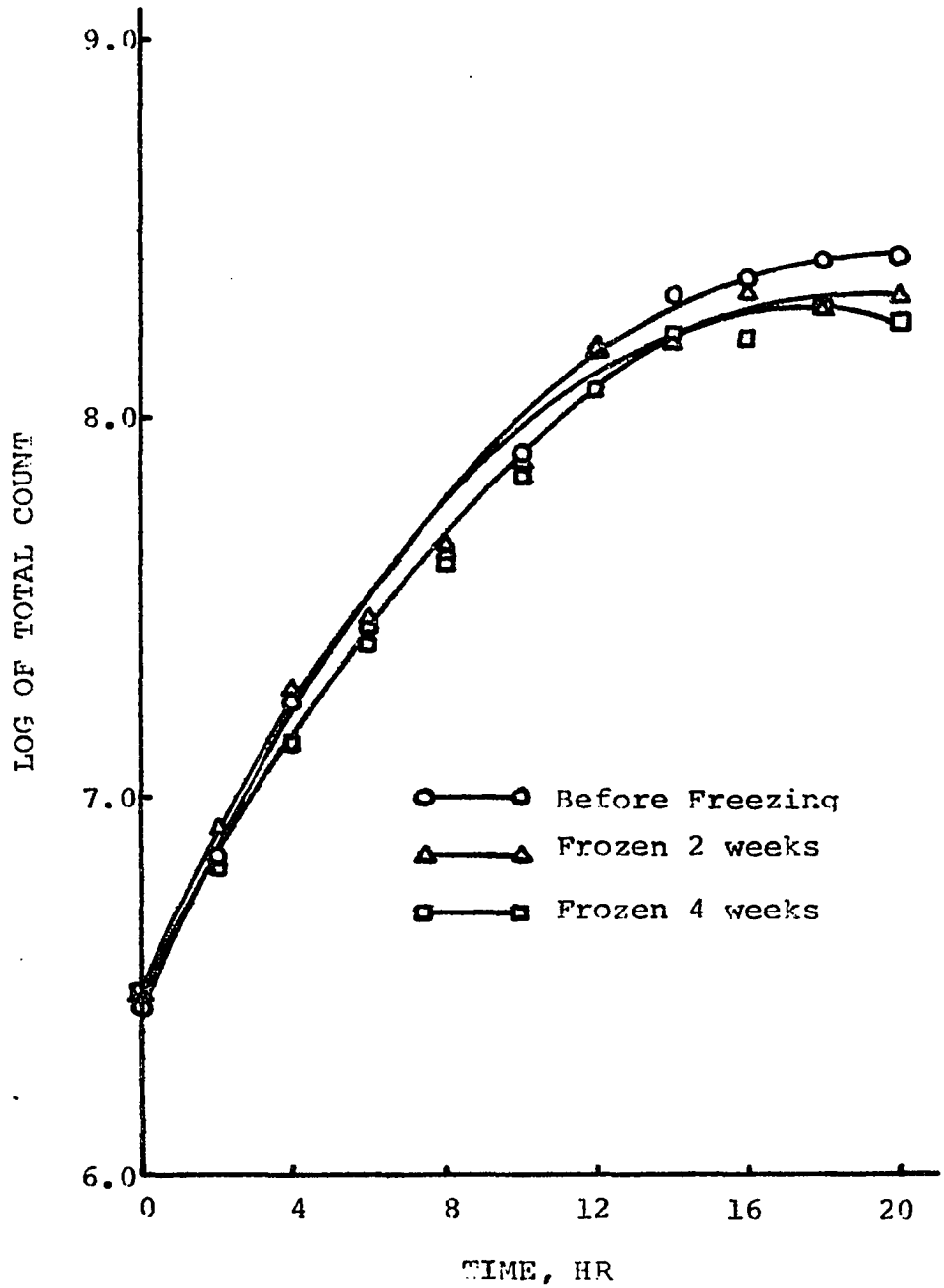

9.0
8.0
7.0
6.0
LOG OF TOTAL COUNT
0
4
8
12
16
20
TIME, HR
Before Freezing
Frozen 2 weeks
Frozen 4 weeks

Table 21. Effect of storage at -196 C for 1 month upon total count and activity of concentrated culture[a]

Before freezing				After frozen storage	
Before concentration		After concentration			
Total count/ml x 10^7	[Activity/cell] x 10^{-8}	Total count/ml x 10^8	[Activity/cell] x 10^{-8}	Total count/ml x 10^8	[Activity/cell] x 10^{-8}
76	6.8	87	5.7	90	5.3

[a]Commercial cheese Culture I was used for concentration.

Manufacture of Cheese with Concentrated, Frozen Culture

Milk used for the manufacture of Cheddar cheese varied in total count from 2.2 to 2.9 x 10^3. Total counts of culture and cheese-milk after thorough mixing are shown in Table 22. Counts of the culture-milk mixtures for Trial 1 were considerably lower than for the other two trials, especially for the 12-hr culture.

Total counts after the 1-hr ripening period of culture and cheese-milk is shown in Table 23. Again, as shown in Table 22, the 12-hr culture used in Trial 1 had a lower count than the other vats. Total counts for the 16-, 20-, and 24-hr culture-milk mixtures were quite similar for each trial, as was also found for the unripened vats in Table 22.

The ability of different aged, liquid-nitrogen frozen cultures to perform in Cheddar cheese manufacture is presented in Table 24. The lowest titratable acidity at milling time was found for the 12-hr culture used in Trial 1. Considering rate of acid production, only the vats with the 16-hr and 24-hr cultures (Trial 3) were milled at the desirable time of 4-1/2 hr.

Table 22. Total counts of cheese-milk after mixing with culture

Vat no.	Age of culture used[a]	Total count/ml x 10^6		
		Trial		
		1	2	3
1	12 hr	1.5	9.2	21.0
2	16 hr	7.7	13.0	20.0
3	20 hr	7.7	14.0	20.0
4	24 hr	7.3	11.0	27.0
5	Control[b]	1.3	7.0	8.3

[a]These figures represent the age at which the culture was concentrated and frozen.

[b]Normal bulk culture was used for Trials 1 and 3; concentrated culture was used for Trial 2.

Table 23. Total counts after 1 hr ripening of culture in cheese-milk

Vat no.	Age of Culture used[a]	Total count/ml x 10^6		
		Trial		
		1	2	3
1	12 hr	2.2	10.0	34.0
2	16 hr	9.3	23.0	50.0
3	20 hr	11.0	24.0	41.0
4	24 hr	8.8	18.0	42.0
5	Control[b]	9.1	11.0	24.0

[a]These figures represent the age at which the culture was concentrated and frozen.

[b]Normal bulk culture was used for Trials 1 and 3; concentrated culture was used for Trial 2.

Table 24. Effect of culture age at the time of centrifugation, freezing, and storage in liquid nitrogen upon subsequent performance in Cheddar cheese manufacture

Vat no.	Age of culture used[a]	Trial	Milling time, hr[b]	Acidity at milling[c]	pH at milling	pH at following morning	Flavor evaluation after 1 month ripening of cheese
1	12 hr	1	5 3/4	0.30	5.6	5.5	Slight acid
		2	4 3/4	-[d]	5.5	5.3	Normal
		3	4 3/4	-[d]	5.5	5.3	Acid
2	16 hr	1	5 1/2	0.51	5.6	5.2	Acid, slight bitter
		2	4 3/4	0.55	5.5	5.3	Normal
		3	4 1/2	-[d]	5.5	5.3	Flat
3	20 hr	1	5 1/2	0.55	5.4	5.4	Acid
		2	5	-[d]	5.5	5.3	Flat
		3	4 3/4	-[d]	5.5	5.3	Flat

[a]These figures represent the age at which the culture was concentrated and frozen.

[b]Milling time includes time from rennet addition until curd milled.

[c]Acidity expressed as % titratable acidity as lactic acid.

[d]Insufficient whey available for titration.

Table 24 (Continued)

Vat no.	Age of culture used[a]	Trial	Milling time, hr[b]	Acidity at milling[c]	pH at milling	pH at following morning	Flavor evaluation after 1 month ripening of cheese
4	24 hr	1	5 1/2	0.54	5.6	5.4	Acid, bitter
		2	5	-[d]	5.5	5.3	Normal
		3	4 1/2	-[d]	5.5	5.3	Normal
5	Control[e]	1	4 1/2	0.56	5.5	5.5	Normal
		2	4 1/2	0.56	5.6	5.5	Flat
		3	3 3/4	0.57	5.4	5.3	Normal

[e]Normal bulk culture was used for Trials 1 and 3; concentrated culture was used for Trial 2.

DISCUSSION

Rate of Freezing

Freezing rates used in this study are depicted in Figures 1 and 2. Slow freezing was employed only when it was desirable to compare effects of cooling rates. Ultrarapid freezing, as obtained here by the use of liquid nitrogen, followed by ultralow storage and fast thawing, has been shown to result in greatest survival of lactic streptococci (6, 19, 31, 49). Although the reasons for high rates of survival following rapid freezing and thawing have not been established, the most tenable explanation relates to protection from exposure to high electrolyte concentration and intracellular ice-crystal formation during freezing and thawing (70, 73). Luyet (61) reported that electron micrographs of S. lactis cells frozen at various rates showed no evidence of intracellular ice under conditions which readily showed ice in other cells. If Luyet's observations (61) are correct, the success of ultrarapid freezing followed by fast thawing may be due to the protection of the cell from high electrolyte concentrations, or to some other unexplained factor, or more likely, a combination of factors.

As culture media or contents of bacterial cells freeze, there is an increase in the concentration of solutes in the remaining fluid as the water freezes. Ultrarapid freezing

and rapid thawing would expose the cell to these deleterious factors for a minimum time period, thus improving freezing survival. The significance of increased electrolyte concentration during freezing and thawing has been demonstrated for cells of E. coli, Serratia marcescens, and L. fermenti (37).

Frozen Storage of Cultures and Culture Activity

Maintenance of culture viability and acid-producing ability are shown in Tables 3, 8, 9, 12, 15, 16, 19, 21; and in Figures 3, 4, 5, 10, 11, 12, and 13. As shown repeatedly throughout this investigation, cultures survive storage remarkably well at -196 C.

The acid-producing activity was consistently better maintained and less variable than were bacterial numbers after frozen storage. Baumann (6) also found that plate-count recoveries were more variable than culture activity after frozen storage. Slight decreases in total count were found after frozen storage of certain cultures, especially after extended periods of storage.

Failure of certain cells to grow after storage (Table 14) may be due to a progressive metabolic injury, as has been reported to occur after frozen storage at temperatures above -196 C by Moss and Speck (77) and Gibson et al. (30). In

this study, although total counts of six mixed-strain cultures decreased slightly after 1 month at -196 C (Table 3), activity remained at the same levels. Based upon activity/cell, an increase in acid production/cell was observed. No adequate explanation for the phenomenon is known. Stimulation based on selection of the more active cells has been offered as an explanation (5). However, stimulation has been observed more frequently when using less active cells (6). To further complicate matters, Garvie and Mabbitt (29) remarked that the common change of fast cells into slow acid-producing variants was not reversible. At present, the most tenable explanation for stimulation is that freezing may cause changes in cellular permeability and nutrients would thus be more readily accessible to the cell. Results of Baumann (unpublished data) indicated that increased permeability of the cell may be responsible for increased activity of cultures stored in liquid nitrogen. In a later study, Baumann (unpublished data) found that the rate of glucose uptake was increased after storage of lactic cultures at -196 C.

Although stimulation was observed several times in these studies (Table 3 and Figure 5), more intensive investigations using numerous single-strain cultures did not conclusively demonstrate stimulation in a consistently reproducible manner. In most cases, no changes in activity of single-strain

cultures were found after frozen storage (Table 15). In fact, slight decreases of activity were observed as often as were instances of apparent stimulation. Slight decreases of activity were probably due to freezing injury.

Age of Cells and Incubation Temperature

Maximum acid-producing activity and total counts of cultures grown at 21 C occurred several hours earlier than cultures grown at 15 C (Figures 6-9) because the higher temperature was nearer the optimum growth temperature for the cultures. These results show that maximum acid-producing activity of cheese cultures occurs several hours after maximum populations are attained. When cells are expending less energy for cell division after attaining high numbers, perhaps greater energy is available for enzyme synthesis responsible for acid production.

Cultures grown for various periods of time before frozen storage (Figures 10-13) satisfactorily maintained their activity after thawing. These findings show that resistance to freezing is equal at any time during the incubation periods used in this study. However, for practical application, freezing cultures late in the life cycle (after 24 hr) would be unadvisable since the cells would have lost much of their viability and activity by that time.

Data presented here showing that cells having reached

their stationary phase of growth produce greater amounts of acid than younger cells is in agreement with observations made by Rahn, Hegarty, and Deuel (88). They found that when transferred to a fresh medium older cells of S. lactis temporarily possess almost double their fermentative ability. The increased fermentative activity in older cells may be due to alteration in the permeability barriers with aging.

Whey Medium for Culture Growth

Milk is generally used for propagating starter cultures. This is true because milk is readily available to those concerned with the trade, cultures grow well in milk, milk components furnish protection and buffer capacity to suspended cells, and milk affords indirect indication of growth and activity by visual and organoleptic examination of the culture. However, for preparation of cellular concentrates for use in bulk or direct-set applications in making cheese, a relatively clear medium is desirable. Readily available whey, with small amounts of various growth-promoting additives, may be used. Results presented here (Tables 6 and 7) show that cultures can be satisfactorily grown in whey medium. When cultures were grown in skimmilk, transferred to whey, and finally grown in skimmilk again (Table 7), culture activity, total counts, and activity/cell did not diminish but were maintained at comparable levels

unaffected by the changes in media.

Culture Proteolysis and Acid-Producing Activity

Fast acid-producing strains of lactic streptococci are known to be more proteolytic than slow acid-producing strains, indicating a correlation between proteolysis and acid production at 21 C (13, 18, 29). Since slow-culture activity can be easily stimulated by addition of compounds of proteolysis, it was postulated that stimulation of slow cultures could occur after frozen storage because of a concomitant increase in proteolytic ability. On the contrary, proteolytic ability declined upon frozen storage, confirming reports of Cowman and Speck (17, 19). At the same time, culture activity and viability remained at high levels. However, on subculturing, the proteolytic ability was partially restored (Table 10). Since it is known that the proteolytic enzymes of S. lactis are inducible (122), it is probable that frozen storage somehow damages or alters the proteinase system which is gradually adapted toward original activity upon subculturing.

Data presented here show that the acid-producing ability of lactic streptococcus cultures is well maintained during frozen storage, whereas the proteolytic activity decreased considerably. These findings are in agreement with Smittle and Koburger (104) who reported that the glycolytic activity

of S. lactis remained constant over a 10-day period at 4 C, whereas the proteinase activity was essentially absent by the 6th day. These authors concluded that proteinase activity apparently was not related to glycolysis, as is generally believed. Williamson and Speck (122) also reported that the rapidity of acid production by lactic streptococcus cultures was not related to proteolytic activity. In view of the contradictory reports, further work is necessary to understand the exact relationship between proteolysis and acid production by lactic streptococci especially at different incubation temperatures.

Daily Comparison of Unfrozen Culture to Frozen Culture after Extended Storage at -196 C

Cultures used in this study had been stored at -196 C for 6 months. This study was planned to determine whether extended storage in liquid nitrogen would cause any diminished culture activity or change in total count after numerous daily transfers, when compared to the same cultures that had not been frozen. No impairment of maintenance of these criteria were observed over the 2-week period of daily transfer (Table 12). These results show that cultures can be thawed after extended storage at liquid-nitrogen temperatures and can be used satisfactorily. Also these findings agree with those of Baumann (6) and Johnson et al. (49), who found no loss of activity of lactic Streptococcus cultures after 7

months and 2 years, respectively. Proteolysis determinations after thawing and after the 2-week transfer period further substantiated the results shown in Table 10 that lost proteolysis was partially restored after subculturing.

Effect of Frozen Storage upon Cellular Integrity of Cultures

Cellular integrity was investigated by means of maximal and minimal media. Cellular injury and death, although not completely eliminated, were markedly lower with freezing and storage at -196 C than at -20 C (Table 14). Also, slow-frozen cells were more susceptible to freezing injury and death than fast-frozen cells. The results of this investigation further indicate the significance of fast freezing, followed by low-temperature storage. Although injury and death were comparatively low with freezing and storage at -196 C, susceptibility to injury and death was not equal for all strains studied. Similar results were obtained by Gibson *et al.* (30) following frozen storage of cultures at -17.8 and -23.3 C. In order to help insure the retention of maximum culture viability following storage at -196 C, proper selection of strains may be advisable.

Effect of Freezing upon Subsequent Carbohydrate Utilization of Cultures

The data shown in Table 17 reveals that freezing and frozen storage at -196 C caused no change in the fermentation of lactose, glucose, or galactose as measured by titratable acidity. Also, the specificity toward these three sugars was not altered. Although only four cultures were studied, these results indicate that cultures can be frozen and stored in liquid nitrogen without changing the fermentative patterns of carbohydrate utilization - an important culture characteristic necessary for use in fermented dairy products.

It was hoped that this study would gain an insight into the mechanism of the phenomenon of stimulation. However, activity tests conducted before and after frozen storage gave no indication of stimulation. In a similarly planned experiment using radioactive glucose, Baumann (unpublished data) presented evidence suggesting a positive correlation between increased acid production and higher glucose uptake by cells of S. cremoris after frozen storage.

Effect of Frozen Storage upon β-Galactosidase Activity of Culture 7962

Since β-galactosidase is the first enzyme responsible for the degradation of lactose, it was thought that perhaps changes in culture activity were linked to this enzyme.

Culture 7962 was chosen for this study because of the stability of its β-galactosidase or lactase activity (14).

It was hypothesized that if lactase activity of whole cells was increased by freezing while the activity of toluene-treated cells remained constant, then permeability may be responsible for stimulation. If freezing caused increased activity of whole and toluene-treated cells, then increased enzyme activity would be indicated. However, the results followed neither pattern (Tables 18 and 19). Neither acid-producing activity nor lactase activity of whole cells was stimulated by freezing. The independent investigation of Baumann (unpublished data) also indicated that increased acid-producing activity after freezing was not due to changes in lactase activity.

Effect of Freezing Cultures upon the Probable Release of Biologically Stimulatory Substances

Results of this study indicated that any stimulatory effect of culture after frozen storage was not due to a release of material during frozen storage (Table 20). Cell-free filtrate from frozen or unfrozen cultures was equally stimulatory to culture growth. Also, it should be noted that if substances responsible for stimulation were released from the cell during storage, the effect would certainly be small since only 1% inoculum is used for the activity test. This 1% inoculum would be considerably less concentrated than the 1%

cell-free extract used in Table 20 as a source of stimulatory material. Results of Baumann (unpublished data) also indicated that freezing did not release cellular materials into the extracellular environment which could stimulate surviving cells.

Growth Curves of Lactic Cultures Before and After Frozen Storage at -196 C

The ideal method of culture preservation would insure that cells would resume their high rate of metabolic activity immediately after removal from the preservative. With most methods of preservation, this ideal is not attained. Preserving cultures in liquid nitrogen does not appear to affect the lag phase of growth, the growth rate, or the maximum populations reached, as seen in Figures 14 through 19. Rapid initiation of growth after preservation is quite significant in cheese manufacture. The importance of ultra-rapid freezing in relation to growth response after liquid-nitrogen storage was illustrated by the work of Sokolski et al. (106). Cultures of L. leichmannii were frozen by immersion in liquid nitrogen and slowly at 1 C/min. After frozen storage, the rapidly-frozen cells showed good growth response, whereas the slow-frozen cells had a long lag period and showed poor growth.

Concentration and Freezing of Cheese Culture

Successful maintenance of activity and viabiltiy of concentrated cheese culture when preserved in liquid nitrogen is shown in Table 21. The results indicate the usefulness of storing concentrated cheese culture in liquid nitrogen for later use in bulk cultures or for direct-set cheese-making. With equipment available in the laboratory, it was only possible to prepare cellular concentrates slightly over 10-fold. Concentrates of this order were also grown in batch quantities by using automatic pH control of whey cultures. However, with the use of a high-speed, continuous centrifuge, it is a simple matter to prepare cell suspensions containing at least 100-times the concentration of cells found in normal culture (58). For the economic application of liquid nitrogen as the refrigerent, it would be advantageous to use highly concentrated cultures for freezing.

Concentrated cultures have been preserved at -20 C for later use. However, after 21 weeks of storage, 14% of initial activity was lost, and growth rates were about 1 hr slower than unfrozen culture (58). Baumann (6) successfully preserved concentrated culture at -196 C without loss of activity. Cheddar cheese made with concentrated, frozen culture was comparable in quality to cheese made with unfrozen culture.

Recent commercial applications have been made using

frozen, concentrated culture for use in fermented dairy products. Use of these cultures can eliminate mother culture, intermediate culture, and may eliminate bulk culture when used as a direct-set inoculum.

Manufacture of Cheese with Concentrated, Frozen Culture

A very reliable and practical means of testing the successful use of concentrated and frozen culture is to use the frozen concentrate to make cheese. Making Cheddar cheese with a direct-set, liquid-nitrogen frozen, concentrated culture offers a new approach to cheese making with distinct advantages over the traditional methods of culture preparation. Various cultures have been concentrated for experimental purposes, but information on the use of concentrated and frozen culture for Cheddar cheese making by the direct-set method is quite limited. Baumann (6) successfully made Cheddar cheese using liquid-nitrogen frozen cultures that were concentrated 35- and 70-fold.

The data in Table 24 indicate the usefulness of using frozen, concentrated cultures as a direct-set inoculum. The practicality of using a frozen concentrate to inoculate bulk culture was also demonstrated, as the control vat of cheese for Trials 1 and 3 were made from bulk starter that was prepared from a frozen concentrate.

Cheese-making data presented in Table 24 indicated that cultures frozen any time from 12 to 24 hr of incubation can successfully be used to make Cheddar cheese. Incubation from 16 to 20 hr on a commercial scale for concentrated culture production would be the most feasable. It appears that the number of organisms in the concentrate is quite important for cheesemaking, as the low population of the 12-hr culture of Trial 1 was not sufficient for a desirable rate of acid production. Culture activity would also be greater for cultures older than 12 hr, as was shown in Figure 9.

Preliminary data on the experimental cheese shows that Cheddar cheese made with frozen, concentrated culture to be of comparable quality to the control. Measurements of pH at milling and the following morning of the experimental vats of cheese were closer to the normal working range of pH 5.1 to 5.3 (119) than were the control vats. Body and flavor evaluations after 1 and 7 months of ripening indicated no abnormalities. More vats of cheese with longer periods of ripening are needed to fully evaluate the applicability of using frozen, concentrated cultures. Evidence at present indicated that the use of concentrated, frozen cultures can and will help establish a sound and unfailing culture program for the cheese and fermented milk industry.

SUMMARY

In order to gain a better understanding of the applicability of liquid-nitrogen frozen cultures for use in cheesemaking, factors influencing culture maintenance were studied. Culture viability was measured by the determination of total counts before and after freezing treatments. Acid-producing ability, one of the more important cheesemaking criteria of desirable cultures, was used throughout the investigation to evaluate numerous effects upon its maintenance.

The relationship between maximum activity and total count of unfrozen cultures grown at 15 and 21 C was studied. Cultures grown at 15 C had a maximum total count at about 20 hr and a maximum activity at 24 to 28 hr. Cultures grown at 21 C had a maximum total count at about 14 hr of incubation, whereas highest activity occurred at approximately 20 hr. No advantage was found by growing cultures at 15 C.

Culture activity and viability of 16-hr cultures were well maintained after 1 month of storage at -196 C. When different aged cells were compared before and after frozen storage, culture activity and viability were well maintained at any age studied from 12 to 36 hr.

The usefulness of using whey, fortified with yeast extract, as a growth medium for cultures was demonstrated.

Activity and total counts were well maintained when cultures were transferred from milk to whey and back to milk.

Activity and viability of fast or slow acid-producing cultures were maintained uniformly during 6 months at -196 C. Maximum activity/cell generally occurred after 1 month of frozen storage. Maximum proteolytic activity was observed before freezing and decreased considerably with storage.

Cultures survived storage at -196 C for 6 months and performed normally when thawed and transferred daily over a 2-week period.

Death and injury of culture cells stored at -196 C were less than when cells were stored at -20 C. Injury and death of slow-frozen cells were greater than for fast-frozen cells. Cultures were not equally susceptible to injury and death at -20 or -196 C storage.

Stimulation of culture activity by liquid-nitrogen freezing was not consistently demonstrated in these studies. It appeared that under certain unknown conditions, certain cultures were capable of producing acid at an increased rate after storage at liquid-nitrogen temperatures.

Storage of cultures at -196 C caused no changes in utilization of, or specificity toward lactose, glucose, or galactose.

β-Galactosidase activity of S. lactis cells was not affected by liquid-nitrogen storage for 1 month.

Cell-free filtrate from frozen cells was no more stimulatory to culture activity than was filtrate from unfrozen culture.

Frozen storage at -196 C of single-strain cultures did not change the lag phase of growth, growth rate during log phase, or maximum counts achieved after thawing. Growth response after storage was comparable for slow acid-producing or fast acid-producing cultures.

Activity and activity/cell of concentrated cheese culture were maintained uniformly after storage at -196 C.

Cheddar cheese was successfully manufactured by the use of concentrated, liquid-nitrogen frozen culture as a direct-set inoculum. Provided that enough bacterial cells were present, cheese could be made from concentrated culture varying in age from 12 to 24 hr (grown at 21 C) before concentration and freezing.

After years of cryobiological research concerning various microorganisms, several problems still plague researchers. Foremost, is the cause of freezing injury and death. When the nature of cellular damage at low temperatures is further understood for lactic cultures, better methods of treatment prior to freezing and freezing techniques can be established to preserve maximum viability and activity. Also concerned is the cause of occasional stimulation of activity by freezing. Understanding and consequent application of this phenomemon could mean use of less inoculum for preparing cultured products.

inoculum for preparing cultured products.

The usefulness of liquid-nitrogen frozen cultures has been well demonstrated. No other known method of preservation is able to maintain cellular activity, viability, and other cultural characteristics as uniformly as low-temperature freezing. Before termination of these studies, liquid-nitrogen frozen cultures had been successfully used for preparation of bulk starters for cheesemaking at Iowa State University for nearly 2 years. Cultures were frozen in quart milk cartons at the rate of approximately 850 ml per quart to prevent breakage of cartons. A carefully sanitized, commercial carton filler was used for filling. A minor difficulty encountered was that chunks of frozen culture were difficult to melt in the milk. This problem could be solved by use of a sterilized or well sanitized ice grinder. Several vats of Cheddar cheese also were made successfully using direct-set, frozen cultures.

There is no reason to believe that concentrated cultures stored at -196 C could not be used in a large-scale operation for cheesemaking. It is the author's contention that concentrated cultures could be prepared in a culture laboratory, placed in suitable cartons or cans, quick-frozen in liquid nitrogen, followed by distribution in special nitrogen refrigerators to the consumers in the dairy industry.

Indeed, concentrated culture has been recently prepared in this manner, and is now available from several commercial culture suppliers.

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