BACTERIOLOGICAL STUDIES ON FROZEN CONCENTRATED MILK

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by

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INTRODUCTION

Freezing has long been regarded as a dependable and economical method of preserving perishable foods. Particularly within the last two or three decades many advances have been made in this field. There is no doubt today that the art and science of freezing technology have advanced to a point where one can obtain many high quality, nutritious and economical frozen foods.

The dairy industry is continually being faced with the problem of seasonal surpluses and shortages. Any technique which would allow surpluses to be carried over into shortage periods would be highly desirable to this industry. Frozen milks may be one such answer. Milk can be produced at lower cost during the spring and summer than during the fall and winter, therefore, it would be an advantage to process milk into such forms that will be suitable for future use. Storage of frozen concentrated milk appears to have possibilities in aiding the needs of the industry and to supply the fluid milk trade with high quality milk. Furthermore, concentrated milk may have a place in non-milk-producing areas, particularly where transportation is an important cost factor.

Though frozen cream has been a standard product in the manufacture of ice cream mixes, the freezing of fluid and concentrated milks for direct consumption has not been practised on any large scale. A frozen three-to-one concentrated

milk is not a new product since experimental work goes back to the early 1930's. However, technical advances, as compared with other industries, were slow, probably due to the unique physical and chemical properties of milk. Protein instability and flavor deterioration have been the main obstacles, either of which limits the storage period. As the storage period is extended this product will undoubtedly develop greater potentials.

Since frozen concentrated milk is a food product, attention must be given to the bacterial flora. Further, to add to the knowledge of a comparatively new product and in hope of obtaining information applicable to the industry, studies have been undertaken to determine the effects of processing and storage on the numbers and types of bacteria in frozen concentrated milk.

REVIEW OF LITERATURE

In reviewing the literature, it became evident that the studies on the microbiology of frozen foods can be divided into two groups; the first group includes investigations of materials which contain a mixed flora of microorganisms such as dairy products, fruit and fruit juices, fish, and meats. The second group centres on pure culture studies in which well known organisms or their products were subjected to low temperatures.

Studies on Foods Containing a Heterogenous Flora

Reduction in the microbial content of frozen fruits and vegetables were reported by Smart et al. (50,51,52,54) to be, generally, over 90 percent. Lochhead and Jones (35) observed that the numbers of bacteria in strawberries packed in a sugar syrup were reduced gradually when stored at 0 F. However, counts on vegetables showed a sharp initial decrease followed by a more gradual decrease. They also concluded that from a bacteriological view point the method of packing vegetables (water, brine and dry-pack) was not an important factor.

Brown (6), in an attempt to correlate types of organisms found in fresh vegetables with those of defrosted samples, found that the percentage of spore-forming rods to other types had greatly increased. She also isolated Flavobacteria, Achromobacter, Diplococci, Streptococci, and colon-organisms. Smart (52) found Sarcinae, Flavobacteria and Bacilli as being most frequently isolated.

In two separate investigations, Lochhead and Jones (34,35) found Micrococci and species of Flavobacterium which survived freezing relatively better than other forms after 8 months storage at 0 F. Differences obtained in the types of organisms could probably be traced to the original flora of the fresh product.

Berry (4), working with frozen packs of berries, observed a less rapid decrease in microbial numbers at -20 C. than at -10 C. Higher death rates were observed in air-tight containers. He claimed that this was due to the accumulation of carbon dioxide which exerts a destructive influence on the microorganisms. Further, work with frozen peas stored at -4 C. indicated that this temperature was insufficient to inhibit microbial growth. Similarly, Prescott (41) observed that a storage temperature of -6.6 C. was more destructive to bacteria than temperatures of -12 to -18 C.

The bacterial flora of frozen meats was somewhat different from vegetables; gram positive cocci and fluorescent types occurred most frequently in the former according to Fellers (15) and also Straka et al (57). Geer and co-workers (17) compared unfrozen to defrosted hamburger steak and found that, like in vegetables, freezing greatly reduced bacterial numbers.

Richardson and Scherubel (45,46), in their work on deterioration of frozen beef, observed abundant microbial growth at 0 C. They estimated that temperatures of -9 to -12 C.

were required to inhibit such growth on stored beef. It was pointed out that there are three temperatures of interest, the freezing point of water, the point at which bacterial growth ceases due to the concentration of meat extractives during the freezing process, and the cryohydric point(when only solid phases are present). They distinguished between the effect of freezing and the effect of temperature and stated, "Bacteria develop and multiply very well in suitable media, provided only that the media are liquid. It is the solid condition which limits bacterial growth absolutely." The authors concluded with a statement that some bacteria can grow in a supercooled liquid at -20 C. However, no experimental data supported the latter presumption.

Hess (23,24), at a Canadian Fisheries Experimental Station, has made valuable contributions to the bacteriology of frozen fish. The majority of these studies were made on a special group of microorganisms, namely the marine bacteria. These bacteria were termed truly "psychrophilic" since the temperature of their natural habitat is about 5°C. A large portion of this work was done at temperatures near freezing and probably is not directly applicable to other frozen foods which are usually stored at a lower temperature.

Castell and Anderson (9) observed that spoilage in cod fillets was produced most rapidly by Pseudomonas, Proteus, and Achromobacter.

According to Pivnick (40), slow freezing of cod muscle at -12 C. and fast freezing at -23 C. did not influence the numbers of bacteria which were destroyed. Storage for six months gave decreases of over 90 percent and the death rate was slightly greater at the higher temperature. He also observed that certain species of the genus Pseudomonas decreased considerably during freezing and storage; no consistent change was noted with other genera. Similar reductions in numbers were obtained by Kiser and Beckwith (32) who studied frozen mackerel.

A temperature of -18 to -20 C. was found by Gibbons (18) to be optimum for storage of fillets. Those stored at -5 C. and even those at -10 C. deteriorated, however, spoilage at -10 C. was probably a combination of chemical action and microbial activity since there were no indications of increases in bacterial numbers. Bacterial growth was evident on the fillets stored at -5 C. by the fiftieth week. Fillets stored at -18 C for one year showed little change in numbers; after this there was a gradual decrease.

Swenson and James (58) observed that quick-freezing of egg melange at -109 F. was much more destructive to bacteria than slow freezing at 0°F. Freezing at such a low temperature with subsequent storage at -4 F. for 8 months gave reductions of about 80 percent. A prolonged investigation by Schneiter et al (49) showed that gradual reduction of bacteria was still evident after continued storage for 6 years. Somewhat different results were obtained by Johns and Berard (27); the freezing

process reduced counts of whole egg melange by one third and subsequent storage at 5 F. for 6 months resulted in little further change. E. coli and Pseudomonas appeared to reduce more readily, while other genera showed variable results.

Pennington (39) held milk at -0.55 C. and observed a steady increase in numbers of bacteria. The predominating species, a liquefying type of organism, was particularly resistant to cold and was frequently present in pure culture. Ravenal et al (43) stored milk at -9°C. and reported a gradual decrease in bacterial numbers. Similar results were reported by Babcock (2) who stored homogenized milk at and below -10°C. This decrease was not affected by changes in freezing and storage temperature, nor by exposure to room temperature for four hours.

Cream was reported by Fabian and Trout (13) to be similar to milk in its effect on bacterial viability; storage at -5°to -10°F. for one year reduced bacterial numbers.

Roadhouse and Henderson (47) quick-froze various milk products at -25°F. with subsequent storage at -5°F. for 6 months. No trend could be established with most products due to limited results, but homogenized evaporated milk showed no change in bacterial count.

Studies on the bacteriology of ice cream produced variable results. In general, the freezing of a mix resulted in an increase in bacterial numbers (22,10,12,14). This increase was undoubtedly due to breaking up of the clumps or colonies of bacteria and should be regarded as an apparent increase.

Stiles and Pennington (56) stored ice cream at a temperature varying between -10 C. and -20 C.,and observed an increase in numbers. They attributed this increase to the development of psychrophilic bacteria. Contrary to this, Hammer (21) reported bacterial counts that showed a decrease or very little change in storage. His finding were confirmed by Esten and Mason (11) and also Fay and Olson (14).

Ellenberger (10) made a comprehensive study of the bacteria in ice cream and a section of this work dealt with qualitative changes during storage. The groups of bacteria, as determined by litmus gelatin plates and litmus milk, did not change noticeably during storage.

Bacterial growth in ice cream at temperatures near freezing was followed by Weinzirl and Gerdeman (63). Results at -3 C. and -6 C. showed a gradual increase up to the 19th day and then a sudden decrease. Counts at -10 C. were erratic at first, followed, after a number of days, by a gradual decline. No explanation was advanced to support these fluctuations.

Bell (3), studying the effects of various treatments on the properties of frozen milk, reported a limited number of bacterial counts. A 2:1 concentrate stored at 0 C. became sour in 4 weeks. At a storage temperature of -7 C. bacterial numbers showed a slight reduction over a period of 5 weeks. Definite destruction was evident at a storage temperature of -17 C; a count of 900 after the first week was reduced to less than 100 in 5 weeks. These results were not in agreement with those reported by Roadhouse and Henderson (47). However, the

latter investigators did not sample the product as frequently as did Bell, and therefore, their results should be interpreted cautiously.

Before leaving the first group of studies, mention should be made of quantitative bacterial examinations which frequently were not too satisfactory due to the uneven distribution of the organisms throughout the sample. This was particularly true of fish and meats, as pointed out by Gibbons (18). Furthermore, both bacteria and solids were shown by McFarlane (37) to be unevenly concentrated in certain areas of the frozen package, and this could result in sampling that was far from representative.

The size of the inoculum has a decided influence on the amount of reduction; Hess (24) exposed varying concentrations of Ps. fluorescens in broth suspensions to freezing temperatures and found that a larger percentage of cells survived in the concentrated suspensions.

The method of preparing food products for analysis is also of considerable importance, since results largely depend on the degree of mixing or agitation. Recent results reported by Jones and Ferguson (28) showed that either increased or decreased counts may be obtained by comminuting the material in a Waring Blendor.

Studies Using Pure Cultures

Studies of pure cultures at lower temperatures have not only been valuable from quantitative and qualitative view points but also have furnished food technologists with a

basis for establishing theories which might explain the mechanism by which bacteria are destroyed at freezing temperatures. Much of this work has been concerned with the destruction-survival phases rather than with the actual growth at lower temperatures.

Considerable evidence exists today that microorganisms can grow below freezing temperatures. Berry and Magoon (5) conducted experiments which showed growth at -4° C. of Torula, Monilia, Penicillium, Ps. fluorescens and species of Lactobacillus. A species of Cladosporium and Sporotrichum grew at -6.7° C. Growth in strawberries and raspberries of Oidium and Torula species at -4 C. was reported earlier by Berry (4).

Reed and Reynolds (44), investigated the effect of low temperatures on the growth and activities of microorganisms in milk and found that at -1 C. thirteen types of bacteria were able to make growth. Considerable variation was noted; for example, Bact. lactis acidi and other acid-producers increased at first but decreased under dontinued storage while others, such as Microspira tyrogena, grew slowly at first but made greater growth with increased storage. Budinov (7) held Bact. lactis acidi in milk at 0 C. for 30 days with no change in numbers. Experimental evidence, from these two investigations, indicated that the lactic acid-producers exhibited no psychrophilic tendencies.

The question of the existence of true psychrophilic bacteria has prompted several investigations. Hess (23),

working with Ps. fluorescens and Flavobacterium deciduosum, noted that practically all cultural characteristics were evident at -3 C. Adaption to low temperatures was possible since prolonged cultivation at 5 C. produced strains that were extremely active at -3 C. Gibbons (18) made similar observations which gave evidence of psychrophilic characteristics of marine bacteria; lengthy storage of fillets at -5 C. for 50 weeks resulted in a relatively larger number of colonies on plates at 5 C. than at 10 or 25 C. Hess (23) pointed out that since maximum crop yields should be considered as the best criterion for optimum growth temperature, marine bacteria may be termed as psychrophilic. On the other hand, Lochhead (33) concluded, from a study on frozen soils, that psychrophilic tendencies did not exist and the bacteria merely exhibited psychrotolerance.

Hilliard et al. (25) made a comprehensive study on the resistance of bacteria to freezing and presented a list of factors which may influence the longevity of bacteria in suspensions: (1) species and strain of bacteria, (2) history and cultural manipulation prior to freezing, (3) chemical and physical composition of the medium, (4)temperature and duration of freezing, and (5) the abruptness of changes in temperature. A similar investigation was carried out by Hess (24); in general, his findings supported the above summary.

The presence of salt had a definite effect on the numbers of bacteria. Bacteriological examination, by Sayer and co-workers (48) of butter in cold storage, showed that the number of acid-producing bacteria is directly related to the salt content. High salt concentrations appeared to preserve or protect such bacteria. Less concentrated solutions would reach a solid state sooner due to a relatively higher freezing point, thus resulting in mechanical crushing by the ice crystals. Somewhat different results were reported by Hess (24); the absence of salt in broth cultures of Ps. fluorescens was more injurious than 1 to 3 percent salt and concentrations of near 10 percent and over were again destructive. Wide differences between the two media and type of organism used probably accounted for the variations in results. However, one may conclude that salt acts as a protective agent in certain concentrations, either directly due to its chemical properties or indirectly by altering the physical state of the medium. Further support to this protective effect was given by Tanner and Wallace (61) who observed a slower death rate by freezing in physiological saline than in broth.

The hydrogen ion concentration was shown by Hess (24), Tanner and Wallace (61) to have a considerable effect on destruction by freezing. Smallest reductions occurred near the neutral point; reactions on the alkaline side were less harmful than corresponding ones on the acid side. McFarlane (38), studying E. coli, noticed that a greater destruction took place at a low pH. In all these

investigations the pH was measured before freezing. It is not known whether the pH remained constant throughout the change from liquid to solid phase since concentration of salts and solid matter may occur during freezing.

Tanner and Wallace (61) also investigated the behaviour of various pure cultures in a large number of frozen fruits and vegetables; considerable reduction was shown but in no instance was sterility reached, even after storage for two years. It is difficult to predict the longevity of any organism in most foods; for example, organisms of the colon-typhoid group were destroyed in two weeks in frozen cherries while in cherry juice they remained viable for five months. Considering the protective effect of the solid content, a greater degree of protection would be expected with the whole berries. No doubt, the survival of microorganisms is complex and involves various interlated factors.

Sucrose, commonly used as a preservative in frozen fruit products, has been shown to affect the survival of microorganisms. McFarlane (38) noted that concentrations of 30 to 50 percent definitely retarded the destruction of E. coli and a species of Saccharomyces.

Sufficient experimental evidence exists to-day to show that extremely low temperatures as such are no more destructive than those just below freezing. Such effects were investigated as early as 1900 by McFayden (36). Exposure of various organisms, including pathogens, to liquid air temperature (-190 C.) for periods up to 6 months did not

affect their pathogenicity, fermentative and pigment-producing powers. Liquid hydrogen temperatures (-252 C.) were found to be equally ineffective. Microbial cells at such low temperatures were believed to be in a state of suspended animation. More recent work by Winchester and Murray (66) showed similar results; common bacteria were able to grow after storage at liquid air temperature.

Attempts were made to determine whether laws governing bacterial destruction by chemicals and heat would be applicable for freezing. Kiser (31), using an Achromobacter sp., found that for the first 300 hours the destruction was proportional to the number of viable organisms present. Similar work was done by Tanner and Williamson (60) who used yeasts as the test organism. They also showed that the death rate followed the curve of a monomolecular reaction.

Just what happens in or to a bacterial cell when the medium is frozen is not fully understood. The manner in which low temperature injury or destruction is produced is extremely complex, and up to now, no tenable mechanism has been worked out.

Weiser and Osterud (64) observed that marked death occurred just below 0 C., during the final stages of ice formation, which supports the theory of mechanical action of the extracellular ice. Intracellular ice is not likely to form at this temperature. Further experiments were designed to show that ice does not form within the bacterial cell under any conditions. Repeated fluctuations

from a high to an extremely low temperature should increase the mortality if intracellular ice forms, assuming that death is caused by some mechanical action. However, fluctuations from -1.5 C. to -195 C. did not increase the mortality, indicating that ice was not formed in the cell.

Haines (20) regarded the mechanical destruction theory as untenable. In the first place, the rate of freezing appeared to have little effect on the proportion of cells destroyed. He reasoned that if bacteria are killed by mechanical crushing, one would expect greater destruction with slow freezing which generally produces larger ice crystals. Further evidence against the mechanical crushing theory was given by actual measurements of yeast cells. Saccharomyces cereviviae cells showed no significant change in size when carefully measured in the frozen and thawed state; the cells appeared to be lying undistorted among crystals of ice about ten times the length of the cell. He suggested a critical temperature of storage at about -2 C. and considered two factors responsible for death; one, some change in one fraction of the cellular protein leading to denaturation and flocculation, and the other, unknown, but apparently not mechanical.

Colloidal particles, such as milk constituents, have been shown by several workers (30,26,65) to exert a protective influence on microbial cells. Intercrystallic films are probably thick enough to accomodate microbial cells, thereby permitting them to escape from the action by ice crystals.

In a later experiment, Weiser and Hargiss (65) compared the effects of vitromelting, devitrification and crystallization on E. coli. In order to obtain vitreous ice formation, 10 percent sucrose was used in preparing the suspensions, hence a new factor was brought into play. Results indicated that vitromelting was more lethal than crystallization and the devitrification treatment was found to be more lethal than either vitromelting or crystallization. This indicated that the intracellular physical state resulting from the vitromelting treatment was more injurious than the crystallization treatment, or in other words, additional "forces" were operative besides mechanical destruction. No mention was made of what these "forces" might be.

Several investigators have tried to either prove or disprove the theory that ice formation is necessary to cause death. Hilliard and Davis (26) noted that the death rate of E. coli is higher in media which are frozen than in liquid media at the same temperature. Similar findings were reported by Berry (4), and as a result the "cold per se" factor was ruled out and the physical crushing concept was generally being accepted. The results reported by Hess (24) do not support these conclusions; three test organisms showed higher reductions in super-cooled sea water than in frozen sea water at the same temperature. Variations in experimental procedures should be noted. Crystallization in the former investigations was prevented by lowering the freezing point, using sugar solutions, while Hess attained true super-cooling without introducing a new factor.

In support of the "cold per se" concept, Hess concluded that cells in a less active state, as in the frozen state, would have more vitality and greater longevity than cells in the super-cooled medium which would be in a more active state. Keith (30) also emphasized the rate of metabolism as a possible factor.

Experimental work on inorganic hydrogels, as discussed by Fisher (16) and Zsigmondy (67), showed that in capillary spaces much of the water failed to freeze even at as low as -78 C., indicating that cells surviving exposure to low temperatures may have little or no intracellular ice.

Weiser and Osterud (64) also supported the concept of concentration of solutes in the intercrystallic films as another factor which could contribute to the lethal action of freezing. In all probability this effect occurs in combination with others mentioned.

Since freezing does not destroy all microorganisms, the survival of pathogens in frozen foods is of considerable consequence. Variable results have been reported. The type of food, storage temperature and species of bacteria have been shown to influence survival periods, as pointed out by Tanner (59). Consequently, there is no assurance that potentially dangerous foods will become safe through continuous storage.

Prucha and Brannon (42) reported E. typhi as surviving in ice cream for over two years, suggesting that such longevity might be expected in other frozen foods.

Thomas (62) believed that variations in resistance of typhoid organisms to freezing were due mainly in a failure to simulate natural conditions. Organisms leading a saprogenic life for years would be more resistant than the same organisms in a highly virulent parasitic condition.

Hahn and Appleman (19) inoculated concentrated orange juice with E. coli, S. typhosa and Sh. paradysenteriae, and Streptococcus faecalis. Within 48 hours none of the organisms except Str. faecalis could be recovered. The increase in the concentration of organic acids, mostly citric, is probably the main factor contributing to the lethal action of freezing. Similar results were reported by Burton (8); fecal streptococci were found to be superior in their ability to survive freezing storage. As a result of these and other investigations, it has been suggested that the fecal streptococci replace coliforms as indicators of fecal pollution in frozen foods.

Closely related is the problem of toxicogenic microorganisms. Although botulism has received considerable attention, Tanner (59) concluded that if frozen foods are kept frozen little danger will be encountered from toxin-producing organisms. However, enterotoxin producing bacteria were readily isolated from frozen foods by Jones and Lochhead (29). It appeared that the organisms do not produce the toxin at frozen storage temperatures.

In summary, the following facts have been more or less established:

1. Some bacteria, probably psychrophiles, grow and function well at freezing temperatures. Others do not grow at these low temperatures but are not easily destroyed at such temperatures.
2. Extremely low temperatures are not any more destructive than temperatures at or just below 0 C. if temperature alone is the only consideration.
3. Destruction appears to be greater in frozen suspensions than unfrozen suspensions at the same temperature, indicating physical crushing.
4. Microorganisms react quite differently towards cold. This appeared to be true to a certain extent even in strains of the same organisms and between cultures of the same strain.
5. Freezing causes chemical changes in foods. The effect of these changes on bacteria is not well understood.

The investigations on the survival of microorganisms in whole milk are not necessarily applicable to concentrated milk since factors which might affect microbial viability are introduced by concentration. Ice cream is similar in total solids to a three-to-one concentrate but differences in physical properties may affect the susceptibility of bacteria to destruction by freezing.

It has been fairly well established that milk constituents exert a protective effect but it is not known to what extent this action will occur after concentration. The metabolic state of the cell at the time of freezing may be another factor influencing survival at low temperatures.

From the foregoing discussion it would be difficult to predict the behavior of the bacterial flora in frozen concentrated milk. Two brief references have been made to the bacterial count of this product stored below freezing temperatures, but from these it was difficult to decide what effect freezing may be expected to have on the bacterial flora. It is hoped that the studies here will provide a more definite answer.

EXPERIMENTAL PROCEDURE

Technological

Fresh, fluid, mixed milk was used in the preparation of the concentrated milk. Six lots were prepared and in each case 160 pounds of raw milk were used, this amount being necessary for normal operation of the vacuum pan. The raw milk was obtained from two sources, the University herd and a commercial source, the latter was generally lower in quality.

All equipment used was carefully cleaned with commercial washing powder. Just before processing the assembled equipment was "sanitized" by using a hot chlorine solution of approximately 200 p.p.m.

Processing procedures were not uniform for all lots; the following table outlines the variations and source of raw milk.

Lot	Source of raw milk	Heat treatment	Concentration	Time required to concentrate
A	Commercial	155 F for 30 min.	3:1	90 min.
B	Commercial	155 F for 30 min.	4:1	45 min.
C	University	155 F for 30 min.	3:1	50 min.
D	University	175 F for 15 min.	3:1	45 min.
E	University	180 F for 15 min.	3:1	45 min.
F	University	180 F for 15 min.	3:1	45 min.

Some difficulty was encountered with the vacuum pan in concentrating Lot A and this accounts for the longer time required to reach the 3:1 ratio.

An attempt was made to obtain raw milk of exceptionally high quality, Lot C was selected for this purpose. Milk cans and pails were sterilized by autoclaving in the laboratory. Extra care was taken in sanitizing the milking machines and cleaning the cows udders. The milk was cooled in a refrigerated water tank.

After pasteurization or heat treatment, the milk was cooled to 140 F. and drawn into the vacuum pan for concentrating. A stainless steel, Majonnier 16 inch Laboratory Vacuum Pan was used. The milk was condensed at 23 inches of vacuum (136 to 138 F.). The degree of concentration was estimated by drawing off a sample with the equipment provided and taking a Baumé hydrometer reading.

The hot concentrate was then run into "shot-gun" type containers, dumped into a small vat connected to the homogenizer, and homogenized at a pressure of 2500 pounds. No attempt was made to standardize any lot to a definite composition. With the exception of one lot, which was a four-to-one concentrate (one-fourth the volume of normal milk), all lots were of a three-to-one ratio. The total solids varied from 35 to 39 percent with the 3:1 concentrate while the 4:1 batch had a solids content of 52 percent.

Following homogenization, the warm concentrate was run into the previously used containers and dispensed directly without initial cooling or freezing into half-pint and pint paper containers (Perga nesting-type). These were sealed with an electrically heated sealer and a metal crimp. The filled

containers were immediately placed in a specially constructed air blast at -15 F; freezing was accomplished in about 25 minutes. The frozen concentrate was stored at -15 to -20 F.

Stabilizers, sodium citrate and ascorbic acid, were added to two lots in hope of extending the storage life of the product. 0.2 percent sodium citrate was added to a portion of Lot D prior to packaging. With Lot F, 0.15 percent sodium citrate was added to the raw milk and 0.01 percent ascorbic acid, dissolved in sterile distilled water, was added to the concentrate just before homogenization.

Bacteriological

Sterility tests were made on a number of paper containers prior to using them for storing the concentrate. Methods and medium used were those recommended in "Standard Methods for the Examination of Dairy Products" (1). In each case the "Estimated Number of Colonies per container" were less than 20, indicating that such containers could be safely used without interfering with experimental results.

Samples, representing a line-run of each lot, were taken throughout the routine of manufacture as follows: (1) raw milk from the vat after thorough mixing, (2) heat treated milk from the vat, (3) concentrated milk leaving the vacuum pan, (4) concentrated milk after homogenization, before freezing, and (5) frozen samples after 3 and 7 days storage. Sterile pipettes and vials were used for this purpose and on completion such samples were kept under refrigeration until plated. Not more than 2 hours elapsed between sampling and plating.

Tryptone Glucose Extract Agar (TGEM) was used for plate counts. Whenever dilutions higher than 1:10 were prepared, one percent of sterile skim milk was added prior to pouring. The pH was adjusted to give a final reaction of pH 7.0. A pressure of 15 pounds (120 C.) for 20 minutes was used for sterilization. This medium had the following composition:

Beef extract	3 gms.
Tryptone	5 gms.
Glucose	1 gm.
Agar	15 gms.
Distilled water	1000 ml.

Frozen samples were removed from storage, thawed within one hour in a 30 C. water-bath, and plated. Bacteriological analyses of the concentrates were made on a gravimetric basis. The purpose of using a gravimetric sample was to overcome the variation in volumetric samples of melted concentrate. The counts, therefore, represent the number of bacteria per gram. Duplicate plates of each dilution were poured with TGEM agar and incubated at 25 C. for 5 days. This somewhat longer incubation period was necessary to facilitate counting of colonies where undiluted concentrate had to be plated. A good source of light and a hand lens were used to count such plates. Plates having dilutions of 1:10 or higher were counted with the aid of a Quebec colony counter

Dehydrated Violet Red Bile Agar (1) was used for plate counts of coliforms. Sterilization was carried out at 15 pounds pressure (120 C.) for 15 minutes. A two gram sample was used and plates were incubated at 32 C. for 18 to 24 hours.

"Thermophilic" plates, poured with TGEM agar, were incubated at 55 C. for 2 days. The same medium was used for psychrophilic bacteria but plates were incubated at 3 C. for 30 days.

Differentiation of physiological types of bacteria was made on a basis of their action on litmus milk. Though a detailed classification was not made, bacteria were grouped into one of four groups. Acid-production included acid with coagulation, gas, and reduction with acid. Organisms affecting no change in litmus milk, except to turn it white, were considered to be reducers. The third group, alkaline-proteolytic, included alkali formers and bacteria producing proteolytic changes. Strains causing no change in litmus milk and those not capable of growth were reported as inert.

Areas, judged to be representative, constituting about 16 colonies were marked on suitable plates and all colonies were picked into litmus milk. These were incubated at 32 C. for 6 days. Cultures typifying each morphological and physiological group were carried on TGEM agar slants.

Litmus milk was prepared from fresh skim milk. A saturated solution of litmus was added to give a lavender color. The medium was sterilized at 15 pounds pressure (120 C.) for 10 minutes.

The Hucker Modification (55) of the Gram Stain was used to group the morphological types. Three general groupings were used, gram positive rods, gram positive cocci and small gram-variable cocci-rods. The latter group included any gram negative types.

Pure culture freezing studies were made on six strains isolated from the concentrated milk. These were selected at random from 20 cultures which had been carried on agar slants. The suspending media were concentrated milk from Lot E, homogenized milk, and a phosphate buffer solution. The milks were sterilized at 15 pounds pressure (120 C.) for 10 minutes while 20 minutes autoclaving was used with the buffer solution.

The phosphate buffer solution was a mixture of potassium dihydrogen phosphate and sodium hydroxide. To prepare this solution 50 ml. of 0.2 M potassium dihydrogen phosphate was added to 29.63 ml. of 0.2 M sodium hydroxide; this mixture was then diluted with distilled water to 200 ml., giving a pH of 7.0.

Preliminary work was necessary in order to prepare an inoculum containing a determined number of organisms. Using 10 ml. of nutrient broth, a series of transfers with a standard loop were carried out; tubes of broth were incubated at 32 C. for exactly 24 hours and then one loopful of this culture was transferred to a tube of fresh broth. The plate count method was used to estimate the number of organisms in 10 ml. of broth. Once the number of cells per ml. had been

established, the amount of inoculum required to give a concentration of approximately 300,000 cells per ml. was calculated.

The prepared suspension was dispensed into sterile test tubes, about 12 ml. per tube; one set of the samples was plated immediately, a tube of each of the buffer suspensions was kept at room temperature for 24 hours and then plated, the remainder were placed in wire racks and frozen in the -15 F. air blast. Freezing was completed in about 5 minutes. TGEM agar was used and duplicate plates were incubated at 32 C. for 2 to 3 days. Counts on the concentrate represent the number of bacteria "per gram".

PRESENTATION OF DATA

Quantitative Studies

Table I shows the quantitative bacterial changes occurring during the various steps in the manufacturing process and after the concentrate had been frozen for 3 and 7 days. The counts of the concentrated milk are given on a whole milk basis; this was necessary for comparative purposes and graphic presentation. A wide range in the raw milk counts is evident. The pasteurization efficiency, in every case, was high, 96 percent for Lot B and over 99 percent for the other lots. The lower count raw milk, in general, produced a finished product with a lower count.

Reductions occurred in the vacuum pan or concentration step, as shown by Lots A,D and E. The same lots exhibited an increase in numbers on homogenization. No apparent change in bacterial numbers occurred in Lots C aand F at either stage in processing. A plate count was not available at concentration with Lot B but the overall effect between pasteurization and after homogenization was a decrease. Slight variations were evident on freezing for 3 and 7 days but no trend could be established.

The effect of freezing and storage is given in Table II. A considerable range in total counts is noticeable among the six lots. Continuous storage of concentrated milk at -15 F.(-26 C.) for periods up to 238 days resulted in no significant change in bacterial numbers. Lot F was the last one to be processed and as a result it was not possible to study

TABLE I

The Effect of the various Processing Steps on Bacterial counts of Concentrated Milk.

Sample	Standard Plate Count per ml. on a whole milk basis					
	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F
Raw milk	16,000,000	340,000	2,300	15,000	15,000	440,000
Pasteurized or heat treated milk	720	13,000	22	63	26	550
Concentrated milk leaving vacuum pan	290		20	29	12	500
Concentrated milk after homogenization (just before freezing)	730	3,500	17	140	16	570
Concentrate frozen 3 days	700	4,500	20	250	24	
Concentrate frozen 7 days	800	4,250		200	17	530

TABLE II

The Effect of Freezing and subsequent Storage on the Bacterial count of Concentrated Milk stored at -15 F.(-26 C.).

Days in Storage	Standard Plate Count per gram					
	Lot A	Lot B	Lot C	Lot D	Lot E	Lot F
0*	2,200	14,000	52	520	49	1,700
3	2,100	18,000	60	750	72	
7	2,400	13,000		600	50	1,600
14	2,500			720		1,600
21	2,100		86	700	58	1,400
28		17,000				1,600
35	2,500		84	850	61	
42		14,000				
49	2,000		79	560	74	
56		20,000	52		56	1,600
63	2,400	17,000	89	460		
70	2,200	15,000		500	46	1,200
77	1,500		74			
84		16,000		490	45	
91	1,700		85		50	
98		17,000		690		
105	1,800		79	390	54	
112		15,000	83			
119	2,100	18,000		480	57	
126	1,600		70		44	
133		15,000		400		
140	1,900		83	420		
147		17,000	71		42	
154	2,300					
161	2,400		74			
168				390	45	
175	1,900		77			
182			75	370		
189	1,900					
196	2,200					
210			65			
224	2,200		72			
238	1,600					

* unfrozen

this lot over a very long period. Due to extreme graininess and thickening of Lot B, it was decided to discontinue further studies after 147 days of storage.

Coliform counts were made on the concentrate from each lot; four of these gave negative results and were not reported while two, Lots D and F, were positive and these counts are shown in Table III. Coliforms were probably introduced during homogenization. Due to small numbers of coliforms present, it was necessary to estimate such counts on a "two-gram basis". No significant change in coliform numbers was noted in either lot while in storage for 70 and 182 days.

Thermophilic and psychrophilic counts are given in Tables IV and V, respectively. These are averages of two counts, one being taken soon after processing and the other near the end of the experiment. Since both types of organisms were represented in very small numbers, it was thought that their presence in frozen concentrated milk was relatively insignificant.

Qualitative Studies - Physiological

Results on the effect of freezing and storage on the main physiological types of bacteria, as determined by litmus milk reactions, are presented in Tables VI to XI, inclusive. Since results are far from uniform in the six lots, it will facilitate interpretation to consider each lot separately.

Lot A: It was decided to pick about 16 colonies from each plate studied. Due to the large number of colonies on the

TABLE III

The Effect of Freezing and Storage on the Coliform Count of Concentrated Milk stored at -15 F.(-26 C.).

Days in Storage	Plate Count per 2 grams	
	Lot D	Lot F
0*	8	5
3	7	
7	5	5
14		1
28	2	3
35	4	
49	2	
63	2	
70	5	4
84	2	
98	1	
105	3	
119	3	
133	1	
140	2	
182	1	

* unfrozen

TABLE IV

Average Thermophilic Counts of Concentrated Milk frozen and stored at -15 F.(-26 C.).

Lot	Plate Count per gram
A	48
B	< 10
C	< 10
D	13
E	< 10
F	< 10

TABLE V

Average Psychrophilic Counts of Concentrate Milk frozen and stored at -15 F.(-26 C.).

Lot	Plate Count per gram
A	20
B	< 10
C	< 10
D	< 10
E	< 10
F	27

plates, it was impossible to pick any more than about 8 percent of the colonies. The majority of the organisms for about the first 63 days storage were acid formers; this was followed by a decrease in such types for the remainder of the period. A corresponding small percentage of inert bacteria in the beginning of the storage period was followed by an increase. A similar effect was noticeable with reducers and proteolytic types, although these were present in smaller numbers; for the first 35 days only reducers were present while from about the 63rd day till the end of the storage period, the reducers disappeared and the proteolytic types became evident. Reduction was often observed to be followed by slow coagulation.

Lot B: This lot was characterized by the apparent absence of reducers and proteolytic types among the colonies picked. Acid formers definitely predominated for the most part. The percentage of inert forms, although erractic at first, were present in equal numbers toward the end of the storage period. Approximately 11 percent of the colonies per plate selected were picked.

Lot C: Since the colony count was lower in this lot, a greater percentage of the colonies could be picked. Inert types comprised the greater portion of the flora. Reducers were absent for the most part. The inert and acid groups were fairly constant throughout the entire storage period, while the other two groups displayed varied results.

Lot D: Acid formers, the largest group represented, remained fairly constant during the 168 days of storage. Inert types

made up about one-third of the total and were fairly constant in numbers. The percentage of reducers and proteolytic types were too erratic to show any trend.

Lot E: Approximately 33 percent of the colonies appearing were picked. These were mainly inert and acid formers, and for the most part they showed slight variations in percentages present. As in previous lots, the reducers and proteolytic types varied considerably during storage.

Lot F: An attempt was made with this lot to determine the physiological types present at each stage in the processing procedure. Unlike previous lots, a large percentage of proteolytic types were present. A high incidence of proteolytic types was noticeable in the raw milk. On pasteurization, a reduction occurred and the inert forms predominated. The percentage of colonies picked is fairly low but, surprisingly, the flora of the concentrate remained uniform during the 56 days of storage. The acid group in this lot consisted mainly of organisms capable of producing only small amounts of acid. Acid with coagulation was evident in a few cases.

Morphological

A total of 935 organisms, isolated from the concentrated milk, were classified into three general morphological groups (Table XII). The types present showed considerable variation, but for the most part gram positive rods and small cocci-rods predominated. Gram positive cocci, although absent or nearly so in Lots A and F, showed no significant change in numbers during storage.

TABLE VI

Lot A: Isolations and Physiological Types of Bacteria found in Concentrated Milk stored at -15 F.(-26 C.)

Days in Storage	Colonies on plate	No. of Isolates	% Isolated	Action on Litmus Milk (percent)			
				Acid	Reduction	Alkaline, Proteolysis	Inert
0*	204	34	17	67	23		9
3	199	16	8	81	19		
7	236	16	7	75	19		6
14	244	12	5	58	25		17
21	212	14	7	93	7		
35	213	16	8	81	6		13
49	194	15	8	93			7
63	228	16	7	69		6	25
77	192	14	7	50			50
105	195	16	8	49		13	38
140	156	16	10	44		13	43
161	238	16	7	37		13	50
196	215	16	7	88		6	6
224	214	16	7	37		19	44

* unfrozen

TABLE VII

Lot B: Isolations and Physiological types of bacteria found in concentrated milk stored at -15 F.(-26 C.)

Days in Storage	Colonies on plate	No. of Isolates	% Isolated	Action on Litmus Milk (percent)			
				Acid	Reduction	Proteolysis, Alkaline	Inert
0*	129	16	12	81			19
3	125	14	9	71			29
7	163	16	10	100			
28	165	15	9	100			
42	139	16	11	94			6
56	188	16	9	100			
70	115	14	12	57			43
98	162	16	10	50			50
133	129	16	12	50			50

* unfrozen

TABLE VIII

Lot C: Isolations and physiological types of bacteria found in Concentrated Milk stored at -15 F.(-26 C.)

Days in Storage	Colonies on plate	No. of Isolates	% Isolated	Action on Litmus Milk (percent)			
				Acid	Reduction	Proteolysis, Alkaline	Inert
0*	48	16	33	25			75
3	50	16	32	32	6	12	50
21	79	14	18	14	14	28	44
35	73	16	22	19	13	18	50
49	66	16	24	19		37	44
63	88	16	18	19		13	68
91	53	16	30	19		31	50
126	62	16	26	13		6	81
147	68	16	24	12		25	63
182	71	15	21	27		66	7
210	68	15	22	14	14	27	45

* unfrozen

TABLE IX

Lot D: Isolations and Physiological types of Bacteria found in Concentrated Milk stored at -15 F.(-26 C.).

Days in Storage	Colonies on plate	No. of Isolates	% Isolated	Action on Litmus Milk (percent)			
				Acid	Reduction	Proteolysis, Alkaline	Inert
0*	49	20	41	60		10	30
3	72	16	22	69			31
7	57	15	26	73			27
21	65	16	25	50		6	44
49	60	16	27	37		13	50
84	47	16	34	44		19	37
105	39	16	41	69			31
140	39	16	41	50		25	25
168	50	16	32	63	19		18

* unfrozen

TABLE X

Lot E: Isolations and Physiological types of Bacteria found in Concentrated Milk stored at -15 F.(-26 C.).

Days in Storage	Colonies on Plate	No. of Isolates	% Isolated	Action on Litmus Milk (percent)			
				Acid	Reduction	Proteolysis, Alkaline	Inert
0*	48	20	42	45		25	30
3	64	16	25	44		19	37
7	48	16	33	44	13		43
21	49	16	33	32		13	55
35	48	16	33	37		6	56
70	48	16	33	44		31	25
91	57	16	28	37	6	13	44
126	55	15	27	33		60	7
154	45	15	33	52	14	21	13

* unfrozen

TABLE XI

Lot F: Isolations and Physiological types of Bacteria found during Processing and in the Concentrated Milk stored at -15 F.(-26 C.).

Days in Storage	Colonies on Plate	No. of Isolates	% Isolated	Action on Litmus Milk (percent)			
				Acid	Reduction	Proteolysis, Alkaline	Inert
Raw milk*	440	8	2	12		75	13
Pasteurized or heat treated milk*	55	8	15	12		38	50
Concentrated milk*	148	16	11	50	6	44	
0*	171	16	9	43		38	19
14	161	16	10	50		31	19
21	127	16	13	31		44	25
56	154	16	10	50		31	19

* unfrozen

TABLE XII

Isolations and percentages of morphological types present in concentrated milk stored at -15 F.(-26 C.).

Lot	No. of Isolates	Gram pos, rods	Gram pos. cocci	Small cocci-rods, stain gram pos or gram-variable
A	234	23	4	73
B	139	5	35	60
C	180	73	20	7
D	147	41	27	32
E	146	59	29	12
F	89	50		50
Total	935	42	19	39

A few observations were noted with regards to morphological and physiological types. It was previously pointed out under Lot A that a particular relationship was noted between the acid formers and inert forms. A corresponding effect appeared with the small cocci-rods; whenever a change occurred in the acid and inert groups a similar change was evident in the percentage of cocci-rods. Morphological types in the other five lots showed no appreciable change during the storage periods.

The cocci-rods varied from small rods, which could scarcely be distinguished from cocci, to slightly larger distinct rods.

The 42 percent of gram positive rods were principally inert or proteolytic types. The litmus milk reactions showing no other change than reduction were nearly always found to be caused by gram positive rods.

Most of the gram positive cocci were acid formers, the remainder generally were inert in litmus milk. Many of the acid formers produced a smooth coagulum and the morphology indicated streptococcal types.

Freezing Studies on Pure Cultures

The following organisms, selected at random from isolated cultures, were used in the pure culture studies:

Culture No. 1 Gram positive rods, abundant glistening growth, gelatin liquefied, litmus milk coagulated with slow proteolysis.

Culture No. 2 Gram positive rods, abundant growth, gelatin liquefied, coagulation of litmus milk with proteolysis.

Culture No. 3 Gram positive cocci occurring singly and in small clumps, moderate growth, gelatin not liquefied, litmus milk turned alkaline.

Culture No. 4 Gram positive cocci occurring singly and in clumps, scant growth, gelatin not liquefied, litmus milk turned acid with coagulation.

Culture No. 5 Slender gram positive rods, abundant glistening growth, gelatin not liquefied, litmus milk reduced with slow coagulation.

Culture No. 6 Gram negative rods, abundant yellowish growth, gelatin liquefied, inert to slight acid in litmus milk.

The results of the effect of freezing and storage on these six cultures in three suspending media are shown graphically in Figures 1 to 6 inclusive. Each culture will be considered separately.

Culture No. 1(Fig.1): This particular organism showed susceptibility to destruction by freezing, especially when suspended in the buffer solution. The effect was less noticeable with increased milk solids content. Furthermore, the greatest destruction occurred during the freezing process or at the point of solidification. No significant decrease in numbers is noticeable once the buffer solution was frozen. The reduction in numbers with the milk suspension was not only smaller but also more gradual. Organisms in the concentrated milk appeared to be unaffected until after the 5th day of storage, counts being fairly uniform up to this time.

Culture No. 2(Fig.2): As with the previous culture, the largest reduction took place in the buffer suspension. However, this effect was somewhat more gradual since a decrease was noticeable up to the 35th day. Reduction in the concentrate was fairly uniform. Milk suspension counts showed the smallest reduction. A significant drop in numbers was shown by the buffer suspension during the freezing process and after storage for one hour.

Culture No. 3(Fig.3): Counts on the buffer solution indicated a gradual reduction in numbers. No appreciable change in numbers was noticeable with the milk. Counts were too erratic with the concentrate to establish a definite trend.

Culture No. 4(Fig.4): Freezing and storage appeared to have no noticeable affect on the viability of this organism. Considerable fluctuations in counts were evident, particularly in the concentrated milk suspension.

Culture No. 5(Fig.5): A milk suspension was not available for this culture. Considerable reduction in numbers was noted in the buffer suspension during the first few days of storage. Results indicated a slight reduction with the concentrate.

Culture No. 6(Fig.6): The results showed no change in numbers in any of the three suspensions.

The effect of the phosphate buffer itself on the cultures studied produced interesting results. After exposure to room temperature for 24 hours, Culture No. 3 showed a slight decrease, No. 4 gave no change, and the remaining four cultures showed definite increases in numbers.

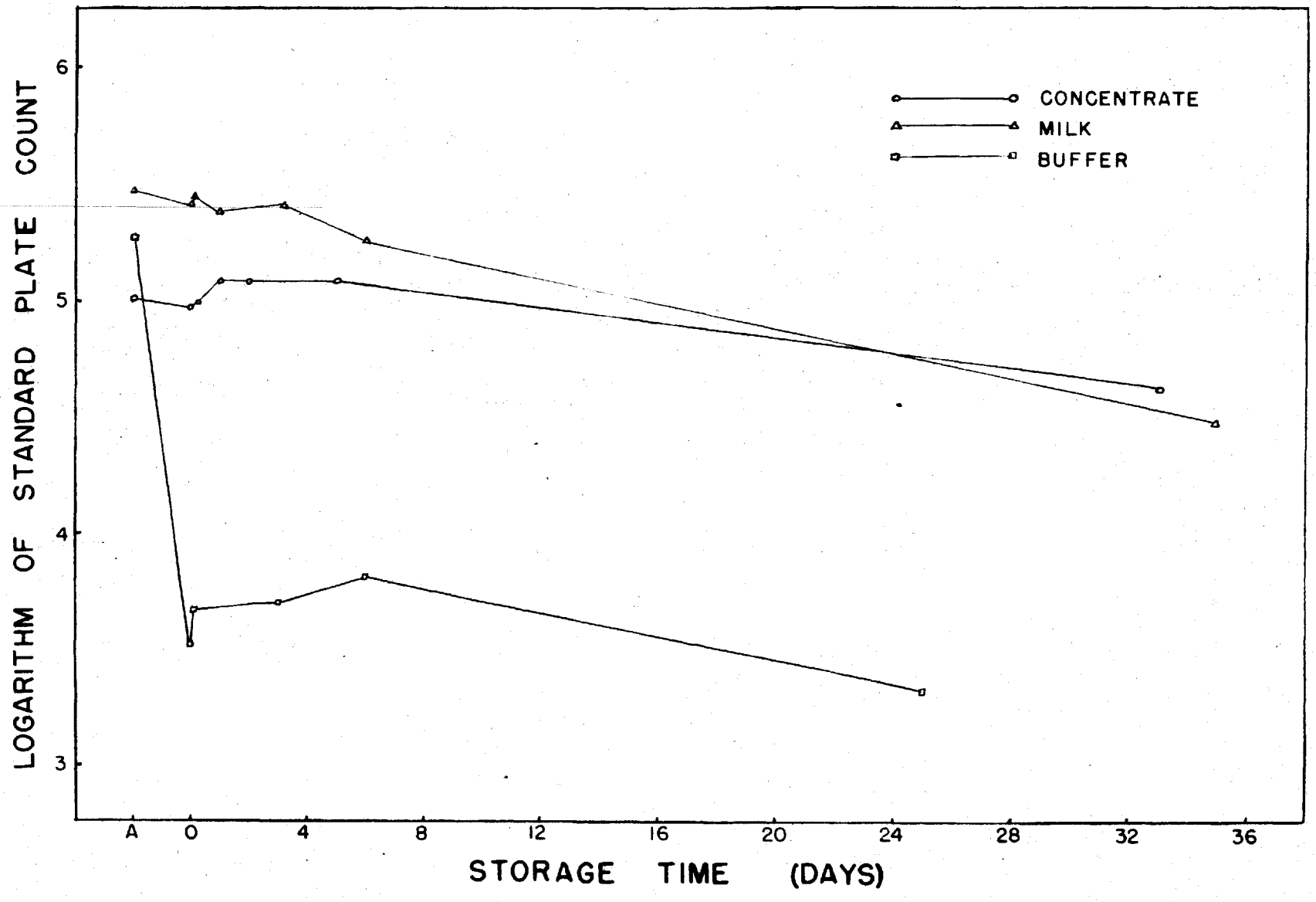

FIGURE 1 The Effect of Freezing and Storage at -15 F.(-26 C.) on suspensions of Culture No. 1(gram positive rod).
A = prior to freezing.

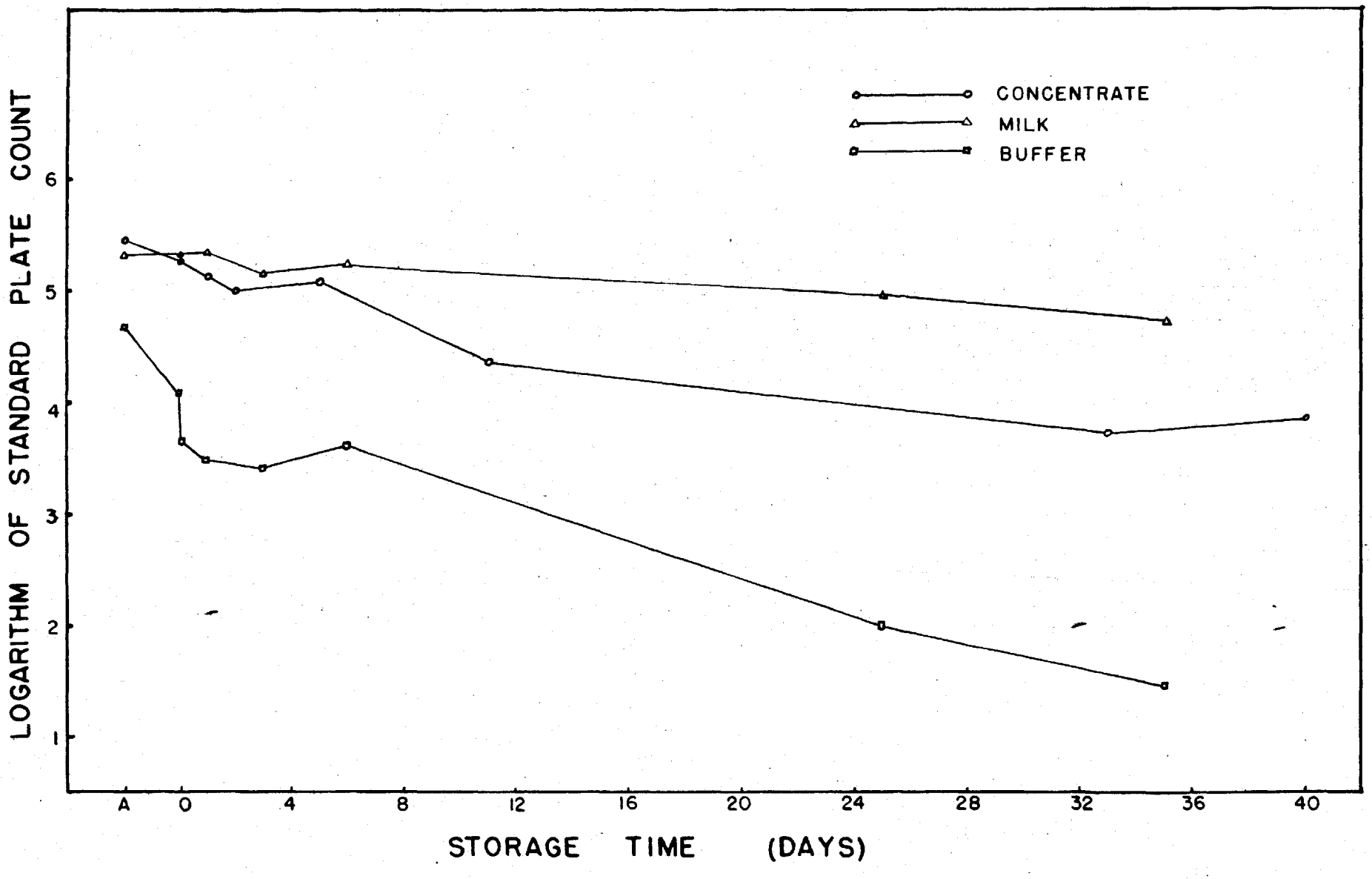

FIGURE 2 The Effect of Freezing and Storage at -15 F.(-26 C.) on suspensions of Culture No. 2(gram positive rods).
A = prior to freezing.

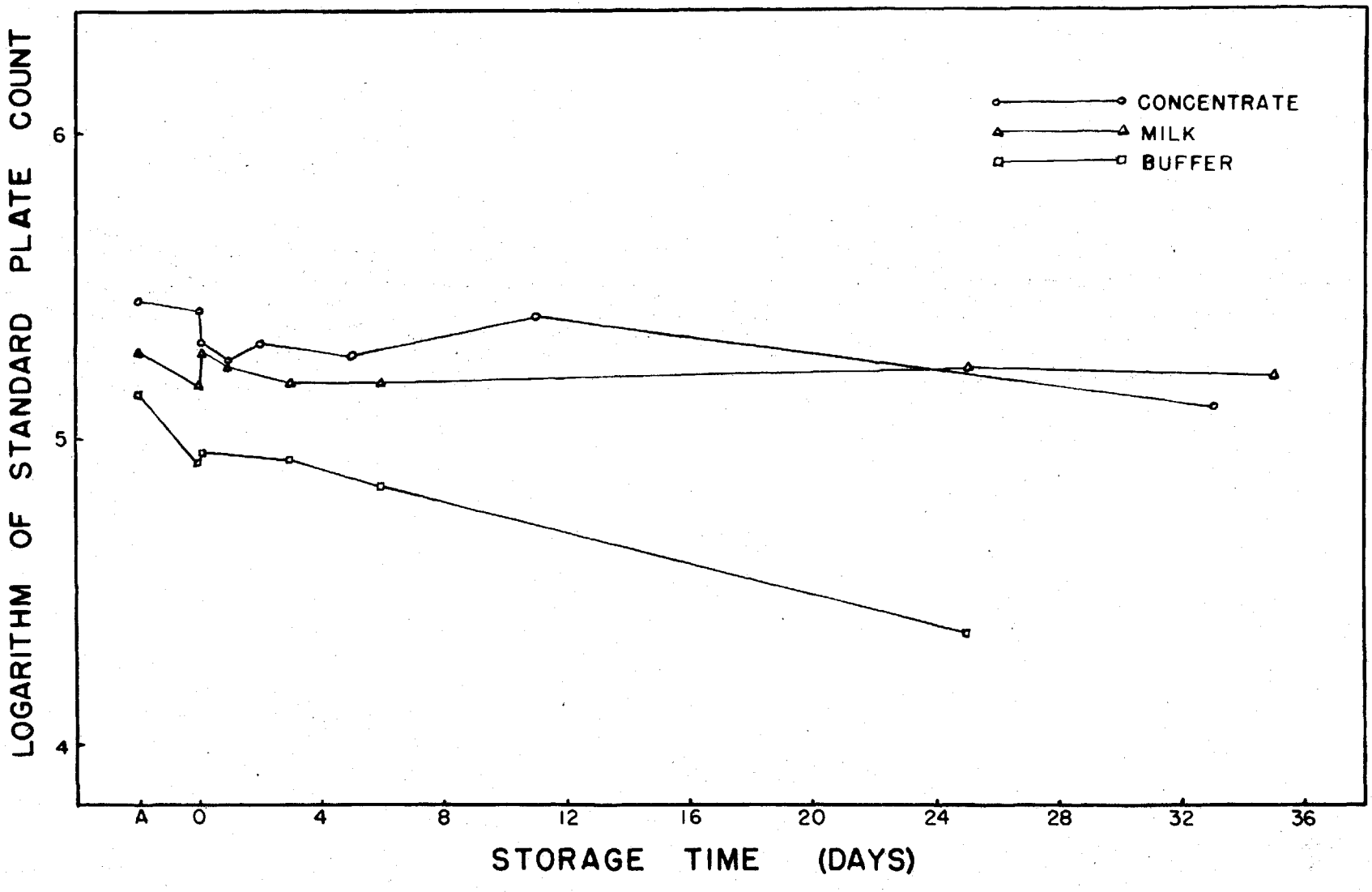

FIGURE 3 The Effect of Freezing and Storage at -15 F.(-26 C.) on suspensions of Culture No. 3(gram positive cocci).
A = prior to freezing.

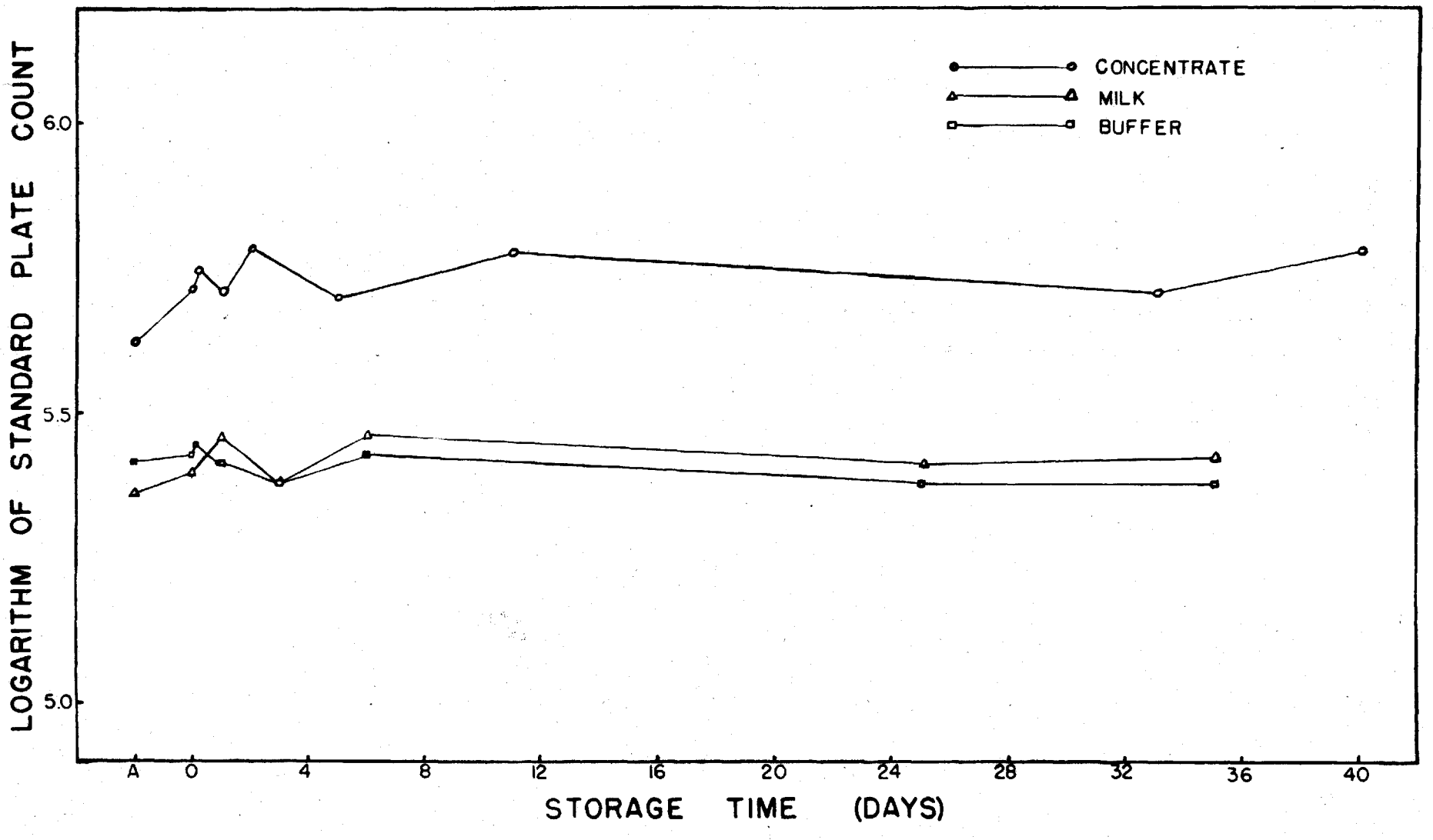

FIGURE 4 The Effect of Freezing and Storage at -15 F.(-26 C.) on suspensions of Culture No. 4(gram positive cocci).
A = prior to freezing.

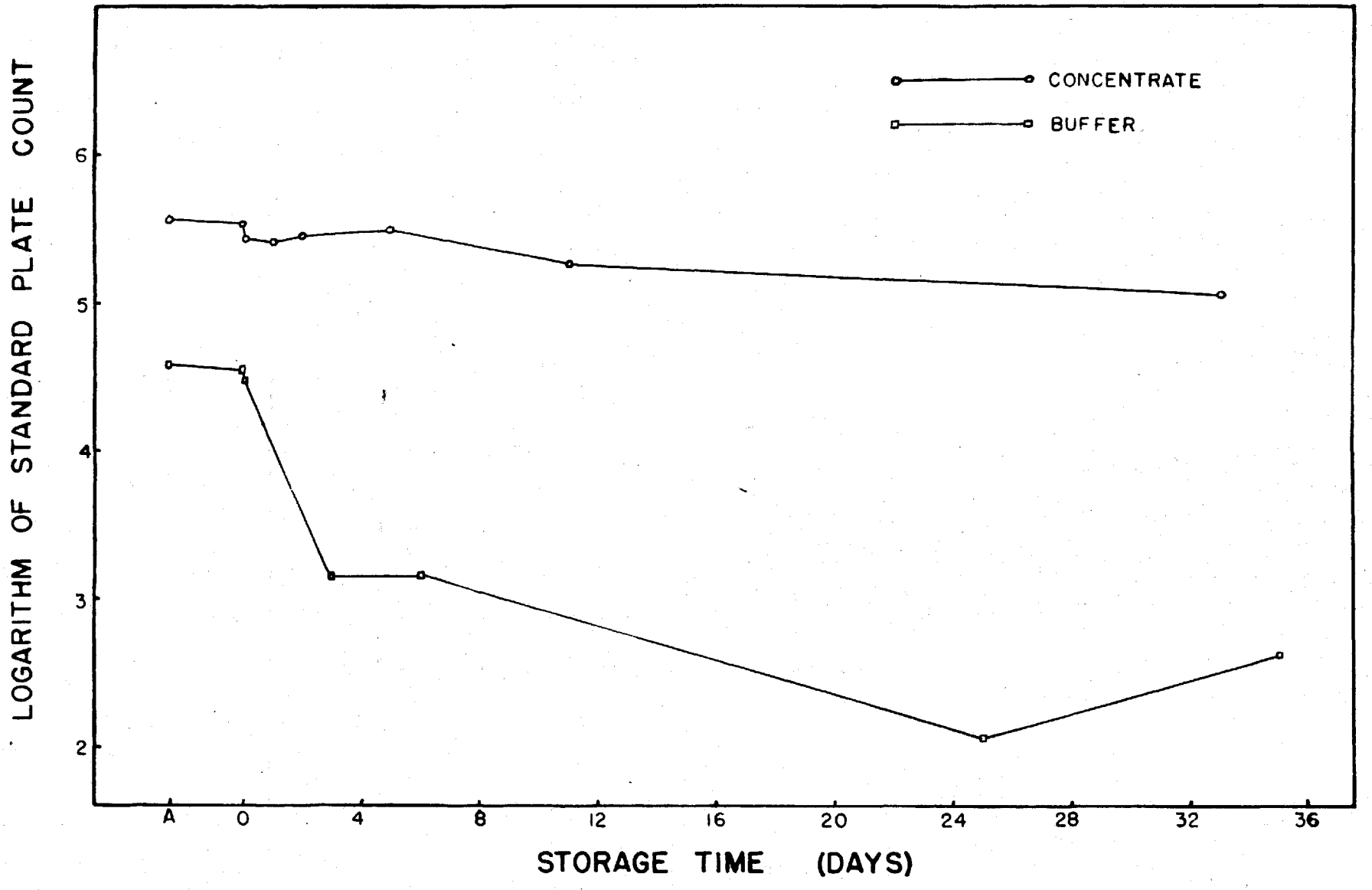

FIGURE 5 The Effect of Freezing and Storage at -15 F.(-26 C.) on suspensions of Culture No. 5(gram positive rod).
A = prior to freezing.

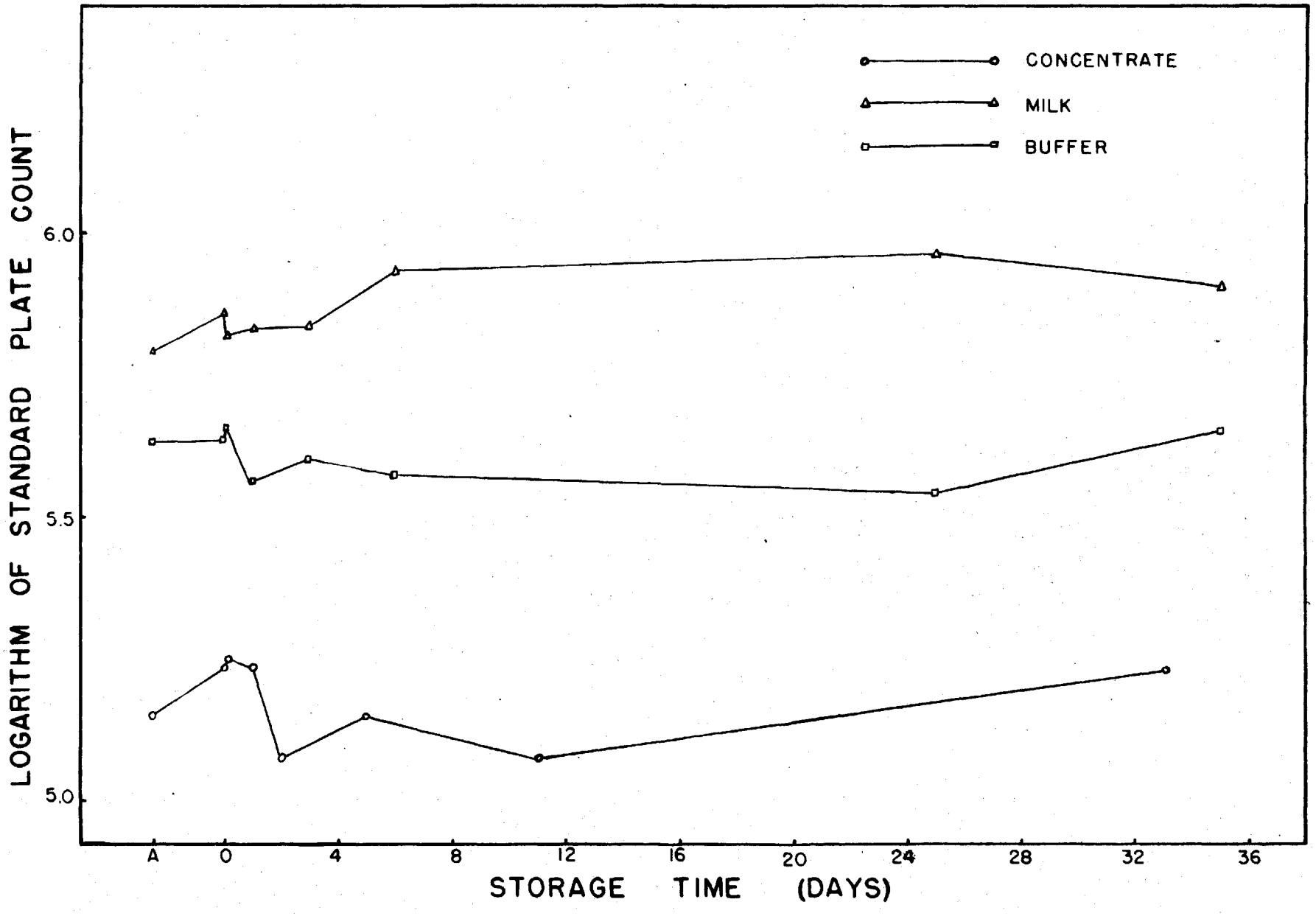

FIGURE 6 The Effect of Freezing and Storage at -15 F.(-26 C.) on suspensions of Culture No. 6(gram negative rod).
A = prior to freezing.

Culture Nos. 1,2 and 5, all gram positive rods, appeared susceptible to destruction by freezing to about the same extent. One of the gram positive cocci cultures was extremely resistant while the other showed a reduction in the buffer solution and concentrate but not in the milk. Culture No. 6, a gram negative rod, also was not affected by freezing temperatures.

Product Acceptibility

The original flavor of the concentrated milk was slightly cooked, however, this cooked flavor gradually disappeared after one to two months storage. Lot B, the 4:1 concentrate did not prove too satisfactory; gelation and graininess, due to crystallization of the lactose, was evident after one month storage and this resulted in poor reconstitution and an abnormal appearance.

Oxidation appeared to be the more serious problem. An objectionable oxidized type of flavor was detectable in Lots C and D after 40 and 15 days storage, respectively.

Lot A had a storage life, that is, acceptable on reconstitution, for about 6 months. After this period thickening and flaking became noticeable. Lot E, in storage for nearly 6 months, had no objectionable flavor but slight flaking was evident. Lot F was of excellent quality, having been in storage for 3 months.

It was too early to determine the effect of ascorbic acid but sodium citrate had a tendency to retard the appearance of flakiness in Lot D.

DISCUSSION

Concentrated Milk Studies

Line-run studies indicated that high quality raw milk was essential for the production of low count concentrated milk. This correlation was not appreciably affected by low or high heat treatments nor by further exposure to near pasteurization temperatures in the vacuum pan. It should be noted that these studies, with the exception of one lot which was processed in spring, were made with winter milk. Similar studies on summer milk may have produced different results, not only in processing but also in subsequent qualitative studies.

It is generally recognized today that homogenization breaks up clumps of bacteria, causing an apparent increase in plate counts. An additional factor complicates matters; some homogenizers are difficult to maintain in good bacteriological condition and this frequently results in being a source of contaminants. Consequently, any increase in numbers may be due to the addition of organisms or due to clumps being broken up. It would be difficult to determine whether increases in bacterial counts on homogenizing in any of the six lots were real or apparent.

Since the majority of frozen food products investigated by other workers showed considerable reduction in numbers, it was surprising to find no change in the six lots of concentrated milk. This is not in agreement with the counts reported by Bell (3) who noted large reductions after five weeks storage at -17 C. This higher storage temperature may be a partial answer to the difference, since various workers have reported greater

destruction at temperatures just below freezing than lower temperatures. However, a difference of 9 C. may not effect a noticeable change. The rate of freezing may be another factor contributing to disparity.

The plate method, when used to estimate numbers in frozen material, is actually a measure of two effects; one, the freezing action which may reduce the number of viable cells, and the other, freezing and thawing which may break up bacterial clumps, thus giving an apparent increase. As a result, it is possible to have these opposing effects operative at the same time with no apparent change in the plate count. However, homogenization of the concentrate prior to freezing probably broke up most of the clumps and lessened the freezing-thawing effect.

Errors arising from inaccuracies of the plate count, although difficult to calculate, nevertheless, are always present. These will not be discussed here. In support of the reliability of these plate counts, two features of the experimental methods require further mention. In order to overcome unrepresentative sampling, arising from possible redistribution of suspended and dissolved substances, it was decided to eliminate sampling of the concentrate in the frozen state. The problem of sampling frozen foods has confronted food technologists for some time and has not yet been completely solved. Due to the viscosity of the concentrate, gravimetric measurements were used to reduce any major inaccuracies introduced in preparing the initial dilution.

As shown by low psychrophilic and thermophilic counts, the predominant flora of the concentrate undoubtedly belongs to the mesophilic group. Further evidence was given by the isolated cultures which displayed abundant growth at 25 and 32 C. It has been reported by Johns and Bérard (27) and also by Lochhead and Jones (34) that organisms in frozen vegetables and egg melange developing at 4 C. were least resistant to freezing. In other words, psychrophiles are readily destroyed by freezing. The low incidence of psychrophiles and no change in numbers on freezing appeared to correspond with findings from the literature.

In designing the qualitative experiments, a reduction in the bacterial flora was anticipated, consequently, it was decided to determine which types are destroyed by freezing and which remain viable. These results need to be interpreted with care since a critical study was not made. In addition, the isolation technique used here, a method nearly always used to study heterogenous flora, was not as accurate as desired. It was only possible to pick a fairly small percentage of the colonies in most cases; this introduced inaccuracies in addition to those generally present on plating. For this reason, large, but not surprising, differences were noted. A value of 100 percent does not necessarily mean that only one group of organisms were present in the concentrate, nor does a zero value indicate the absence of a particular group. Conclusive statements cannot be made on one or two isolations.

Since quantitative studies have shown constancy in counts, it can more or less be expected that the various groups of bacteria, either on a physiological or morphological basis, should remain fairly uniform. This, for the greater part, has been found to be true, hence confirming standard plate counts.

The striking results obtained in Lot A are probably an artifact rather than actual changes in the flora present. Since nearly all of the litmus milk reactions which showed reduction and proteolysis were caused by gram positive rods, it would appear from the data that their reducing ability was transformed during storage to proteolytic types. Since it was also observed that reduction was often followed by slow coagulation and proteolysis, this would tend to support the fact that there were no real changes in the actual types present. The trend of the acid formers and the inert group could also be a mere artifact, however, slight attenuation is perhaps suggestive. These small cocci-rods were weak acid formers originally, therefore, it may be possible to have had a partial or temporary loss of this characteristic. From the literature on this subject, as far as is known, a loss of a certain cultural characteristic has never been reported.

It was previously mentioned that potentially dangerous foods cannot be rendered positively safe through freezing because viable and virulent pathogens have frequently been recovered. Although pathogens were not used

in this study, the results would tend to indicate that no appreciable reduction could be expected with most of the bacterial types in concentrated milk. This is further emphasized by the presence of coliforms which were still recoverable after six months storage. As in processing and handling other dairy and most frozen food products, sanitation cannot be over-looked.

Investigations indicate that concentration and freezing may have possibilities as a method for preserving milk for future use. The factors which affect the storage life are numerous. With continued research a concentrate that fully satisfies market requirements should not be too far in the future

Pure Culture Freezing Studies

Pure culture freezing studies were prompted by the absence of any significant changes as shown by the quantitative and qualitative results. Therefore, it seemed reasonable to expect that certain factors must contribute to their survival in the frozen state. The most likely ones would be: (1) the organism itself, either a resistant type or strain, (2) the protective effect exerted by the milk constituents, and (3) the metabolic state of the cell at the time of freezing.

Identification of cultures was not carried out any further than that given by the brief description of each culture used in the freezing experiments. One or two of the pure cultures may be of the same type or strain, however, a wide range in susceptibility to destruction by freezing is quite evident. Regardless of the suspending medium, the

varied resistance among organisms was still noticeable. The low resistance of the gram positive rods and the greater resistance of the gram positive cocci and gram negative rods is probably a characteristic of the bacteria themselves.

The main purpose in using the phosphate buffer solution was to confirm findings of other workers, namely that suspended and dissolved substances in milk are protective to bacteria. This buffer could be safely used since no appreciable lethal effect was noted by exposing the suspensions to room temperature for one day.

Since freezing and storage was identical in all cases, the experimental evidence is conclusive in pointing out that milk constituents exerted a definite protective effect. Assuming that destruction of bacteria by freezing is caused by physical crushing, films produced by the milk particles were probably thick enough to accomodate microbial cells, thereby allowing them to escape the crushing action of the ice crystals.

A comparison of the whole milk and concentrate milk can be made since both were homogenized, the only variable being the total solids. There appeared to be a maximum protective effect obtainable with milk constituents since organisms in the whole milk were somewhat less readily destroyed. In other words, additional lethal forces were operative in the frozen concentrate.

Some of the organisms, grown in nutrient broth and reintroduced into the concentrate, were found to be quite sensitive to freezing. Bacteria present in the concentrate have been subjected to extremely adverse conditions during the heat treatment and concentration stages; such cells would exist in a comparatively inactive state of metabolism, consequently, one would expect less sensitivity towards abrupt changes in environment. Cells in the broth were, no doubt, in a very active state of metabolism and more sensitive to low temperatures. It is not implied here that the state of metabolism solely determines survival, but rather that it is a factor influencing destruction at freezing temperatures. The importance of the rate of metabolism as a factor determining sensitivity to low temperature has been pointed out by Keith (30) and Hess (24).

The rate of freezing of the concentrate in paper containers differed somewhat from the inoculated concentrate. Even though freezing was accomplished in a -15 F. air blast in both cases, the rate of freezing was more rapid in the test tubes than in the half-pint containers. Hess (24) found that greater destruction took place on rapid freezing; crystallization would be rapid enough to trap bacteria between the ice crystals. In slow freezing, the bacteria are extruded from the solid into the liquid portion of the medium where a smaller reduction occurs.

However, these studies were done with sea water and the findings may not be applicable to material with a much greater total solids content.

Freezing studies were not designed to provide a mechanism of destruction, however, a few observations were fairly evident. The reduction in numbers in the buffer solution was largest in the earlier stages of freezing, which appeared to favor the mechanical destruction theory. Decreases in the milk and concentrate suspensions were more noticeable after three days storage; this could mean that additional factors were operative, a logical one being the concentration of solutes in the intercrystallic films. These indirect effects were probably caused by the salts and lactose.

SUMMARY AND CONCLUSIONS

1. High quality raw milk appeared to be necessary for the production of low count concentrated milk.
2. From the studies on changes during processing, it was difficult to decide whether or not homogenization broke up clumps or colonies of bacteria.
3. Freezing and subsequent storage of concentrated milk at -15 F.(-26 C.) for periods up to 238 days resulted in no appreciable change in bacterial numbers. The physiological and morphological types also remained fairly uniform, which supported the quantitative studies.
4. The low incidence of thermophiles and psychrophiles made their presence in frozen concentrated milk relatively insignificant.
5. Bacteria varied in their resistance to freezing. Gram positive rods appeared less resistant than other types.
6. The metabolic state of the cell at the time of freezing should be regarded as a possible factor influencing destruction at low temperatures.
7. Milk constituents definitely exerted a protective effect. Survival was slightly greater in the milk suspension, indicating that an additional lethal action was present in the concentrate.
8. Two mechanisms of destruction have been suggested, one, mechanical crushing, and the other, concentration of solutes.

9. Since most of the bacterial types, including coliforms, were recovered after periods up to 6 months storage, a high standard of sanitary procedures should be maintained.
10. Two lots of frozen concentrated milk were stored for 6 months without appreciable deterioration in flavor or body.
11. Low temperature storage of concentrated milk for future reconstitution may offer definite possibilities as a method for marketing fluid milk.

APPENDIX A

The Effect of Freezing and subsequent Storage at -15 F.(-26 C.) on pure cultures suspended in Concentrated Milk.

Storage Time	Culture No. (Standard Plate Count per gram)					
	1	2	3	4	5	6
Unfrozen	100,000	280,000	280,000	420,000	360,000	140,000
Frozen	94,000	190,000	260,000	520,000	340,000	170,000
Frozen: 1 hour	97,000	170,000	210,000	560,000	260,000	180,000
1 day	120,000	130,000	180,000	510,000	250,000	170,000
2 days	120,000	99,000	210,000	610,000	280,000	120,000
5 days	120,000	120,000	190,000	500,000	300,000	140,000
11 days		23,000	250,000	600,000	180,000	120,000
33 days	42,000	5,300	130,000	510,000	110,000	170,000
40 days		7,200		610,000		

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APPENDIX B

The Effect of Freezing and subsequent Storage at -15 F.(-26 C.) on Pure Cultures suspended in Homogenized Milk.

Storage Time	Culture No. (Standard Plate Count per ml.)				
	1	2	3	4	5
Unfrozen	290,000	200,000	190,000	230,000	610,000
Frozen	250,000	200,000	150,000	250,000	720,000
Frozen: 1 hour	270,000	190,000	190,000	250,000	660,000
1 day	240,000	210,000	170,000	290,000	670,000
3 days	250,000	140,000	150,000	240,000	680,000
6 days	180,000	170,000	150,000	290,000	850,000
25 days		93,000	170,000	260,000	930,000
35 days	30,000	50,000	160,000	270,000	640,000

APPENDIX C

The Effect of Freezing and subsequent Storage at -15 F.(-26 C.) on Pure Cultures suspended in phosphate buffer solution (pH 7.0).

Storage Time	Culture No. (Standard Plate Count per ml.)					
	1	2	3	4	5	6
Unfrozen	180,000	48,000	140,000	260,000	38,000	430,000
Unfrozen 1 day*	200,000	60,000	34,000	250,000	46,000	3,000,000
Frozen	3,300	12,000	83,000	270,000	36,000	440,000
Frozen: 1 hour	4,600	4,400	89,000	280,000	30,000	460,000
1 day		3,100		260,000		360,000
3 days	5,000	2,500	86,000	240,000	1,400	400,000
6 days	6,400	4,000	70,000	270,000	1,400	370,000
25 days	2,100	100	23,000	240,000	110	350,000
35 days		< 30		240,000	410	450,000

* exposed to room temperature for 1 day