THE VIABILITY OF MICROORGANISMS ISOLATED FROM FRUITS AND VEGETABLES WHEN FROZEN IN DIFFERENT MENSTRUA

BY

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INTRODUCTION

The effect of low temperatures on microorganisms in fruits and vegetables has been a subject of study by many investigators. It is now well known that the microbial content of these products undergoes considerable change in the process of freezing and subsequent freezing storage, and it is generally agreed that freezing temperatures result in a reduction of the initial microbial load. The rate of reduction varies tremendously, both in similar and different products, and is dependent upon a variety of conditions associated with the freezing process. The present study is concerned with a number of phases of the problem, to determine the extent which the various factors contribute either to the protection or the destruction of microorganisms during freezing.

The initial microbial load on fruits and vegetables is comprised of many species of bacteria, yeasts and moulds. Primarily, these organisms are soil types and have little, if any, public health significance. However, if the products are held, either before or after freezing, under conditions favourable for the development of microorganisms, undesirable changes take place in the product and in a comparatively short

period of time the product has spoiled. The rate of spoilage is dependent to a large extent on the numbers and types of microorganisms present. Thus, any measures taken with the initial product to reduce the microbial load will control, to some extent, the rate of spoilage following defrosting of the product. Such practices as washing and blanching, particularly the latter, have a very great influence on the numbers and types of microorganisms present on the product going into the freezer. Properly conducted, the blanching process should reduce the microbial load in excess of 99 per cent. However, even although the blanching is adequate, faulty practices in many commercial plants are responsible for recontamination of products following the blanch process. Recontamination occurs largely from conveyances of the product, such as flumes or belts, or by the handling of the products by workers during inspection or packaging.

Pure culture studies of microorganisms which have been made by many workers have, unfortunately, been limited to comparatively few species of bacteria such as Escherichia coli, Staphylococcus aureus, Salmonella typhosa and a few other well known organisms. While such studies are of interest from an academic viewpoint the writer has found that results of these studies have little, if any, bearing on the effect of freezing

on the heterogeneous microbial flora found on fruits and vegetables.

The present investigation is a continuation of previous studies conducted by the writer on the microbiology of frozen fruits and vegetables with particular emphasis on pure culture studies of organisms found on fruits and vegetables

REVIEW OF THE LITERATURE

FROZEN FRUITS AND VEGETABLES

Previous to 1930 there were few investigations conducted on the microbiology of frozen fruits and vegetables. The earliest reference to studies on these products which provides any authentic information is a paper by Prescott, Bates and Highlands (24). Among other products they examined samples of commercially frozen strawberries, raspberries, orange juice and spinach. These authors found a distinct reduction in the initial microbial load when the products were subjected to freezing temperatures. They also made the observation that higher storage temperatures e.g. - 10°C. (14°F.) were more destructive to microorganisms than lower temperatures.

Smart (30) (31) (32) examined a number of fruits and vegetable products before and after freezing. She found considerable variation in the microbial content of different lots of the same product. Scalding, freezing and storage for periods of 5 to 7 months reduced the average microbial content 94.6 to 99.8 per cent. Even with these striking reductions some frozen products were found to contain up to 1,000,000 microorganisms per gram.

In a later publication (33) this same author reported that storage of blueberries for 9 months at 0°F. resulted in the reduction in microbial content of 59.7 per cent., while a higher storage temperature 6.67°C. (20°F.) for the same period resulted in 99.9 per cent reduction. This agrees with the earlier findings of Prescott, Bates and Highlands (24).

McFarlane (20) studied the distribution and survival of microorganisms in frozen sugar-packed raspberries and frozen brine-packed peas. Among other results McFarlane noted greater decreases with raspberries at higher storage temperatures while the reverse was noted with peas. With raspberries he found the microbial content at -20°C. (-4°F.) greater in sections from the lower half of the container and this corresponded with a higher soluble solids content in this area. Similarly, the microbial content of frozen peas stored at -20°C. (-4°F.) appeared to be correlated with the soluble solids content with the highest count being obtained from the central, cone-shaped area and in the surface.

Pederson, (23) in a study on plate counts on stored, quick frozen vegetables, in some instances, found increased counts as the period of freezing

progressed. Microscopic examination indicated that the increased counts were due to the breaking up of bacterial clumps during freezing, a point which has been suggested by other workers (19) (36). Pederson makes no mention of the method used for the preparation of products prior to analysis. Recent studies made by the writer (12) have indicated that if the Waring Blendor is used for comminution of products the grinding action of this machine may result in increased counts due possibly to the disintegration of bacterial clumps. Van Eseltine et al. (36) also noted increased counts resulting from the use of the Blendor for preparing products for analysis. These same authors found that freezing products in liquid air and in an air blast at -60°F. resulted in such quick freezing that the bacterial content of the foods was essentially unchanged. Luyet and Gehenio (17) also noted this fact and stated that during such rapid freezing the water in the cell is not changed to ice crystals but to a glass-like amorphous mass which they term the "vitreous state", and this results in less injury than freezing at slower rates. Van Eseltine et al., summarizing their results, state that freezing at slower rates allowed multiplication of bacteria before the freezing temperature was reached. This

statement is not substantiated by data presented in graphs nor by other material in the text since with peas they show a marked increase in count during the early hours of freezing storage followed by a levelling off and finally, after 30 hours, a decided decrease. However, with corn the trend was entirely different with a decided decrease during the early hours of freezing followed by a levelling off and finally a more gradual decrease. These findings are somewhat unusual since corn, which is relatively high in starch, might be expected to provide greater protection to microorganisms against freezing and also, in the event of multiplication of microorganisms before the product is actually frozen, corn might provide a greater amount of food material for microbial growth.

Previous studies by the writer (15) on frozen vegetables and fruits showed that the microbial load of freshly packed product may be subject to wide variations which tend to become obliterated during freezing storage at -17.8°C. (0°F.). With the vegetables studied there was a pronounced decrease in numbers of microorganisms during the first weeks of storage after which they declined slowly or remained stationary. With fruits the decrease appeared to be more gradual. Bacteria were the predominant organisms in frozen vegetables while yeasts and moulds were more numerous in the acid

fruits. Even after 9 months storage at -17.8°C. (0°F.) frozen vegetables and fruits contained an appreciable number of microorganisms.

Micrococci and species of Flavobacterium were found to be relatively more resistant to freezing than other types of bacteria encountered in frozen vegetables (16) (10).

More recently a study by Burton (3) of this Division showed that the coliform test was more efficient for detecting contamination in foods prior to freezing and storage while the fecal streptococci are the superior indicator in frozen foods, as the coliform organisms seem less able to survive the storage temperatures.

The fact that the microbial content of fresh fruits and vegetables harvested from the same area of particular fields showed wide variations from day to day led to a study (11) to determine reasons for these variations. It was found that the variations in the microbial content were largely due to weather factors, definite correlation being obtained with bacteria counts and the temperature and humidity prevailing and preceding the time of harvesting. Products harvested during a

period of relatively high temperatures and high humidity generally showed a high microbial content. Material from low growing plants generally carried a heavier microbial load than that further removed from the soil.

Pure Culture Studies

The literature on the effect of subjecting cultures of microorganisms to freezing temperatures is very extensive. Since our main interest is concerned with the range of commercial freezing temperatures the review of the literature, with some exceptions, will cover studies conducted within the temperature range of -28.89°C. (-20°F.) and 0°C. (32°F.).

Wallace and Tanner (38, 39) have presented an excellent historical review dealing with early work on the freezing of pure cultures of microorganisms. Unfortunately, as these authors point out, there was considerable contradiction from one author's work to another and considerable confusion exists even at the present time. As a result of some of the earliest studies recorded it was generally believed that bacteria were quite resistant to freezing. About 1900 another group

of studies indicated that bacteria were easily destroyed by low temperatures. As these authors point out the trend of thought resulting from more recent studies has changed again and it is now generally accepted that bacteria are resistant to low temperatures.

Probably mention should be made of the work of Keith in 1913 (13) since it was concerned with the freezing of Escherichia coli, an organism studied extensively in the present investigation. Keith froze this test organism solidly in tap water at -20°C. (-4°F.) and found that only a fraction of one per cent of the original number remained alive at the end of 5 days. This finding confirmed that of Sedgewick and Winslow (27). However, when the same test organism was placed in tap water and cooled as "water ice or sherbet" (not solid) and held in this condition at -20°C. (-4°F.) a large percentage remained alive for many months. This latter test is far from clear. The writer is at loss to understand how it would be possible to hold this test material, "water ice or sherbet" at -20°C. (-4°F.) without it being frozen solidly. If it is assumed that the temperature is incorrectly stated or that some other factor was introduced to maintain

the test material in a semi-frozen condition then the results are at variance with an earlier study by Prudden (25) who obtained the opposite results.

The most authentic work published in the early years is that reported in a paper by Hilliard, Torossian and Stone (7). They found that suspensions of Escherichia coli frozen in tap water showed a reduction in total number of cells of 93 per cent within 3 hours. A spore-forming bacterium, Bacillus subtilis, in the vegetative stage, showed a variable resistance to freezing while the spores were decidedly more resistant to freezing. They also found that intermittent freezing was only slightly more injurious to bacterial cells than suspensions held continuously in the frozen state. In a later work Hilliard and Davis (8) modified earlier conclusions and stated that in the later studies they found intermittent freezing of bacterial cells much more injurious than continuous freezing. In this later paper they also found that the degree of cold was insignificant insofar as reduction in numbers of microorganisms was concerned. This finding was directly opposed to the earlier report by the same senior author. Like other workers Hilliard and Davis found that freezing bacteria in menstrua other than tap water, such as milk or cream, resulted

in appreciably less destruction to bacteria, indicating that the organisms were afforded protection by these foods.

Berry (2) studying the destruction and survival of microorganisms in frozen pack foods, presented data indicating that lactobacilli and to some extent coliform organisms in peas remained viable for at least 2 years at -10°C. (14°F.). Data are presented on the destructive effect of ice formation on cells of Saccharomyces sp. in wort. The number of viable cells in liquid wort was greater over a period of 6 days at -10°C. than in frozen wort at the same temperature. A table presented in this paper indicates that viable cells in the frozen wort had practically disappeared.

Wallace and Tanner (40,41,42) studied pure cultures of microorganisms isolated from frozen fruits and vegetables. In all instances they found a rapid drop in numbers during the first months of freezing. After the eighth month the numbers dropped slowly. They also noted that spore formers were the most resistant to freezing and that yeasts and moulds were generally more resistant than non-spore forming bacteria. In fruit juice and broth with a decidedly acid reaction, the organisms died

out quite rapidly. These authors, in contrast to work by other authors, present data showing that alternate freezing and thawing probably is no more destructive to microorganisms than continuous freezing, in fact, the data given in tables indicate that it is even less so. They suggest that in view of the fact that this finding conflicts with those of other workers, previous studies had been based on tests in which alternate freezing and thawing were done repeatedly at intervals of just a few hours; their studies consisted of thawing the menstrua at daily intervals and immediately refreezing. They also found that the degree of cold had very little effect on microorganisms although in some instances the lower temperature did not seem as lethal as did -16°C. (-2°F.).

One of the most interesting studies in recent years was conducted by the late R. B. Haines (5) who studied the effect of freezing pure cultures of bacteria. Cultures of Pseudomonas aeruginosa, Escherichia coli and Staphylococcus aureus in aqueous suspensions were used in this study. Using temperatures ranging from -20°C. (-4°F.) to -1°C. (30.2°F.) Haines, like other workers, found the higher storage temperatures more destructive to bacterial cells. Of the 3 organisms studied P. aeruginosa was the most sensitive

to freezing with Staphylococcus aureus the most resistant. Escherichia coli was intermediate between the two. Spores of Bacillus mesentericus were resistant to all temperatures used showing no reduction over a period of 150 days storage. Haines suggests that two factors are responsible for the death of bacterial cells on freezing; one unknown, but apparently not mechanical, and the other, some change leading to flocculation of the cellular proteins; or else there is in reality one process, leading to coagulation, but with a time lag, so that coagulation is not found on immediate freezing and thawing.

Smart and Brunstetter (29), in a study of the types of organisms found on fresh and frozen spinach and kale, state that the predominating types of bacteria on these two vegetables are Achromobacter, Bacillus, Flavobacterium and Pseudomonas species. According to data presented, the microbial flora members of the genus Achromobacter had disappeared during the process of freezing. However, in a later paper by Smart (30) Achromobacter types were prominent in frozen peas, beans and corn. No mention is made of the proportion of types in either the fresh or frozen products.

Hegarty and Weeks (6) conducted an interesting

study on the sensitivity of Escherichia coli to cold shock. They confirmed earlier findings of Sherman and Albus (28) that young cells of E. coli are susceptible to an initial cold shock of 0°C. (32°F.). This sensitivity to cold shock extends throughout the entire logarithmic phase of growth. Mature cells were not affected by either an initial cold shock or prolonged holding at 0°C.

McFarlane (21) studied the effect of freezing Saccharomyces spp. of yeasts and Escherichia coli in distilled water and in concentrations of sucrose solutions varying from 1 to 50 per cent and adjusted to different pH values ranging from pH 3.6 to 6.5. He found that when the hydrogen-ion concentration was the only variable, a reaction of pH 6.5 was more favorable than one of pH 5 for hastening the destruction of yeast cells in some of the sucrose media. With Escherichia coli destruction was greatest in those samples which possessed the greater hydrogen-ion concentration. When temperature was the only variable greater kills tended to occur after several weeks storage, at -10°C. (14°F.) than at -20°C. (-4°F.).

McFarlane and Goresline (22) studying microbial destruction in water and sugar syrup stored at -17.8°C.

(0°F.) over periods of from 1 to 60 weeks, found that greater destruction of microbial cells occurred in water than in any of the syrups used. Escherichia coli, Saccharomyces cerevisae, Saccharomyces ellipsoideus Hansen, Schizosaccharomyces octosporus, a strain of brewer's yeast and an unidentified yeast exhibited greater cold resistance in sucrose than in invert syrup. A Torula sp., Aspergillus nidulans, Zygosaccharomyces pastori and Zygosaccharomyces japonicus exhibited greater cold resistance in invert than in sucrose syrup.

Kiser (14) studied the rate of destruction of an Achromobacter sp. by freezing at -28°C. (-18.4°F.). He found a slight increase in count up to a period of 10 hours following which there was a steady decrease up to a period of 300 hours. After this time there was a distinct decrease in the rate of death of bacteria.

Weiser and Osterud (44) in a recent publication on the death of bacteria at low temperatures, provide some useful information on the mortality of Escherichia coli exposed to freezing temperatures. They report that the mortality due to immediate death by freezing is marked but does not vary with the intensity of the freezing temperature. Data are presented which indicate that immediate death occurs at a brief stage in the freezing process during which extracellular ice formation is

being completed. They report that the rate of storage death at the higher freezing temperatures is very rapid and is much greater at temperatures above -30°C. (-22°F.) than at lower temperatures. Repeated freezing was more lethal than a single freezing. They also found that freezing is much more lethal than supercooling and that repeated fluctuations of temperature of frozen suspensions do not exert a lethal action additional to that of the storage.

Gunderson and Rose (4) studying the survival of bacteria in pre-cooked fresh-frozen chow mein, found that pathogenic enteric bacilli are killed rapidly during the first 5 days at -25.5°C. (-14°F.). After that the rate of death drops off until a more or less resistant population remains. The normal saprophytic flora appears to be killed less rapidly. In either instance the numbers remaining after any given storage time depend primarily upon the initial contamination. When this is high even storage for as long as nine months does not assure a low count.

Studies on food poisoning bacteria, in particular Clostridium botulinum, indicate that spores of this organism survive freezing for long periods. Wallace and Park (37), Straka and James (34, 35), James (9) showed that spores and toxin of this organism

survived freezing for as long as 14 months.

McCloskey and Christopher (18), in a study of some pathogenic bacteria in cold-pack strawberrries, found that in sliced, sweetened strawberries held at -18°C. (-4°F.) Salmonella typhosa could be recovered after 6 months; Staphylococcus aureus after 5 months; Salmonells typhimurium and Salmonells schottmuelleri after one month. Salmonella paratyphi could not be recovered at any time from the frozen berries.

DISCUSSION OF THE LITERATURE REVIEW

Many of the studies reported in the literature have added a great deal to our knowledge of the effect of freezing on microbial cells. There appears to be agreement that freezing generally effects a reduction in numbers either during the initial freezing process or during subsequent freezing storage. However, results of studies on the rate of reduction of microbial cells are extremely variable. If we compare the results with one test organism, Escherichia coli, an organism which has been used extensively by numerous workers, it will serve to illustrate this point. Wallace and Tanner (43) report freezing cells of E. coli in water at -16.11°C. (3°F.) for periods of 1, 5, 6, 9 and 12 weeks and showed reductions in numbers of 20, 50, 60, 80 and 94 per cent respectively. Weiser and Osterud (44), using this test organism in a 0.5 per cent peptone at -15°C. (5°F.), showed a 76 per cent reduction in a period of 3 to 10 minutes. Freezing for 2 minutes at -10°C. (14°F.) effected a reduction of 54 per cent. McFarlane (21) froze the same test organism at -20°C. (-4°F.) and showed a reduction of 99 per cent after 1 week storage. Hilliard, Torrossian and Stone (7) froze this organism in water at -15°C. (5°F.) and effected a reduction of 99.9 per cent in 3 hours. Haines (5)

studied the effect of freezing E. coli in aqueous suspensions and found that after 150 days at -20°C. (-4°F.) a very slight reduction in numbers occurred and at -10°C. (14°F.), a temperature comparable to that used by Weiser and Osterud, a reduction slightly over 50 per cent in the same period of time. The time required to effect a reduction of approximately 99 per cent was between 50 and 140 days.

Contradictions on the effect of freezing micro-organisms in fruits and vegetables are also numerous. Berry (1) states that after one year's storage the microorganisms in frozen foods were so changed that they could not cause spoilage. This statement has not been supported by any other worker. A year after the first paper the same author writes about spoilage of frozen pack peas (2).

These discrepancies do not reflect in any way on the studies conducted by the various workers but rather point to the extreme variability encountered with microorganisms subjected to the freezing process, and also to the fact that, in may instances, experiments were conducted by the various authors under entirely different sets of conditions. The variability of microorganisms will be discussed at greater length in a later portion of the present work.

INVESTIGATIONAL

FRUIT AND VEGETABLE PRODUCTS

The studies reported in this section were conducted over a period of years and are included to demonstrate the degree of variation in the microbial content in similar and different frozen vegetables which had been initially harvested from the same general area of the Central Experimental Farm, Ottawa. All products were packed by the Division of Horticulture and were given essentially the same treatment each year. Vegetable products were blanched, usually until a catalase negative reaction was obtained, and were packed in pint-size waxed cartons in either a 2 or 3 per cent brine, depending on the product.

Procedure of analysis for fresh and frozen products:

The procedure for the analysis of fresh and frozen products follows exactly the same pattern. The frozen product is removed from the freezer, placed in an incubator at 37°C. (98.6°F.) and defrosted to a point where the ice could be separated from the product. One hundred grams of the vegetable are weighed into sterile pint-size sealers and 50 grams of ice or brine solution added. The product is then macerated, using either a flamed, 6-prong cutting knife or a flamed pestle or both, depending on the nature of the product. To this

ground-up mixture is added 100 grams sterile water. This provided the first dilution with a ratio of 1 part product to 2.5 parts water. The whole was then shaken for 5 minutes using a reciprocating type mechanical shaker. Further dilutions were prepared in the usual manner by pipetting 1 millilitre of the first dilution into a 99 millilitre sterile water blank.

A number of tests were made to determine different groups of bacteria but for the studies reported in this section only the method used for determining total numbers of mesophilic bacteria will be given. Duplicate plates were prepared from each dilution. The plating medium was tryptone glucose extract agar and plates were incubated at room temperature, 20 to 25°C., for a period of 4 days.

Isolation of colonies for the purpose of determining the proportion of types consisted of picking 50 colonies from plates of suitable dilution. Where there were more than 50 colonies on a plate the plate was divided into sections and 50 colonies picked from one section. Colonies picked were transferred to tryptone glucose extract agar slopes and incubated for a period of 4 days at 20 to 25°C. Classification into genera was made by macroscopical,

microscopical, and, when required, by biochemical tests.

RESULTS

Data are presented in Table 1 and in Figs. 1, 2, 3 on plate counts obtained with 4 individual samples of asparagus, 7 samples of peas and 5 samples of corn. All of these products were prepared and frozen during the same or in different years from 1935 to 1949. From these data it is evident that there are wide differences in the initial microbial content of the different products. Perhaps more striking are the wide variations in the rate of survival of microorganisms on these products at the different periods of analysis over a period of 30 weeks. In Fig. 1, showing the results of analyses of four samples of asparagus, one sample (No. 4) shows almost complete destruction of microorganisms at the six-week period of analysis while three other samples in the same period show survivals varying from 33 to 84 percent. One other point of interest from the data presented in this graph is the fluctuation in the microbial content with two of the samples (No's 2 and 3) following the initial freezing process. Since these fluctuations have been noted with some samples of all products it might be well to discuss them at this point.

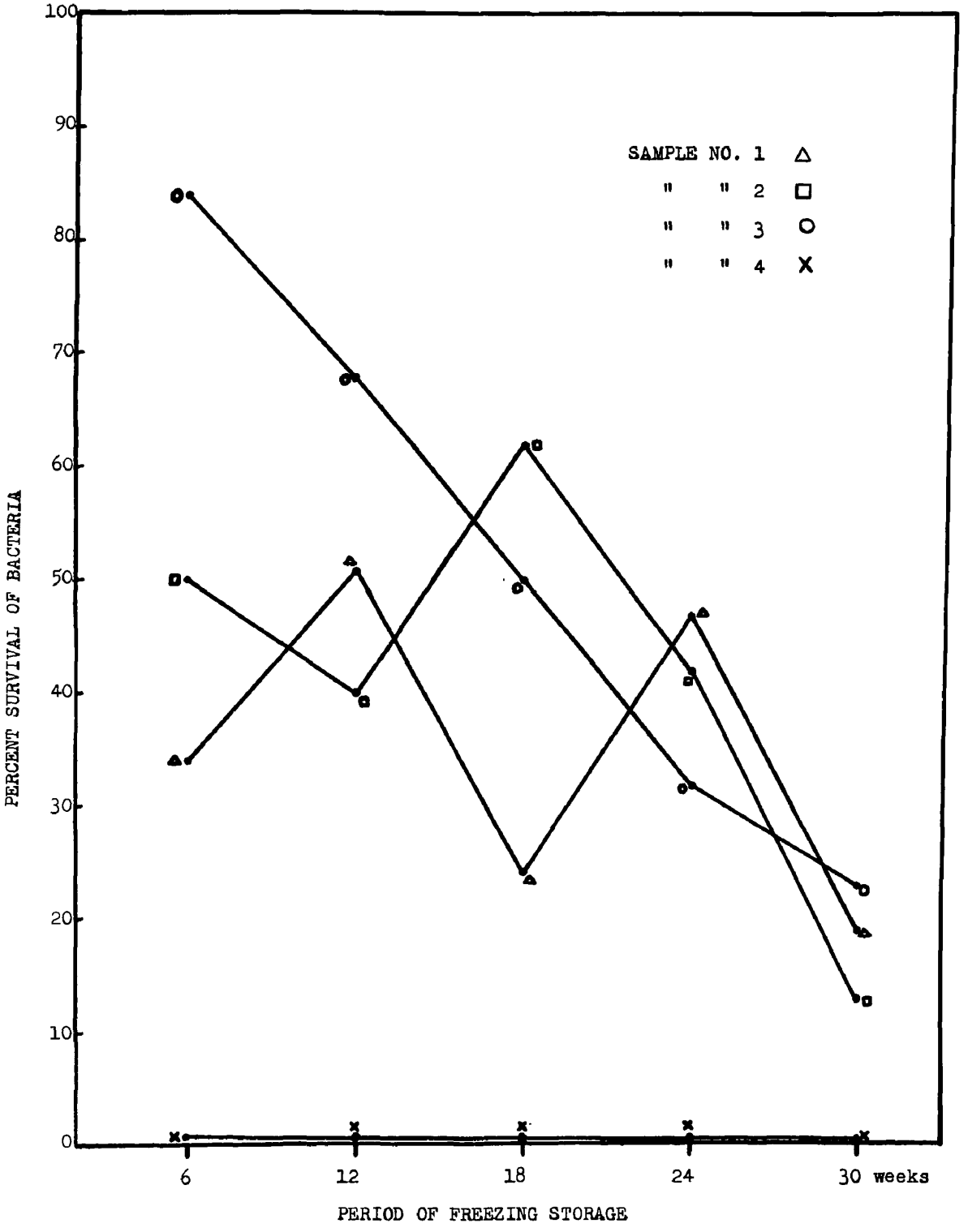

FIG. 1. SURVIVAL OF BACTERIA IN ASPARAGUS FROZEN AND STORED AT 0° F.

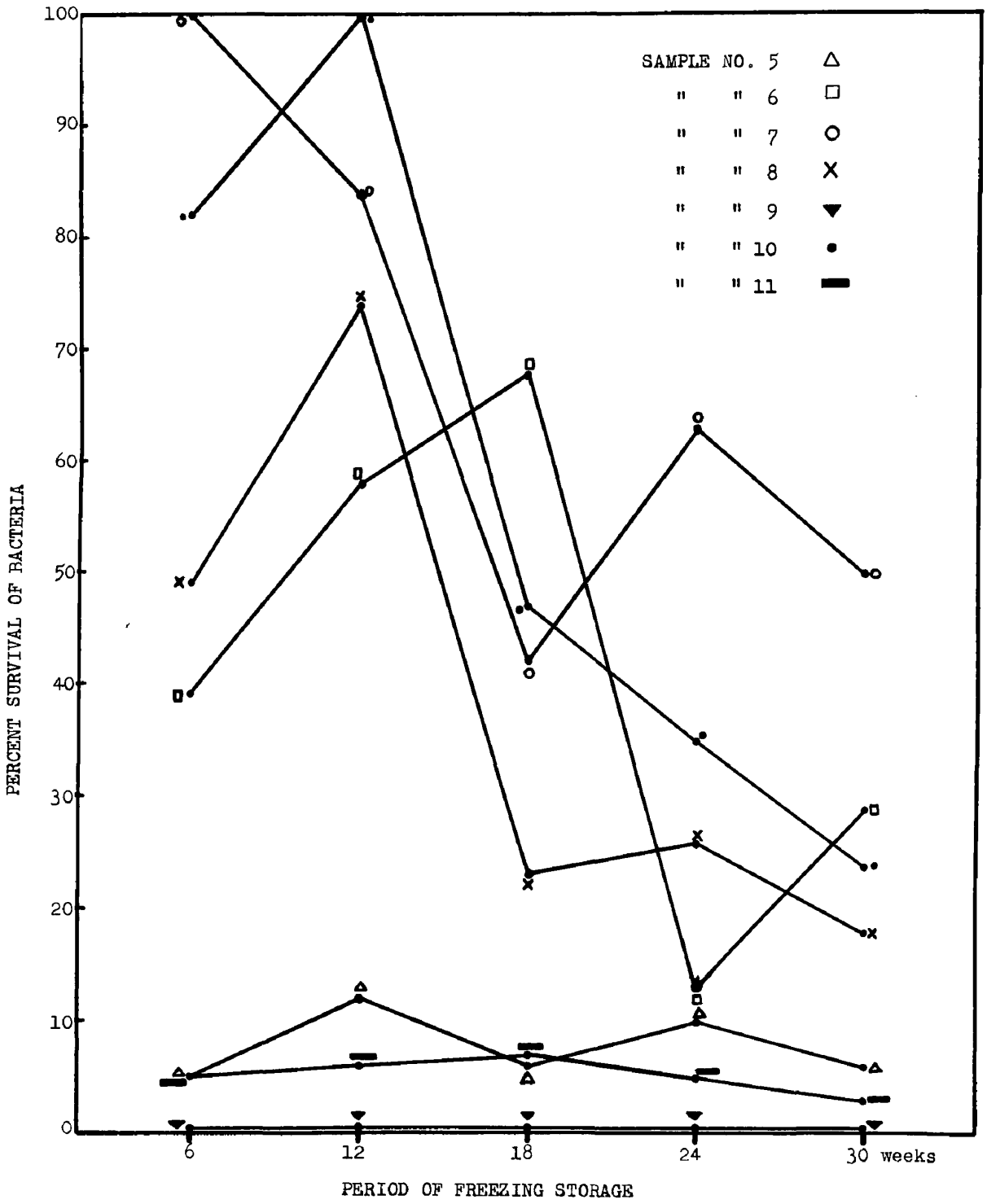

FIG. 2. SURVIVAL OF BACTERIA IN PEAS FROZEN AND STORED AT 0° F.

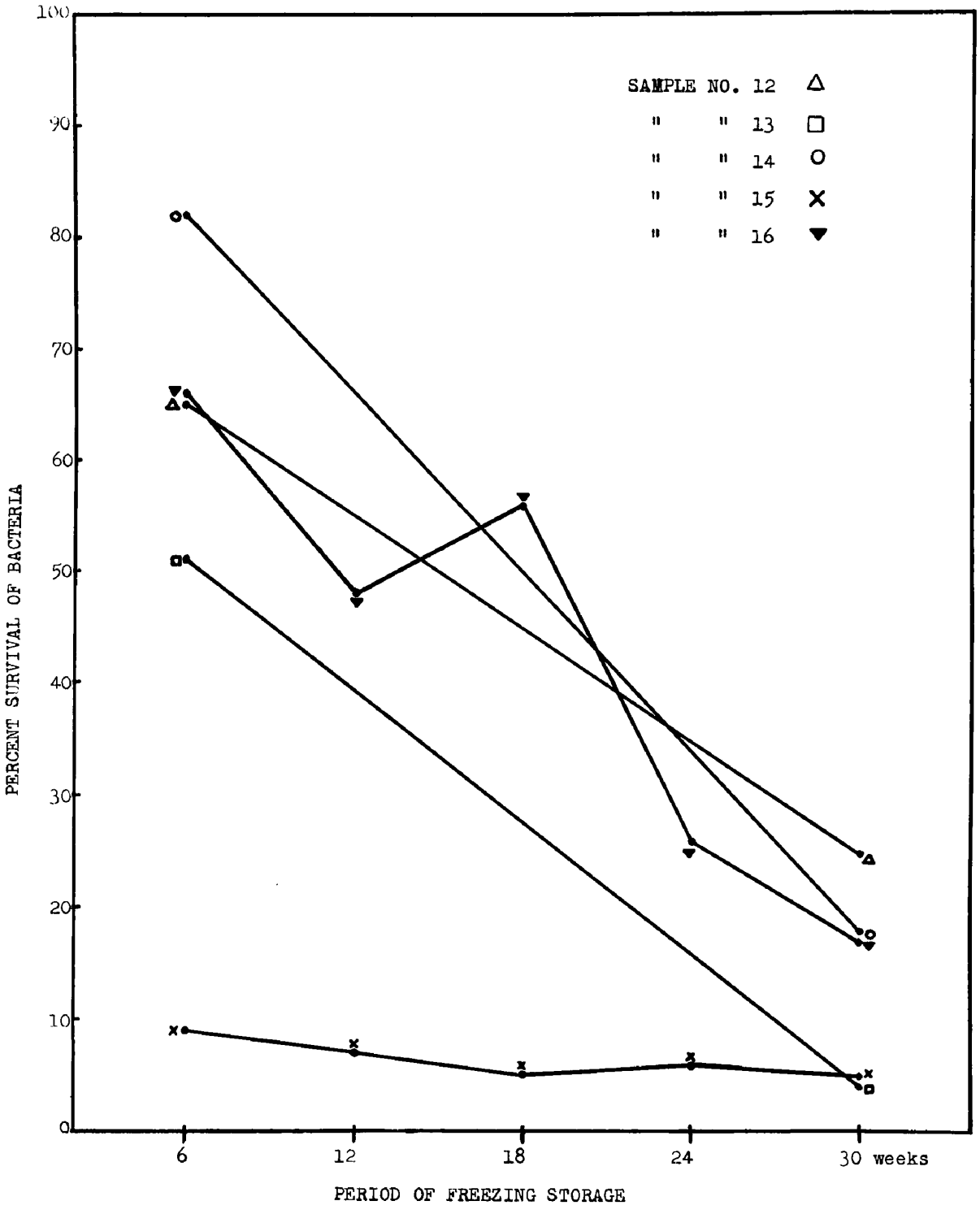

FIG.3. SURVIVAL OF BACTERIA IN CORN FROZEN AND STORED AT 0° F.

Table No. I

Survival of Bacteria in Frozen Vegetables

Product	No.	Year	Fresh	Bacteria Count per Gram and Percentage Survival Period of Freezing 6 weeks Count	6 weeks % survival	12 weeks Count	12 weeks % survival	18 weeks Count	18 weeks % survival	24 weeks Count	24 weeks % survival	30 weeks Count	30 weeks % survival
Asparagus	1	1935	4,500	1,535	34	2,300	51	1,100	24	2,100	47	860	19
"	2	1935	18,000	9,000	50	7,200	40	6,800	62	8,600	42	2,400	13
"	3	1936	38,000	32,000	84	26,000	68	19,000	50	12,000	32	8,600	23
"	4	1940	5,500	40	0.7	30	0.5	30	0.5	20	0.4	20	0.4
Peas	5	1935	15,000	750	5	1,750	12	900	6	1,550	10	950	6
"	6	1935	62,000	24,000	39	36,000	58	42,000	68	8,000	13	18,000	29
"	7	1936	3,800	4,600	100	3,200	84	1,600	42	2,400	63	1,900	50
"	8	1938	8,700	4,300	49	6,400	74	2,000	23	2,300	26	1,600	18
"	9	1940	12,000	20	0.2	20	0.2	20	0.2	20	0.2	10	0.1
"	10	1949	34,000	28,000	82	34,000	100	16,000	47	12,000	35	8,000	24
"	11	1949	80,000	4,000	5	4,800	6	5,200	7	3,600	5	2,400	3
Corn	12	1935	55,000	36,000	65	-		-		-		14,000	25
"	13	1935	156,000	79,000	51	-		-		-		6,500	4
"	14	1935	78,000	64,000	82	-		-		-		14,000	18
"	15	1949	172,000	16,000	9	12,000	7	8,000	5	9,600	6	8,200	5
"	16	1949	96,000	63,000	66	46,000	48	54,000	56	25,000	26	16,000	17

Pederson (23) noted these increased counts following the initial freezing process and attributed them to the breaking up of bacterial clumps during freezing. He based his findings on microscopic examinations of the organisms in the products before and after freezing. However, in the opinion of the writer, while these findings might be partly true, they fail to explain how these fluctuations persist beyond the initial freezing process when the breaking up of bacterial clumps might be expected. Sample No. 2, Fig. 1, demonstrates this point. After 6 weeks' storage at 0°F. the microbial content of this product showed a survival of 50 per cent of the initial count. After 12 weeks there was a further reduction in count and the survival was approximately 40 per cent. At the 18-week period of analysis the count suddenly jumps and 62 per cent of the organisms are found to have survived the freezing process. From this period on there is a steady decrease in the microbial content through to the 30 week period. These fluctuations are more likely due to the uneven distribution of microorganisms on the product or to some properties peculiar to the particular types of organisms present. This latter statement will be discussed at greater length in connection with the pure culture studies which follow.

With the seven samples of peas the trend of counts

was similar to those of the asparagus (Fig. 2). Three samples showed a rapid reduction in counts during the first 6 weeks of freezing storage followed by a more or less stationary count which persisted throughout the entire storage period. Four other samples of peas showed survivals varying from 100 per cent to 39 per cent during the first 6 weeks of storage. These same four samples show considerable fluctuation in counts following the six-week analysis. After 30 weeks storage the seven samples of peas showed survivals of bacteria varying from 1 to 51 per cent.

While the results are not as complete as for asparagus and peas, five samples of corn, show the same degree of variation in the rate of survival of bacteria at the 6-week period of analysis (Fig. 3). After 30 weeks' storage survivals varied from 4 to 27 per cent.

Results of a study of the types of bacteria from 6 samples, both from the fresh products and from those frozen for 30 weeks, are given in Table 2. Sample No. 3, which showed a steady decline in numbers of bacteria from 84 per cent survival after 6 weeks' storage at 0°F. to 23 per cent after 30 weeks, showed a predominance of micrococci (60 per cent) with Flavobacterium,

Table No. 2.

Survival of Types of Bacteria in Frozen Vegetables

Identification No. #	Product	No. of Colonies Examined	Classification -- Percentage of Types										
			Micrococcus	Bacteriaceae							Lactobacillus	Bacillus	No Growth on Transfer
				Serratia	Flavobacterium	Chromobacterium	Achromobacter	Escherichia	Aerobacter	Proteus			
3	Asparagus (Fresh)	100	19	-	11	-	23	12	16	1	14	4	-
3	Asparagus (Frozen)##	100	60	-	15	-	10	3	2	-	9	1	-
4	Asparagus (Fresh)	100	11	-	14	-	53	5	8	-	3	2	4
4	Asparagus (Frozen)##	8	12	-	-	-	50	-	12	-	26	-	-
7	Peas (Fresh)	50	17	1	16	2	8	25	16	-	12	2	1
7	Peas (Frozen)##	50	44	-	22	-	3	1	-	-	30	-	-
9	Peas (Fresh)	100	4	-	7	1	74	2	1	-	5	4	2
9	Peas (Frozen)##	4	50	-	25	-	25	-	-	-	-	-	-
15	Corn (Fresh)	100	6	1	4	-	46	15	18	-	2	4	4
15	Corn (Frozen)##	100	58	-	24	-	8	-	-	-	4	-	6
16	Corn (Fresh)	100	14	-	31	-	28	12	6	-	8	1	-
16	Corn (Frozen)##	100	32	-	46	-	3	1	-	-	12	-	6

Taken from Table 1.

30 weeks plus or minus 6 weeks.

Achromobacter and lactobacilli following with 15, 10 and 9 per cent respectively. Escherichia, Aerobacter, and Bacillus were present in small numbers.

Sample No. 7, which showed no reduction in numbers after 6 weeks' storage and 50 per cent after 30 weeks, showed a large proportion of micrococci (44 per cent) with lactobacilli and Flavobacterium following with 30 and 22 per cent respectively.

Sample No. 15, which showed a very rapid decline in numbers after 6 weeks of freezing storage, showed 58 per cent of the colonies isolated from the frozen product to be micrococci, 24 per cent were Flavobacterium, 8 per cent were Achromobacter and 4 per cent were lactobacilli.

Sample No. 16, which showed a survival of 66 per cent in numbers after 6 weeks and 17 per cent after 30 weeks, showed 46 per cent of the colonies in the frozen product to be Flavobacterium and 32 per cent micrococci. Twelve per cent wer lactobacilli with Achromobacter and Escherichia forming a small part of the microflora with 3 and 1 per cent respectively.

Too few colonies were examined from the frozen samples of Nos. 4 and 9 to permit making comparisons with other samples.

While it is impossible to state definitely from the data presented that the types of bacteria influence the survival of numbers of bacteria in frozen products, there is some indication that the types present play an important role. Of particular interest is the fact that the number of types in the frozen product is considerably reduced when compared with those present in the fresh product. From the data presented in Table 2 it will also be noted that in the frozen product of all samples studied bacteria belonging to the genera Micrococcus and Flavobacterium formed a higher percentage of the total microflora than did the corresponding fresh sample.

SLOW FREEZING VS. QUICK FREEZING ON NUMBERS OF MICROORGANISMS IN FROZEN PACK FRUITS AND VEGETABLES

Until a comparatively few years ago the usual method of freezing consisted of placing products to be frozen in a refrigerated room maintained at temperatures varying from -12.22°C. (10°F.) to -28.89°C (-20°F.). Although the air within such a room will circulate to some extent by convection, no provision was made for forced air circulation. The relatively still air is a poor conductor of heat and products are frozen at a comparatively slow rate, many hours or days being required before the products are completely solidified. This method is commonly known as the slow or "static" method of freezing. The term "quick freezing" refers to any method which incorporates some feature which speeds up the rate of freezing such as "contact" or "air blast" methods.

Few comparisons have been made of the effect of slow and quick freezing on microorganisms in frozen pack fruits and vegetables. Van Eseltine et al. (36), studying the effect of freezing on the bacterial content of frozen vegetables, subjected peas and corn to temperatures of liquid air, -51.1°C. (-60°F.), and -17.8°C. (0°F.). At the latter temperature products were frozen in "still air" both exposed freely to the atmosphere and in insulated boxes. These authors

state that freezing in liquid air and in an air blast at -60°F. resulted in such quick freezing that the bacterial content of the foods was essentially unchanged. However, this statement is not substantiated by the data presented since pronounced increases in counts are recorded in the -51.1°C. series. It is further stated that freezing at slower rates allowed multiplication of bacteria before the freezing temperature was reached. The Data presented show no greater increase after 1 and 2 hours' freezing at -17.8°C. than after 2 hours at -51.1°C. Substantial increases in counts are recorded in the series frozen in insulated boxes at -17.8°C. but this might be expected under such unusual circumstances since the rate of freezing is retarded considerably as these authors have demonstrated by the use of thermocouples.

Experimental

The present study was made with three products, asparagus, peas and strawberries. The products were packed dry in rectangular cartons. Some were frozen by the "static" method at both -28.89°C. (-20°F.) and -17.8°C. (0°F.), others by placing the cartons of the product between refrigerated coils at -20°F. Some products frozen by this contact method were also stored at 0°F. Initial analyses were made of the fresh products and again after one and six months' freezing storage.

The results of these studies shown in Table 3 indicate a higher percentage survival of bacteria in vegetable products frozen contact at -20°F. and stored at the same temperature when compared with the static frozen products stored at either -20°F. or 0°F. The only exception to this was with the psychrophilic bacteria in peas which showed about the same percentage survival with both methods of freezing. Vegetable products frozen by contact (-20°F.) and stored at 0°F. compared with those frozen and stored at 0°F. showed variable results.

With strawberries the rate of freezing or temperature of freezing storage appeared to have little, if any, influence on the percentage survival of microorganisms.

A summary of the data presented in this table of both vegetable products shows an appreciably higher percentage survival with the contact method of freezing.

Table 3. Effect of Slow vs. Quick Freezing on Numbers of Microorganisms in Frozen-Pack Vegetables and Fruits.

Product	Microorganisms	Fresh (Count per gram)	Contact - Frozen -20°F. Percentage Survival Stored 1 month	Contact - Frozen -20°F. Percentage Survival -20°F. 6 months	Contact - Frozen 0°F. Percentage Survival Stored 1 month	Contact - Frozen 0°F. Percentage Survival 0°F. 6 months	Static - Frozen -20°F. Counts of Original Stored 1 month	Static - Frozen -20°F. Counts of Original -20°F. 6 months	Static - Frozen 0°F. Counts of Original Stored 1 month	Static - Frozen 0°F. Counts of Original 0°F. 6 months
Asparagus	Bacteria 20°C.	5,525	64.9	50.5	24.7	20.8	36.2	26.4	28.2	20.8
	Bacteria 4°C.	1,245	50.6	57.8	58.2	57.8	20.9	11.2	15.7	17.7
Peas	Bacteria 20°C.	12,140	21.7	20.1	18.0	16.7	12.6	9.7	10.3	13.5
	Bacteria 4°C.	13,260	3.8	3.5	6.7	5.5	3.4	6.8	3.1	4.7
Strawberries	Bacteria 20°C.	478,000	48.4	5.2	60.8	2.1	52.5	9.7	64.2	10.2
	Bacteria 4°C.	315,400	63.5	12.4	86.0	5.0	63.3	15.0	79.0	12.8

Summary

	All Contact Packs	All Static Packs
Asparagus and Peas	30.1	15.1
Strawberries	35.4	38.3
All Products	31.8	22.8

PURE CULTURE STUDIES

The types of bacteria found on fruits and vegetables are a heterogeneous assortment representing many different genera. Many are soil types since these products are usually in close contact with the soil, either during the actual period of growing or through contamination with soil organisms in the process of harvesting and subsequent handling.

Previous studies on the effect of freezing on pure cultures, as pointed out in the literature review, have been limited to pathogenic bacteria or to a few other well-known species. While these studies have added a great deal to our knowledge on the effect of freezing of bacteria, results of analyses of frozen fruits and vegetables have suggested that little, if any, relationship exists between some of these pure culture studies and the total microbial flora of fruits and vegetables. For this reason the present project was undertaken to make a detailed study of bacteria isolated from fruit and vegetable products.

Experimental

For the purpose of this study approximately 2500 colonies were isolated from a variety of fresh products including asparagus, peas, beans, raspberries and strawberries. The procedure of isolation consisted of plating portions of product, as previously outlined, on

tryptone glucose extract agar. Plates were incubated at three temperatures, 55°C. (131°F.) for thermophiles, 20 - 25°C. (68° to 77°F.) for mesophiles and 4°C. (39.2°F.) for psychrophiles. Following a period of incubation, (2 days for thermophiles, 4 days for mesophiles and 2 to 3 weeks for psychrophiles) 50 colonies or, if the number on the plate were less than 50, all colonies, were picked from the plates and transferred to tryptone glucose extract agar slopes. These were incubated at temperatures and periods corresponding to those of the initial plates. Following incubation the test tube cultures were separated on the basis of macroscopical and microscopical observations. Cultures which could not be classified generically by this separation were further inoculated into various test media for determination of biochemical characteristics which would facilitate classification to genera. The biochemical method of separation was used with organisms belonging to genera which were Gram negative rods and showed no chromogenesis such as Achromobacter, Escherichia, Aerobacter and Proteus. This method of isolation provided a number of representative cultures of the three groups, thermophiles, mesophiles and psychrophiles, which were used as test cultures to study the effect of freezing. All final cultures were purified by the usual plating and re-isolation technique, and before using for study were given at least 4 consecutive transfers in the case of thermophiles and mesophiles and 2 for psychrophiles.

PREPARATION OF CULTURES FOR FREEZING STUDIES

In the initial studies attempts were made to grow the cultures, prior to freezing, in nutrient broth. This would have greatly facilitated the problem of diluting the organisms and of obtaining a more even distribution of the test organism. However, for some unknown reason many organisms grown in a nutrient broth, particularly thermophiles, disappeared after the second or third transfer. Accordingly, all cultures used in this work were grown on tryptone glucose extract agar slopes and just before use suspensions were prepared by adding sterile water to the agar culture. The growth was scraped from the surface of the agar and the heavy suspension of organisms was transferred to a sterile tube and shaken for 15 minutes on a mechanical shaker. In the early experiments these mixed suspensions were put through a fine grade filter paper with the object of breaking up any clumps of bacteria. However, subsequent tests showed this filtering process to be of no value for this purpose and the practice was discontinued. Trials with finer filters resulted in a loss of organisms.

The standard method adopted following shaking was to dilute the cultures to a point where a satisfactory plate count was subsequently obtained. A number of methods were tried to facilitate the preparation of

suitable dilutions. Microscopical estimations of numbers were disappointing since not only was considerable time required to make counts of bacteria but the final results showed no correlation with the plate counts.

The most satisfactory method found was to dilute the test culture to a definite turbidity which would provide a final plate count, in one of the four dilutions used, between 30 and 300 colonies. It is impossible to describe the "desired turbidity" but with constant practice it is possible to obtain satisfactory plate counts with very few "misses". The one group of organisms which proved the most difficult with this turbidity method was the spore formers. Generally it was necessary to use a different turbidity factor with this group of bacteria due to the presence of flaky particles in these suspensions. When a satisfactory dilution was prepared it was shaken mechanically for 15 minutes and the turbidity again checked.

The final dilution was prepared by pipetting 1 ml. of the above-prepared dilution into carefully calibrated 25 ml. portions of sterile water or other substrate in large 1" x 8" test tubes. Since many hundreds of these carefully calibrated 25 ml. substrate

were required it was necessary to devise a means of measuring the quantity accurately in minimum time. The procedure used which has proved satisfactory is shown in Fig. 4.

The inoculated substrate was shaken mechanically for 5 minutes and 1 ml. withdrawn for the initial analysis to determine the number of organisms in the suspension going into the freezer.

A separate tube of inoculated substrate was required for each period of analysis; usually 9 or 10 tubes were prepared for each series. To give some idea of the scope of this phase of study, for each organism tested in one substrate for 9 periods of freezing an outlay of 72 petri plates was required. The usual practice was to make a study of 8 test organisms at one time in one substrate. This involved a total outlay of 576 petri plates. While this number was not excessive for tests on thermophilic or mesophilic bacteria where the return of plates into service was only a matter of days, the time required for the return of plates from tests with psychrophilic bacteria was 3 weeks. In view of the time factor with this latter group it was necessary to curtail some of the studies with psychrophiles.

The time required from the inoculation of one

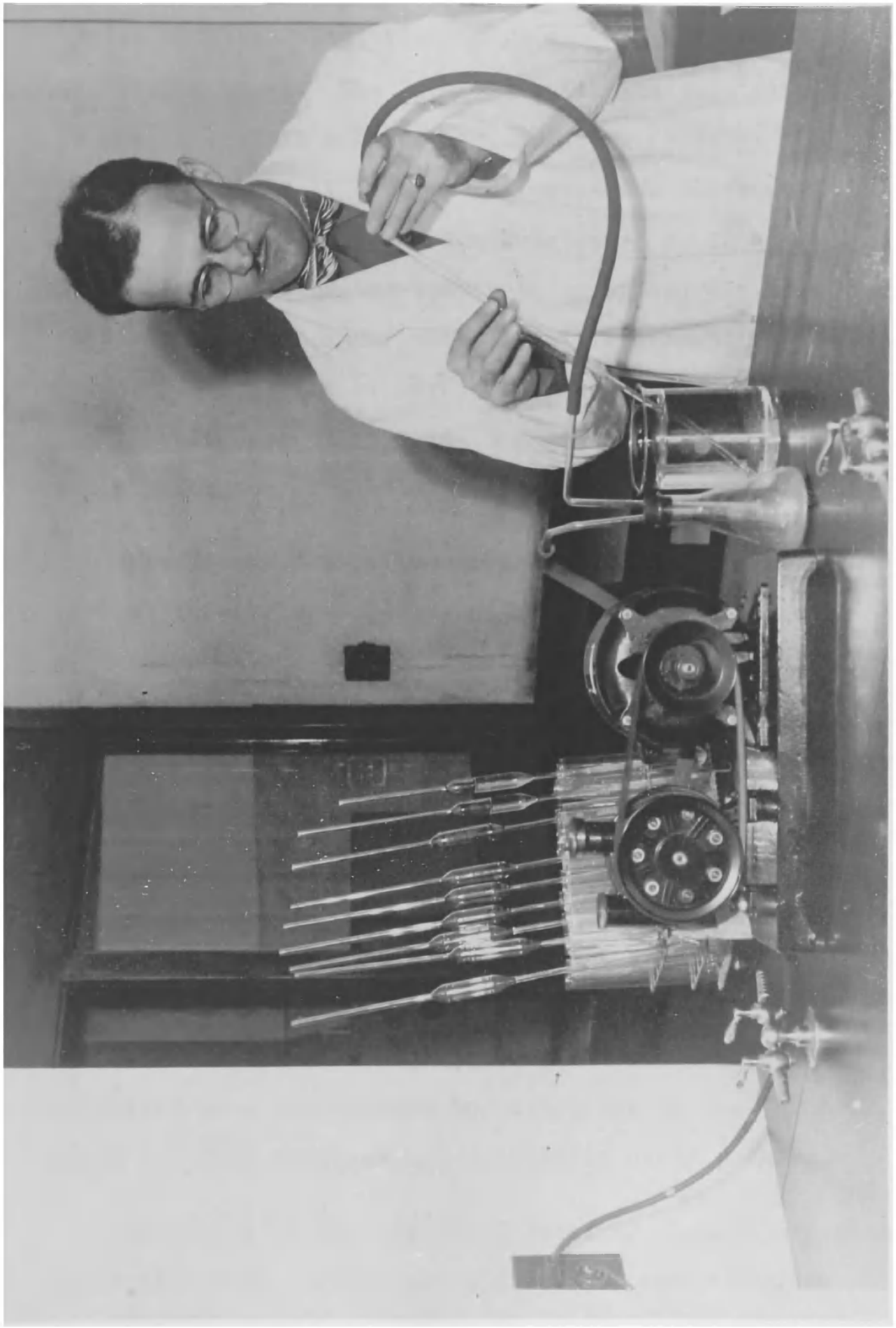

Fig. 4. APPARATUS AND METHOD OF MEASURING SUBSTRATES USED FOR FREEZING

series of tubes until placing in the freezer never exceeded 2 hours. The tubes of substrate were placed in the freezer in a horizontal position (Fig. 5) for two purposes: 1, if frozen vertically the tubes will break due to expansion of the substrate; 2, in a horizontal position the substrate is spread out in a relatively thin layer which facilitates faster freezing. Freezing of pure cultures was at -17.8°C. (0°F.), with air blast using a large fan placed directly in front of the rack holding the tubes.

When removed from the freezer for analysis at various intervals the tubes of suspension were placed in a water bath at approximately 100°F. The tubes were continually agitated and just before the last trace of ice disappeared they were removed and shaken mechanically for 5 minutes. One dilution was made from the substrate by pipetting 1 ml. into a sterile 99 ml. water blank. Four quantities of the substrate were used to inoculate duplicate plates of tryptone glucose extract agar (1.0 and 0.1 of the dilution from the substrate and 1.0 and 0.1 ml. from the substrate itself). The plates were incubated at a temperature corresponding to that from which the test organism had originally been isolated.

The study of the effect of freezing of test organisms under different conditions is discussed under various headings.

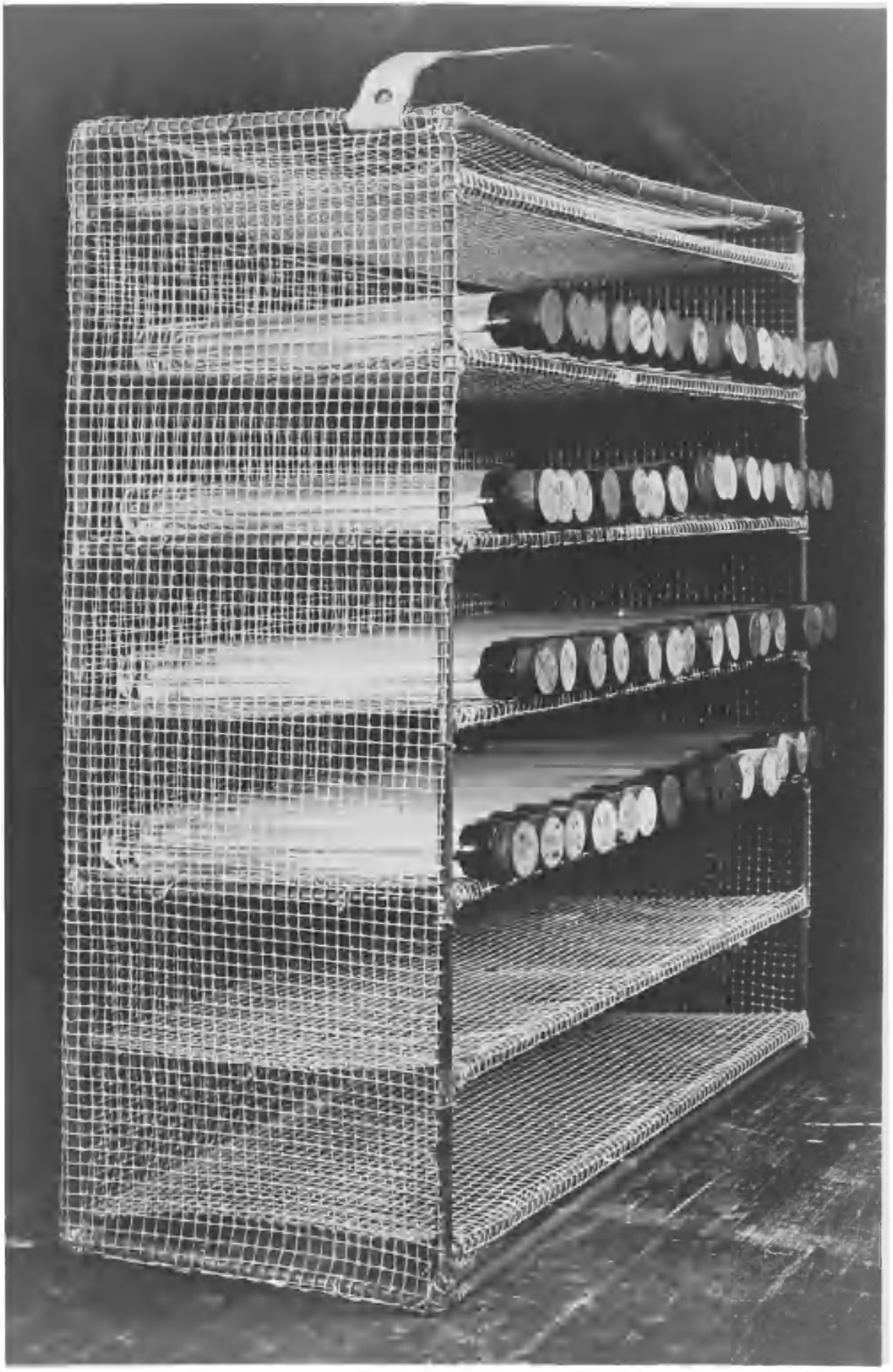

Fig. 5. SPECIAL RACK USED FOR HOLDING TUBES OF SUSPENSIONS IN FREEZING STORAGE

EFFECT OF FREEZING ON BACTERIAL CELLS

The late Dr. Haines assumed that freezing might result in an increased cell size or in actual bursting of cells due to expansion during the freezing process but reported that he was unable to observe any change in the size or shape of microbial cells due to freezing (5).

In the present study, as a matter of interest, microscopical measurements were made of 50 cells of each of 5 bacteria and one yeast culture before freezing, and again after freezing for one week at -17.8°C. (0°F.). The results substantiated the earlier work of Haines since there was no evidence of increased cell size nor were any broken or distorted cells observed. Plans were made to conduct electron microscope studies to determine if there were any differences in the unfrozen and frozen cells which could not be observed under the magnification of the usual microscope. However, the writer was advised that the treatment the cells would be subjected to in preparation for electron microscope studies would probably eliminate any differences which might exist between the fresh and frozen cells.

The rate of growth of six bacterial cultures and one culture of yeast was determined before freezing and after freezing for 72 hours at 0 F. The rate of growth

was determined using a 0.5 per cent peptone solution inoculated with a suspension of the test organism. Plate counts were made every 2 hours up to 12 hours and a final count made after 24 hours. Similar tubes of 0.5 per cent peptone were inoculated and frozen for 72 hours at 0°F. and the rate of growth again determined by plating similar to the series described above. While the results showed some variation in counts the averages for the two series were similar.

The ability to ferment dextrose broth was also studied with the same seven cultures before and after freezing, using the peptone broth cultures of the above series. Following inoculation of the dextrose broth 2 ml. of sterile petrolatum was placed on the surface. This seal provided a reliable means of measuring the amount of gas produced during fermentation. The results indicated that there were no important differences in either the rate or amount of gas produced in cultures subjected to freezing.

The effect of freezing on the chromogenic properties of three species of bacteria was studied using cultures of Serratia marcescens, Chromobacterium violaceum and Sarcina lutea. Freezing in water at 0°F. for a period of one week resulted in a loss of colour

for almost 50 per cent of the colonies of S. marcescens and 72 per cent of the colonies of C. violaceum. However, when these colourless colonies were transferred for 4 consecutive days, 20 per cent of the Serratia and 3 per cent of the Chromobacterium colonies again produced chromogen. The yellow chromogen of S. lutea was not affected by freezing.

These data suggest that freezing has little, if any, effect on the metabolism of microbial cells which survive the freezing process. One possible exception to this statement might be the chromogenic properties of some microorganisms. However, this phase requires further study since it has been noted from other experiments in which freezing has not been involved that chromogenic bacteria frequently lose their power to produce pigment.

EFFECT OF FREEZING DIFFERENT SPECIES OF BACTERIA IN WATER AT -17.8°C. (0°F.)

Preliminary studies indicated that there was no difference between freezing cultures in tap or distilled water. However, since the pH of Ottawa tap water varies considerably from one period of the year to another distilled water was used as substrate throughout this study.

Eighty cultures of bacteria representing different

genera of bacteria as listed in Tables 4, 5 and 6 were subjected to freezing at 0°F. Twenty-eight of these cultures were thermophiles, 35 were mesophiles and 17 were psychrophiles. Some of the latter cultures were not obligate psychrophiles since many were found to grow as well at 20 - 25°C. as at 4°C. However, all 17 cultures grew well at the lower temperature. An initial analysis was made of prepared cultures before freezing and after 4, 8, 12, 24, 48, 72, 96, 120 hours and finally after 6 weeks storage at 0°F.

The results of these studies are shown in Tables 4, 5 and 6. Probably the most significant feature of these results is the enormous differences which exist between various species of bacteria subjected to freezing temperatures. The results of freezing cultures of thermophilic spore formers whown in Table 4 indicate that at the 4-hour period the rate of survival varied from 1 to 100 per cent. The survival after 8 hours ranged from 8 to 100 percent; after 12 hours from 3 to 100 per cent; after 24 hours from 1 to 100 per cent; after 48 hours from 1 to 98; after 72 hours from 1 to 52; after 96 hours from 1 to 38; after 120 hours from 1 to 30 and finally after 6 weeks from 1 to 22 per cent. With few exceptions the thermophiles followed a more definite pattern than the mesophiles or psychrophiles.

In other words, most species of thermophiles showed a steady reduction in numbers throughout the freezing period. After 6 weeks' freezing storage 25 of the 28 cultures (89 per cent) showed less than 10 per cent survival.

Results of a study with mesophilic bacteria frozen in water at 0°F. are shown in Table 5. These results indicate that mesophiles are considerably more resistant to freezing than thermophiles since a greater percentage survival is noted at the various periods of analysis of freezing storage. After 4 hours the percentage survival varied from 15 to 100; after 8 hours 5 to 100; after 12 hours 4 to 100; after 24 hours 1 to 100; after 48 hours 1 to 88; 72 hours 1 to 94; 96 hours 1 to 87; 120 hours 1 to 92 and finally after 6 weeks 1 to 75. Only 20 of the 35 mesophilic cultures showed a survival of less than 10 per cent after 6 weeks freezing storage.

With the mesophilic bacteria studied, considerable fluctuation in counts from one period of analysis to another may be noted with many individual cultures. These fluctuations are considerably more pronounced with mesophilic than with the thermophilic bacteria.

The psychrophilic bacteria subjected to freezing followed a pattern similar to that of the mesophiles although during the first 72 hours there was a decidedly greater resistance to freezing snown by the

Table No. 4

Survival of Thermophilic Bacteria Frozen in Water at 0°F.

Count per ml. Substrate in Thousands and Percentage Survival.

Period of Freezing at 0°F.

Cult. No.	Initial Count	4 hours Count	4 hours % survival	8 hours Count	8 hours % survival	12 hours Count	12 hours % survival	24 hours Count	24 hours % survival	48 hours Count	48 hours % survival	72 hours Count	72 hours % survival	96 hours Count	96 hours % survival	120 hours Count	120 hours % survival	6 weeks Count	6 weeks % survival
72	2,000	560	28	320	16	360	18	400	20	500	25	240	12	160	8	200	10	120	6
74	1,600	160	10	128	8	96	6	16.1	1	16	1	16	1	16.5	1	15.4	1	14	1
76	110	112	100	112	100	110	100	110	100	88	80	44	44	31	28	15	14	13	12
78	14,000	14,000	100	12,300	88	11,800	84	10,900	78	9,800	70	7,300	52	1,500	11	1,300	9	1,100	8
80	1,800	700	40	700	39	470	26	200	11	24	1	20	1	20	1	34	2	20	1
82	92	54	59	40	44	22	24	17	19	7.4	8	3.7	4	9	10	10	11	1.8	2
84	2,000	220	11	220	11	160	8	22	1	20	1	36	2	20	1	14	1	12	1
86	4,000	1,800	45	1,800	45	1,400	34	880	22	440	11	360	9	240	6	32	1	28	1
88	1,200	1,000	88	996	83	384	32	132	11	385	32	72	6	24	2	34	3	24	2
91	600	510	85	504	84	132	22	96	16	95	16	114	19	60	10	36	6	30	5
93	12,000	2,400	20	1,900	16	2,600	22	960	8	1,000	9	1,700	14	120	1	480	4	94	1
94	132	136	100	132	100	134	100	136	100	129	98	21	16	13	1	11	1	10	1
95	96	31	32	32	32	27	28	13.4	14	7.7	8	3.8	4	1.9	2	3.8	4	19	2
96	1,200	264	22	432	36	228	19	156	13	120	10	119	10	48	4	11	1	8.5	1
97	575	35	6	58	10	12	2	23	4	23	4	63	11	12	2	24	4	10	2
99	184	158	86	151	82	18	10	11	6	7.4	4	12.9	7	7.5	4	15	8	5.5	3
101	100	56	56	22	22	11	11	8	8	32	32	35	35	9	9	6	6	5	5
103	15,000	3,600	24	1,350	9	3,150	21	1,350	9	150	1	122	1	108	1	42	1	58	1
105	158	16	10	16	10	16	10	17	11	17	11	13	8	17	11	21	13	11	7
107	225	238	100	234	100	232	100	18	8	11	5	25	11	18	8	25	11	23	10
109	246	143	58	128	52	153	62	106	43	93	38	44	18	93	38	74	30	54	22
182	200	76	38	32	16	8	4	20	10	18	9	12	6	16	8	1.8	1	.6	1
184	2,000	20	1	220	11	220	11	100	5	80	4	40	2	220	11	20	1	20	1
186	124	9.9	8	9.9	8	17	14	.85	1	.72	1	94	1	1	1	.65	1	.5	1
187	168	22	13	59	35	5	3	5	3	3.4	2	10	6	-	-	6.8	4	1.2	1
246	510	85	17	85	17	164	32	63	12	49	10	57	11	70	14	22	4	8	2
272	7	8	100	4	57	7	100	3	43	3	43	2	29	2	29	2	29	.3	4
278	233	39	17	43	18	31	13	43	18	26	11	32	14	30	13	30	13	16	7
	Average		46		41		33		21		19		13		9		7		4

Table No. 5

Survival of Mesophilic Bacteria Frozen in Water at 0°F.

Count per ml. Substrate in Thousands and Percentage Survival

Cult. No.	Generic Classification	Initial Count	Period of Freezing: 4 hours Count	4 hours % survival	8 hours Count	8 hours % survival	12 hours Count	12 hours % survival	24 hours Count	24 hours % survival	48 hours Count	48 hours % survival	72 hours Count	72 hours % survival	96 hours Count	96 hours % survival	120 hours Count	120 hours % survival	6 weeks Count	6 weeks % survival
42	Achromobacter	5,500	1,600	29	1,500	28	1,200	22	830	15	260	5	230	4	114	2	139	3	72	1
48	"	3,900	1,700	44	2,100	54	160	4	710	18	2,300	59	760	19	1,200	31	3,600	92	240	6
35	Bacillus	6,000	4,500	75	3,000	50	3,000	50	1,200	20	420	7	360	6	120	2	80	1	12	1
37	Flavobacterium	5,300	3,600	68	2,800	53	3,000	57	3,300	62	4,000	75	5,000	94	4,600	87	4,400	83	2,100	40
50	"	2,700	3,400	100	2,500	93	2,500	93	2,000	74	1,200	44	1,000	37	1,300	48	800	30	900	33
39	Bacillus	28,000	10,000	36	1,400	5	1,200	4	200	1	600	2	92	1	76	1	88	1	22	1
52	Aerobacter aerogenes	16,000	18,000	100	7,200	45	1,600	10	240	2	200	1	89	1	40	1	28	1	9.8	1
53	Achromobacter	4,000	4,100	100	1,800	45	3,600	90	4,000	100	870	22	120	3	430	11	560	14	12	1
56	"	280	120	43	180	64	240	86	82	29	116	41	41	15	68	24	12	4	2.8	1
67	Lactobacillus	18,000	18,000	100	16,000	89	12,000	67	7,000	39	4,200	23	1,800	10	190	1	190	1	140	1
64	Achromobacter	26,000	26,000	100	8,900	34	14,600	56	22,000	85	12,200	47	4,000	15	2,600	10	820	3	98	1
41	Flavobacterium	14,600	12,300	84	10,200	70	12,100	83	9,300	64	10,000	68	8,600	59	6,900	47	4,200	29	1,800	12
51	"	140	92	66	120	86	68	49	62	44	86	61	48	34	29	21	36	26	24	17
54	"	5,800	2,900	50	3,600	62	1,300	22	920	16	900	16	1,200	21	1,100	19	410	7	189	3
63	Micrococcus	22,000	20,000	91	18,600	85	20,000	91	16,600	75	15,200	69	16,200	74	14,100	64	12,900	59	8,100	37
66	"	2,600	2,900	100	2,100	81	1,900	73	2,000	77	1,800	69	1,100	22	1,200	46	820	32	780	30
70	"	2,000	1,230	61	590	30	570	29	700	35	640	32	670	39	530	27	520	26	470	24
69	Achromobacter	12,400	1,800	15	6,500	52	4,400	35	820	7	2,400	19	1,900	15	600	5	260	2	76	1
43	Flavobacterium	8,600	6,200	72	4,100	48	2,200	26	3,100	36	2,000	23	1,800	21	1,800	21	1,600	19	1,100	13
55	"	24,000	26,000	100	24,000	100	20,200	84	8,900	37	10,100	42	6,400	27	2,200	9	840	4	172	1
36	Bacillus	11,000	2,100	19	2,800	25	860	8	124	1	76	1	32	1	8	1	6.2	1	.2	1
38	Bacillus mycoides	4,100	4,000	98	1,200	29	320	8	300	7	182	4	94	2	36	1	24	1	8.2	1
62	Micrococcus	13,300	16,000	100	15,400	100	14,000	100	12,600	95	9,200	69	8,800	66	4,900	37	2,600	20	1,800	14
40	Serratia	16,000	16,000	100	16,000	100	16,000	100	14,000	88	14,000	88	9,000	56	12,000	75	13,000	81	12,000	75
68	Sarcina lutea	1,200	1,000	83	1,200	100	860	72	920	77	940	78	820	68	870	73	790	66	800	67
65	Lactobacillus	1,800	1,300	72	1,100	61	920	51	148	8	120	7	120	7	88	5	76	4	38	2
44	Achromobacter	4,300	5,200	100	4,300	100	1,400	33	2,600	60	94	2	186	4	82	2	100	2	68	1
47	Bacillus	33	26	79	24	73	13	39	9	27	10	30	8	24	8.5	26	1.2	4	3.2	10
49	Clostridium	12,600	7,800	62	5,100	40	6,200	49	2,800	22	820	7	600	5	128	1	92	1	8.8	1
58	Flavobacterium	140	140	100	128	91	116	83	112	80	102	73	108	77	96	69	98	70	86	61
60	"	1,600	1,100	69	1,300	81	1,000	63	840	53	920	58	640	40	680	43	420	26	388	24
59	Achromobacter	14,000	14,300	100	8,600	61	10,600	76	9,900	71	1,200	86	5,400	39	980	7	1,200	9	640	4
117	Escherichia coli	49,000	25,000	51	16,000	33	9,000	18	3,000	6	490	1	1,500	3	320	1	900	2	480	1
490	Staphylococcus aureus	22,000	24,000	100	19,000	86	15,000	68	15,000	68	12,000	55	11,000	50	3,000	14	12,000	55	2,600	12
57	Flavobacterium	980	600	61	650	66	590	60	520	53	360	37	480	49	220	22	186	19	82	8
	Average			75		63		53		44		38		30		24		23		14

Table No. 6

Survival of Psychrophilic Bacteria Frozen in Water at 0°F.

Count per ml. Substrate in Thousands and Percentage Survival

Cult. No.	Generic Classification	Initial Count	Period of Freezing: 4 hours Count	% survival	8 hours Count	% survival	12 hours Count	% survival	24 hours Count	% survival	48 hours Count	% survival	72 hours Count	% survival	96 hours Count	% survival	120 hours Count	% survival	6 weeks Count	% survival
2	Flavobacterium	170	90	53	67	39	56	33	47	28	39	23	31	18	22	13	18	11	9	5
3	Lactobacillus	1,300	936	72	885	68	675	52	625	48	720	15	130	10	26	2	7.5	1	4.6	1
5	Micrococcus	6,200	5,950	96	6,400	100	5,700	92	5,000	82	4,300	70	3,780	61	2,200	36	1,490	24	1,100	18
7	Achromobacter	400	450	100	136	34	420	100	250	62	375	94	325	76	90	22	56	14	3.2	1
8	Flavobacterium	1,600	1,800	100	1,600	100	1,900	100	1,700	100	1,180	74	990	62	624	39	655	41	255	16
9	"	120	125	100	118	98	110	92	89	74	36	30	50	42	36	30	25	21	13	11
14	Achromobacter	3,000	2,300	76	3,100	100	2,800	94	3,000	100	1,900	64	1,400	48	630	21	360	12	27	1
18	Flavobacterium	205	125	61	77	38	46	22	53	26	64	31	43	21	38	19	32	16	17	8
19	"	1,200	1,400	100	1,300	100	1,100	94	910	76	890	74	420	35	550	46	230	19	120	10
20	"	800	670	84	655	82	610	76	480	60	495	62	345	43	305	38	280	35	175	22
21	Chromobacterium	12,000	10,700	89	9,400	78	5,000	42	4,300	36	2,000	17	960	8	16	1	4.2	1	.3	1
22	Lactobacillus	4,300	5,200	100	5,000	100	3,700	86	2,240	52	1,000	24	775	18	775	18	345	8	130	3
24	Flavobacterium	270	300	100	300	100	265	98	203	75	105	39	127	47	32	12	70	26	24	9
26	"	158	89	56	98	62	93	59	43	27	62	39	49	31	72	46	56	36	34	22
27	Achromobacter	14,000	14,000	100	13,700	98	5,600	40	3,600	26	15,000	100	10,000	72	3,900	28	5,000	36	128	1
29	Lactobacillus	2,100	1,930	92	2,000	96	2,000	97	2,200	100	1,155	55	965	46	609	29	735	35	590	28
31	Flavobacterium	22,000	26,000	100	24,000	100	16,700	76	13,600	62	3,960	18	5,700	26	3,000	14	1,760	8	960	12
	Average			87		82		74		61		49		39		24		20		10

psychrophilic bacteria. However, following the 72 hour period the results were similar to those of the mesophiles. With the psychrophiles, 9 of the 17 cultures (53 per cent) showed a survival of less than 10 per cent after 6 weeks' freezing storage.

A graph showing the effect of freezing on the three groups of organisms is presented in Fig. 6. The figures for this graph are the average percentage survivals for each period of freezing for all organisms studied in each of the three groups. These results indicate that the thermophiles are the least resistant to freezing, showing the least survival of the three groups at the 4 hour period. This marked reduction during the initial freezing process was followed by a still further sharp reduction up to the 24-hour period, following which the reduction was more gradual.

Mesophiles and psychrophiles, as indicated in this graph, are considerably more resistant to freezing than the thermophiles. The mesophiles and psychrophiles follow a similar pattern throughout the freezing storage although the psychrophiles show a faster rate of destruction after 24 hours. However, towards the end of the storage period there is practically no difference in the rate of survival between these two groups.

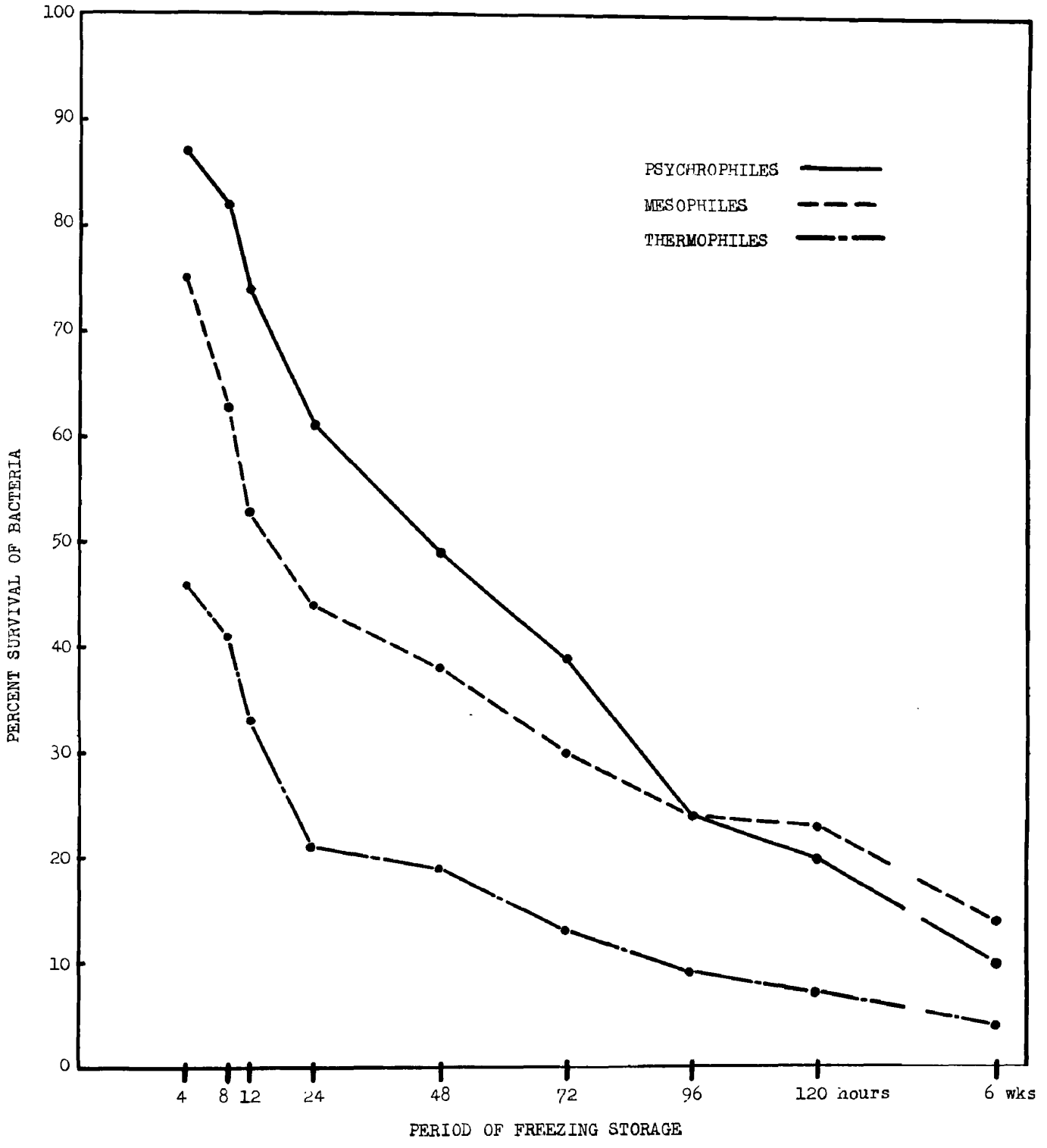

FIG. 6. SURVIVAL OF PSYCHROPHILIC, MESOPHILIC, AND THERMOPHILIC BACTERIA FROZEN IN WATER AT 0° F.

Table No.7

Survival of Different Types of Bacteria Frozen in Water at 0°F.#

Cult. No.		Percentage Survival Period of Freezing 4 hours	8 hours	12 hours	24 hours	48 hours	72 hours	96 hours	120 hours	6 weeks
42	Achromobacter	29	28	22	15	5	4	2	3	1
44	"	100	100	33	60	2	4	2	2	1
48	"	44	54	4	18	59	19	31	92	6
53	"	100	45	90	100	22	3	11	14	1
56	"	43	64	86	29	41	15	24	4	1
59	"	100	61	76	71	86	39	7	9	4
64	"	100	34	56	85	47	15	10	3	1
69	"	15	52	35	7	19	15	5	2	1
	Average	66	55	50	48	35	14	12	16	2
35	Bacillus	75	50	50	20	7	6	2	1	1
36	"	19	25	8	1	1	1	1	1	1
38	"	98	29	8	7	4	2	1	1	1
39	"	36	5	4	1	2	1	1	1	1
47	"	79	73	39	27	30	24	26	4	10
	Average	61	36	22	11	9	7	6	2	3
37	Flavobacterium	68	53	57	62	75	94	87	83	40
41	"	84	70	83	64	68	59	47	29	12
43	"	72	48	26	36	23	21	21	19	13
50	"	100	93	93	74	44	37	48	30	33
51	"	66	86	49	44	61	34	21	26	17
54	"	50	62	22	16	16	21	19	7	3
55	"	100	100	84	37	42	27	9	4	1
57	"	61	66	60	53	37	49	22	19	8
58	"	100	91	83	80	73	77	69	70	61
60	"	69	81	63	53	58	40	43	26	24
	Average	77	75	62	52	50	46	39	31	21
62	Micrococcus	100	100	100	95	69	66	37	20	14
63	"	91	85	91	75	69	74	64	59	37
66	"	100	81	73	77	69	42	46	32	30
70	"	61	30	29	35	32	39	27	26	24
490	"	100	86	68	68	55	50	14	55	12
	Average	90	76	72	70	59	52	38	38	23
65	Lactobacillus	72	61	51	8	7	7	5	4	2
67	"	100	89	67	39	23	10	1	1	1
	Average	86	75	59	24	15	9	3	3	2
40	Serratia	100	100	100	88	88	56	75	81	75
52	Aerobacter aerogenes	100	45	10	2	1	1	1	1	1
49	Clostridium	62	40	49	22	7	5	1	1	1
117	Escherichia coli	51	33	18	6	1	3	1	2	1
68	Sarcina lutea	83	100	72	77	78	68	73	66	67

- Data taken from Table 5

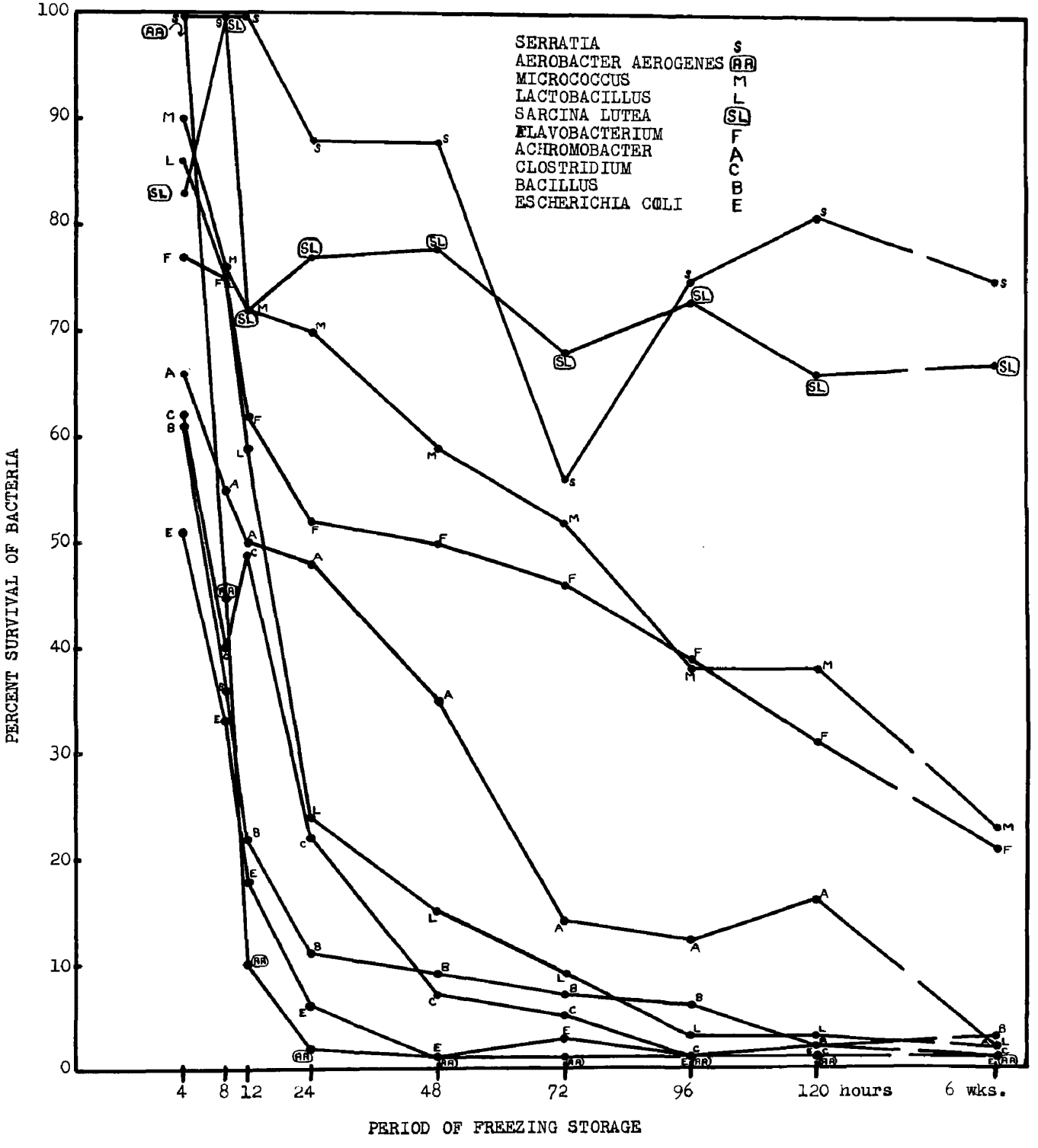

FIG. 7. SURVIVAL OF DIFFERENT TYPES OF BACTERIA FROZEN IN WATER AT 0° F.

A further analysis of the data on mesophilic bacteria taken from Table 5 on the survival of different types is shown in Table 7 and Fig. 7. These data show that the cultures of Serratia and of Sarcina lutea, followed by members of the genera Micrococcus and Flavobacterium, were more resistant to freezing than any of the other groups studied. Although enormous differences in the rate of survival are evident in the early hours of freezing, members of the genera Aerobacter, Escherichia, Lactobacillus, Bacillus, Clostridium and Achromobacter all showed approximately the same survival after 6 weeks' freezing storage. Even after 24 hours' freezing storage the difference in survival rates with these groups had narrowed considerably so that by 72 hours there was very little difference.

EFFECT OF FREEZING ON THREE CULTURES OF ESCHERICHIA COLI.

The fact that there was considerable variation in the rate of survival of different species of bacteria when subjected to freezing led to a study with three cultures of Escherichia coli to determine whether or not similar variations existed within the species. The cultures used in this study were isolated from widely different sources. Culture No. 1 was obtained from sewage, No. 2 from water and No. 3 from fresh beans. All three cultures produced biochemical

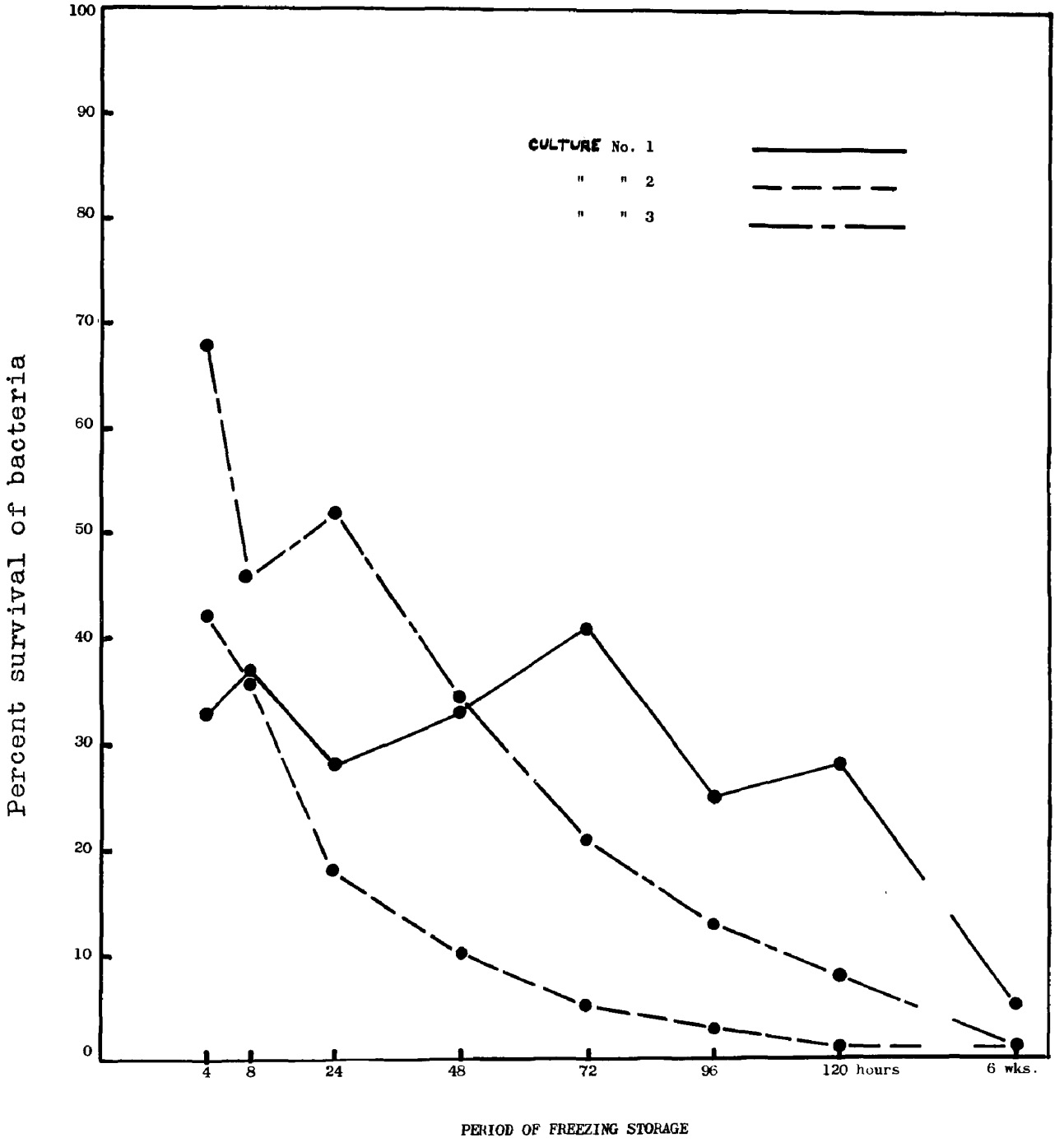

FIG.8 SURVIVAL OF THREE CULTURES OF ESCHERICHIA COLI FROZEN IN WATER AT 0° F.

reactions in various test media typical for Escherichia coli.

Aqueous suspensions of the three cultures were prepared as previously described and following an initial analysis were frozen at 0°F. Further analyses were made after intervals of freezing storage from 4 hours to 6 weeks.

From the results presented in Fig. 8 it will be observed that even cultures of the same species exhibit considerable variability when subjected to freezing. The culture isolated from sewage, (No. 1) which showed the greatest destruction in numbers during the first 4 hours of freezing, also showed the greatest fluctuation throughout the freezing storage. Between cultures Nos. 2 and 3 there was considerable difference in the rate of survival during freezing storage up to the 120-hour period. However, after 6 weeks' storage both showed the same percentage survival.

EFFECT OF FREEZING BACTERIA IN DIFFERENT SUBSTRATES

It has been known for many years that the rate of microbial destruction due to freezing is greatly influenced by the character of the medium in which the organism is suspended. Keith (13) found that when E. coli was frozen in milk diluted to various degrees with water the death rate of this organism increased with the dilution, the largest number surviving in

the undiluted milk and the fewest in that containing the most water. No figures are given in Keith's paper of the survival of E. coli with the various concentrations of milk.

Hilliard and Davis (8), comparing the effect of freezing E. coli in glucose and in tap water solutions, found considerable variation in the percentage of organisms killed in these two substrates. With glucose solutions frozen solidly at -10°C. (14°F.) for 3 hours the percentage killed varied from 77.5 to 99 while in tap water solution the percentage killed ranged from 98.1 to 99.8.

Wallace and Tanner (43) mention that they froze a number of cultures of bacteria, yeasts and moulds in concentrations of 1 to 6 per cent sodium chloride and in concentrations of sucrose varying from 10 to 50 per cent. However, no results are given for any of these experiments in the published paper.

McFarland (21) froze cultures of a Saccharomyces sp. and of Escherichia coli in distilled water and in concentrations of sucrose varying from 1 to 50 per cent. He found considerable variation in results. At -10°C. (14°F.) in all concentrations of sucrose and in distilled water the destruction was greater than at -20°C. (-4°F.). The yeast at -10°C. showed a 99.5 per cent kill on the 28th week in distilled water while

in the 50 per cent sucrose in the same period of time it showed only a 15.4 per cent kill. However, the 20 per cent sucrose showed a kill about the same as in distilled water in the same period. Even more peculiar results were evidenced after the first week of freezing when the results with the 5 and 10 per cent sucrose showed no kill and yet the heavier concentrations which might be expected to provide more protection showed kills varying from 4 to 25 per cent.

With Escherichia coli frozen in distilled water and various concentrations of sucrose at -10°C., all showed a high percentage kill after 32 weeks, indicating that even the highest concentration of sucrose used (50 per cent) afforded little protection to this organism. At the lower temperature (-20°C.) the percentage kill varied considerably after 32 weeks of freezing storage. Suspended in 30 per cent sucrose this organism showed a kill of 74.8 per cent while with 50 per cent sucrose a kill of 85.7 per cent was observed.

The present investigation was concerned with studying the effect of freezing on cultures of bacteria isolated from vegetable products. In all, seven cultures were used, three of which were thermophilic

spore formers (Bacillus spp.) the remaining four were mesophiles, one belonging to the genus Achromobacter, two Micrococcus spp. and one Escherichia sp. Eight different substrates were used, including distilled water, 3 per cent salt, a vegetable extract prepared by boiling green beans and using the filtered extract (ph of the vegetable extract 5.60; specific gravity 1.014), and 5, 10, 20, 30 and 40 per cent sucrose solutions. These substrates were chosen for their similarity to the liquid portion of frozen fruit and vegetable products. The substrates were prepared and accurately measured using a volumetric pipette as previously outlined. The periods of analysis ranged from 4 to 120 hours, while a final examination was made after 6 weeks at $0^{\circ}F$.

Results of these studies are shown in Tables 8 to 15 and in Figs. 9 to 16. These data indicate the extreme variability which may be encountered with certain species of microorganisms when subjected to freezing. Of particular interest are the results presented in Tables 8 and 9 and Figs. 9 and 10 on the effect of freezing in different substrates a species of Achromobacter (culture No. 59) and a species of Micrococcus (culture No. 70). With the Micrococcus species considerable fluctuations in the rate of survival were evident with all substrates with the possible exception of the water series. In the water

Table No. 8

Survival of Bacteria Frozen in Different Substrates at 0°F.

Org. No. 70 -- Mesophile -- Micrococcus sp.

Count per ml. Substrate in Thousands and Percentage Survival

Substrate	Initial Count	Period of Freezing: 4 hours Count	% survival	8 hours Count	% survival	24 hours Count	% survival	48 hours Count	% survival	72 hours Count	% survival	96 hours Count	% survival	120 hours Count	% survival	6 weeks Count	% survival
Water	3,700	1,400	38	1,300	35	1,200	32	820	22	1,100	32	1,100	30	760	21	640	17
3%NaCl	1,200	1,100	92	870	73	870	73	820	68	1,200	100	930	78	660	55	156	13
Veg. Ext.	12,000	8,000	69	7,000	58	3,000	25	5,400	45	6,500	54	8,000	67	6,700	56	5,900	49
5% Sucrose	1,800	700	39	1,600	89	810	45	1,000	56	1,500	83	1,200	67	570	32	270	15
10% "	1,700	800	47	1,400	82	610	36	820	48	1,300	76	1,600	94	820	48	680	40
20% "	1,900	800	42	800	42	690	36	930	49	1,200	63	1,100	58	1,200	63	940	49
30% "	1,700	600	35	900	53	460	27	900	53	1,000	59	860	51	820	42	790	46
40% "	1,870	650	33	1,000	51	440	22	700	36	880	45	900	46	710	36	630	32

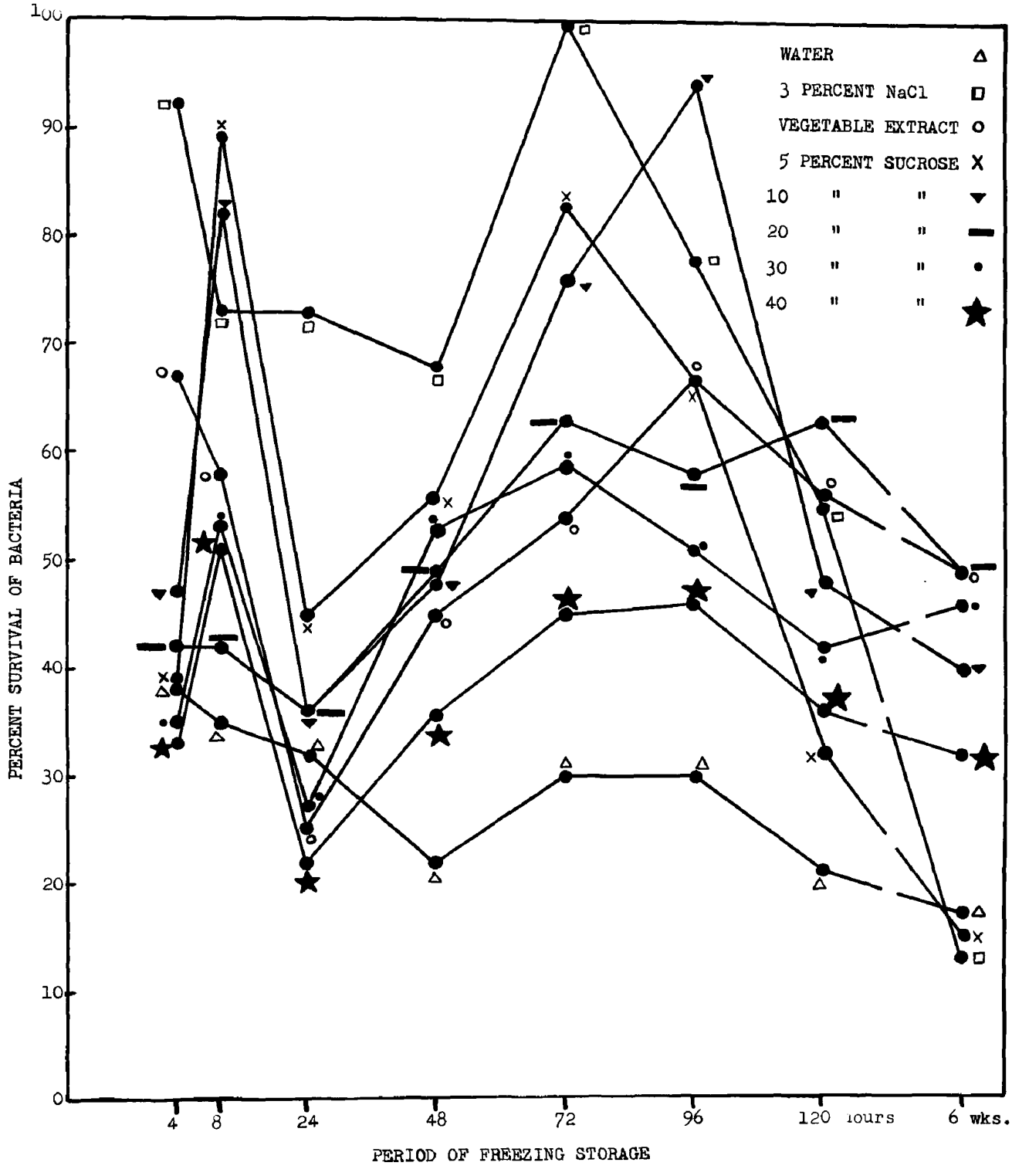

Fig. 9 . SURVIVAL OF MESOPHILIC BACTERIA (ORG. NO. 70 - MICROCOCCUS SP.) FROZEN IN DIFFERENT SUBSTRATES AT 0° F.

Table No.9

Survival of Bacteria Frozen in Different Substrates at 0°F.

Org. No. 59 -- Mesophile -- Achromobacter sp.

Count per ml. Substrate in Thousands and Percentage Survival

Substrate	Initial Count	Period of Freezing: 4 hours Count	% survival	8 hours Count	% survival	24 hours Count	% survival	48 hours Count	% survival	72 hours Count	% survival	96 hours Count	% survival	120 hours Count	% survival	6 weeks Count	% survival
Water	5,200	3,740	72	3,640	70	3,100	56	4,200	80	4,100	78	2,960	57	2,300	45	570	11
3% NaCl	5,200	3,540	68	2,800	53	3,200	62	3,950	76	4,900	94	4,580	88	4,370	84	830	16
Veg. Ext.	1,800	1,550	86	1,760	98	1,620	90	1,420	79	1,400	74	1,200	68	845	47	612	34
5% Sucrose	6,000	1,700	28	4,300	72	2,000	34	4,100	69	2,900	48	1,100	19	2,200	36	1,100	18
10% "	6,000	5,800	96	2,200	37	5,200	87	5,500	92	1,400	24	4,000	67	1,100	19	3,700	62
20% "	5,200	1,980	38	3,500	68	3,600	70	4,470	86	4,900	94	2,400	46	2,700	52	2,400	46
30% "	4,000	3,400	86	1,700	42	3,600	90	3,700	92	3,200	81	1,520	38	1,500	37	1,000	26
40% "	1,000	980	98	1,300	100	12,00	100	670	67	430	43	620	62	390	39	160	16

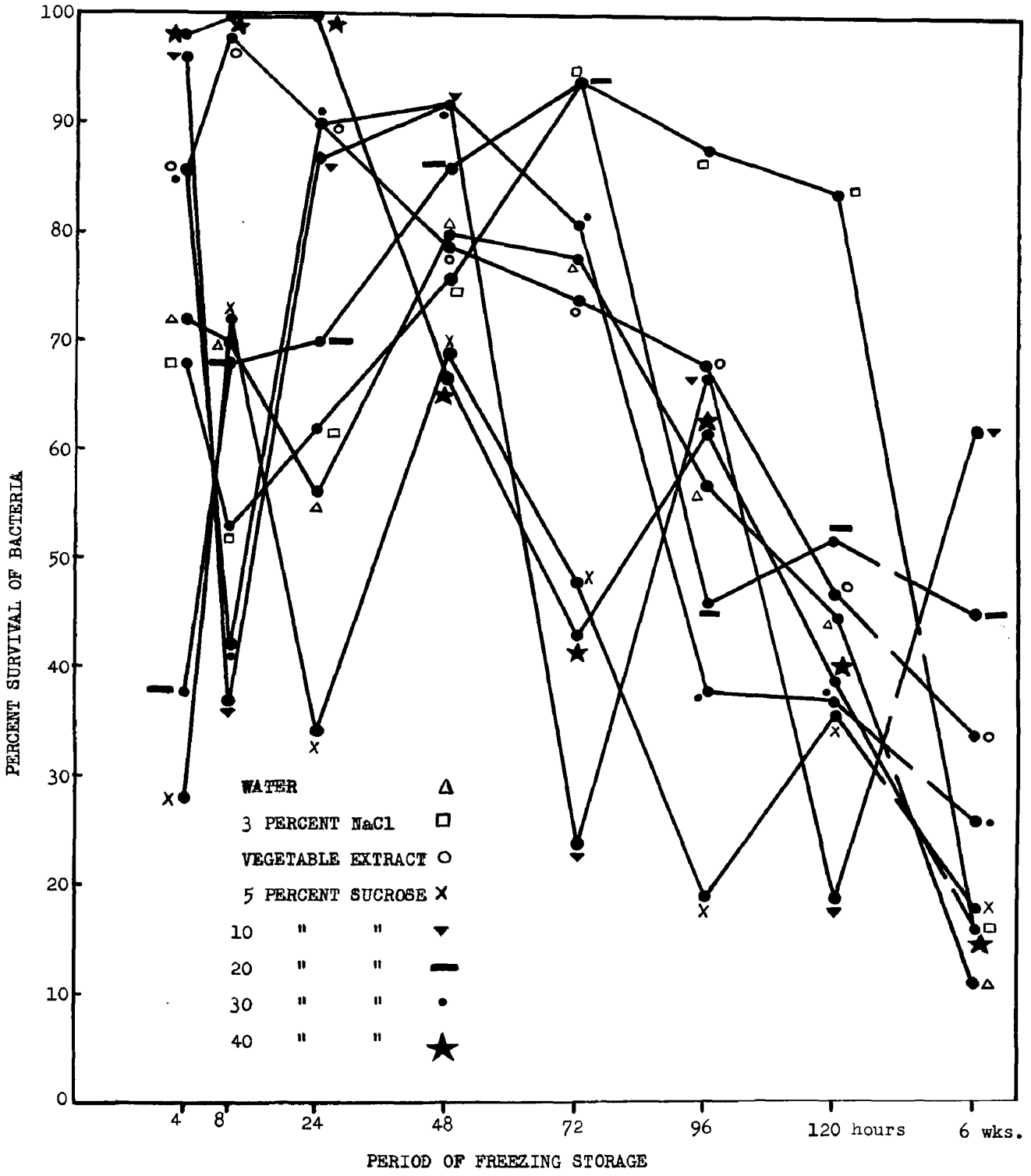

FIG.10. SURVIVAL OF MESOPHILIC BACTERIA (ORG. NO. 59 - ACHROMOBACTER SP.) FROZEN IN DIFFERENT SUBSTRATES AT 0° F.

Table No. 10

Survival of Bacteria Frozen in Different Substrates at 0°F.

Org. No. 490 -- Mesophile -- Staphylococcus aureus

Count per ml. Substrate in Thousands and Percentage Survival

Substrate	Initial Count	Period of Freezing: 4 hours Count	4 hours % Survival	8 hours Count	8 hours % survival	24 hours Count	24 hours % survival	48 hours Count	48 hours % survival	72 hours Count	72 hours % survival	96 hours Count	96 hours % survival	120 hours Count	120 hours % survival	6 weeks Count	6 weeks % survival
Water	21,000	1,680	8	1,890	9	1,260	6	840	4	840	4	630	3	420	2	190	1
3% NaCl	12,000	14,000	100	10,560	88	8,280	69	6,840	57	6,240	52	1,920	16	800	5	300	2
Veg. Ext.	26,000	21,000	80	22,400	86	21,800	84	17,000	65	18,700	72	20,300	78	17,200	66	12,500	48
5% Sucrose	24,000	8,200	34	6,700	28	7,700	32	3,800	16	2,900	12	1,200	5	720	3	216	1
10% "	18,000	14,800	82	14,000	78	11,500	64	8,800	49	11,000	61	5,000	28	6,500	36	5,200	29
20% "	22,000	24,000	100	23,000	100	19,000	86	17,200	78	14,100	64	12,300	56	5,100	23	7,900	36
30% "	16,000	19,000	100	16,000	100	11,500	72	11,200	70	7,400	46	7,000	44	5,800	36	4,500	28
40% "	10,000	13,000	100	12,000	100	8,400	84	7,600	76	8,800	88	7,900	79	6,400	64	4,200	42

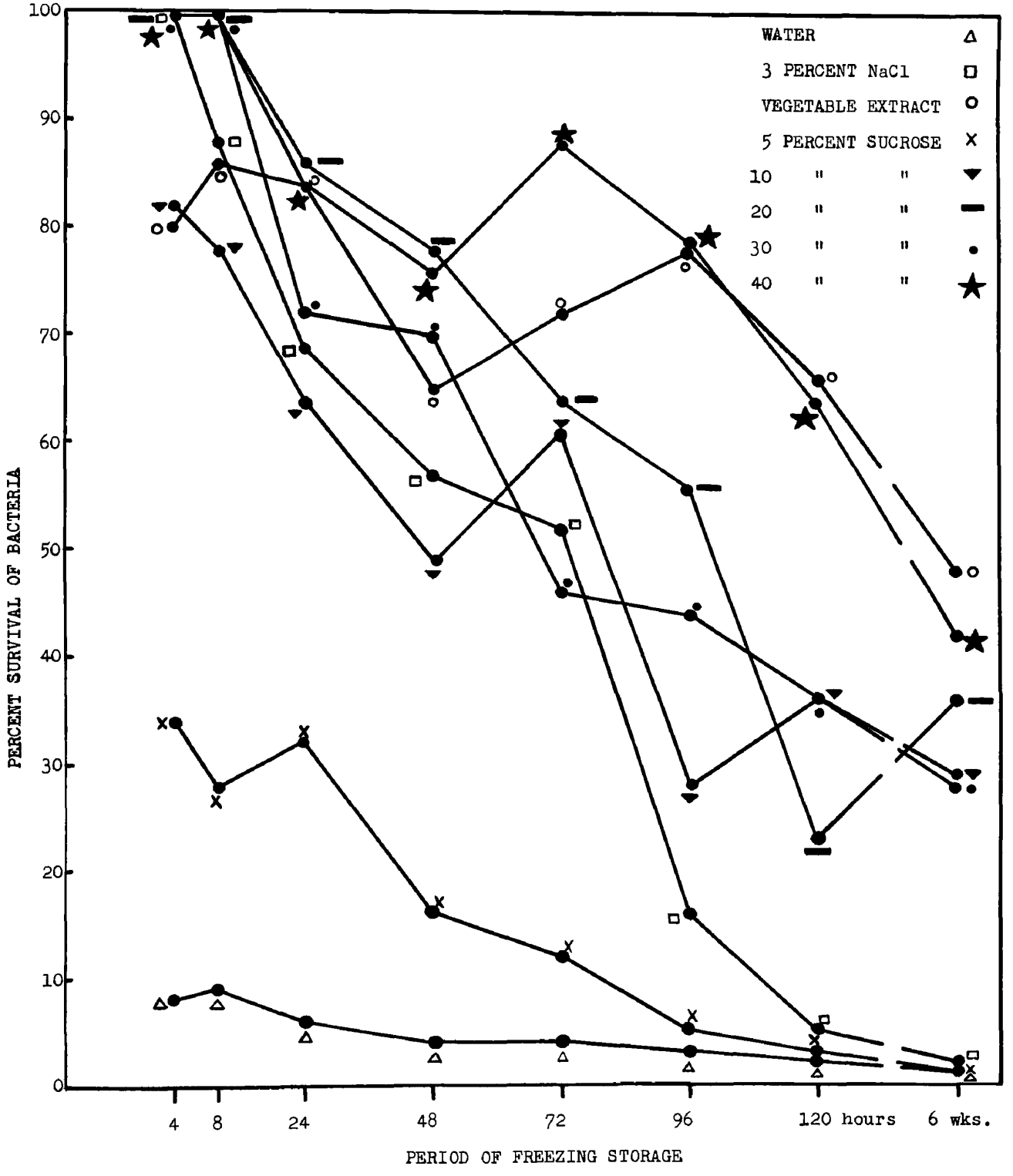

Fig. 11. SURVIVAL OF MESOPHILIC BACTERIA (ORG. NO. 490 - STAPHYLOCOCCUS AUREUS) FROZEN IN DIFFERENT SUBSTRATES AT 0° F.

Table No. II

Survival of Bacteria Frozen in Different Substrates at 0°F.

Org. No. 117 -- Mesophile -- Escherichia coli

Count per ml. Substrate in Thousands and Percentage Survival

Substrate	Initial Count	Period of Freezing: 4 hours Count	% survival	8 hours Count	% survival	24 hours Count	% survival	48 hours Count	% survival	72 hours Count	% survival	96 hours Count	% survival	120 hours Count	% survival	6 weeks Count	% survival
Water	26,000	8,600	33	9,600	37	7,300	28	8,600	33	10,700	41	6,500	25	7,300	28	1,300	5
3% NaCl	24,000	12,000	50	7,700	32	1,400	6	480	2	960	4	475	2	480	2	220	1
Veg. Ext.	26,000	23,900	92	19,500	75	20,300	78	16,100	62	15,600	60	13,500	52	12,500	48	9,400	36
5% Sucrose	14,000	5,500	39	3,200	23	2,200	16	2,000	14	2,000	14	1,100	8	980	7	280	2
10% "	12,000	5,000	42	5,400	45	3,400	28	2,400	20	1,900	16	1,400	12	1,200	10	360	3
20% "	8,000	6,900	86	5,100	64	3,800	48	2,300	29	1,900	24	2,000	25	900	11	480	6
30% "	10,000	12,000	100	12,000	100	9,600	96	6,200	62	5,600	56	4,800	48	2,800	28	1,800	18
40% "	9,000	10,000	100	8,300	92	7,000	74	6,100	68	5,600	62	4,500	50	2,900	32	1,900	21

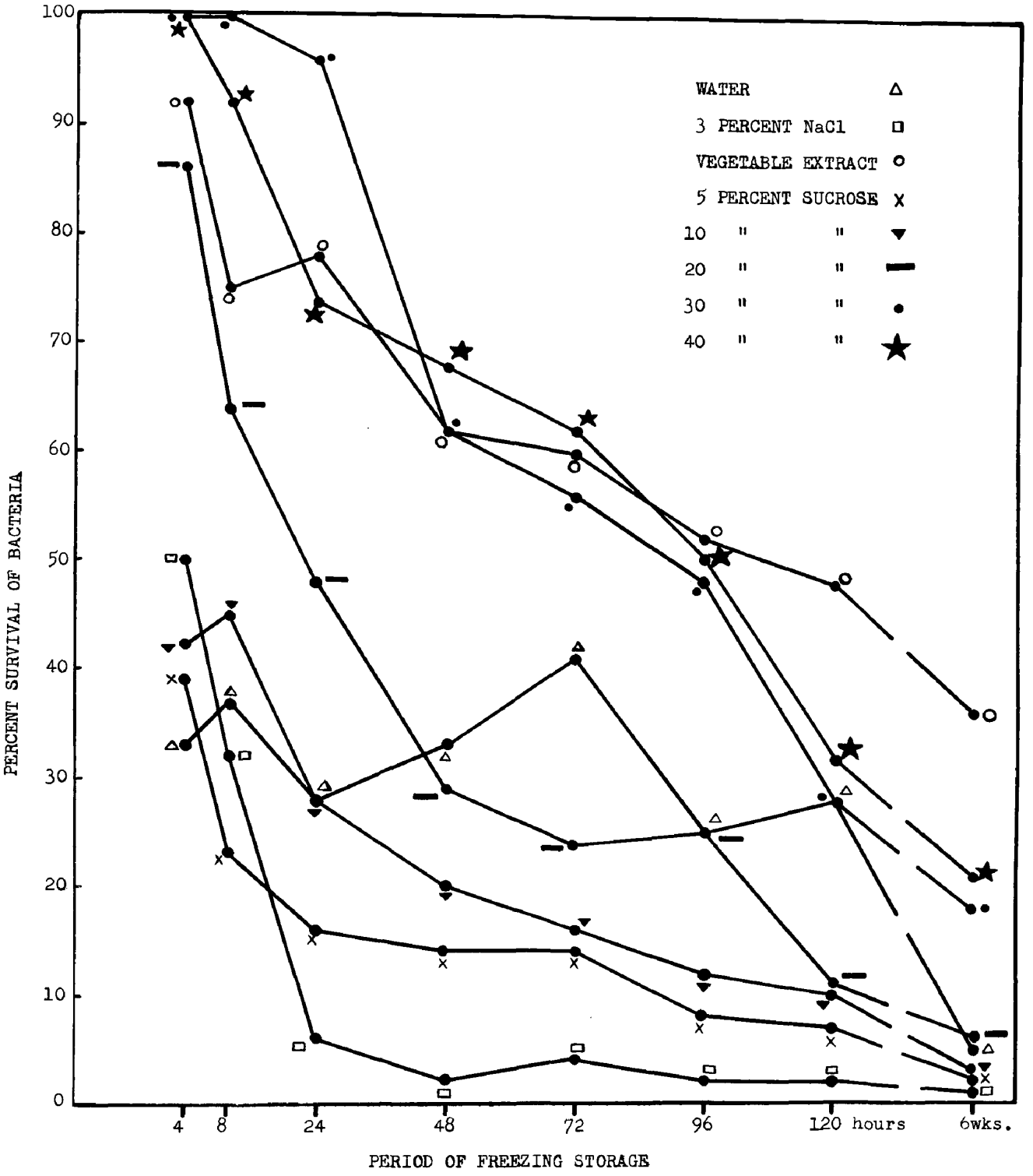

FIG.12. SURVIVAL OF MESOPHILIC BACTERIA (ORG. NO. 117 - ESCHERICHIA COLI) FROZEN IN DIFFERENT SUBSTRATES AT 0° F.

Table No. 12.

Survival of Bacteria Frozen in Different Substrates at 0°F.

Org. No. 97 -- Thermophile -- Bacillus sp.

Count per ml. Substrate in Thousands and Percentage Survival

Substrate	Initial Count	Period of Freezing: 4 hours Count	% survival	8 hours Count	% survival	24 hours Count	% survival	48 hours Count	% survival	72 hours Count	% survival	96 hours Count	% survival	120 hours Count	% survival	6 weeks Count	% survival
Water	2,300	1,660	72	550	24	300	13	161	7	160	7	70	3	16	1	7.2	1
3% NaCl	2,800	2,460	88	736	62	530	19	336	12	224	8	168	6	21	1	6.4	1
Veg. Ext.	1,800	1,920	100	1,690	94	1,100	61	775	43	396	22	198	11	54	3	14.2	1
5% Sucrose	8,200	7,540	92	6,230	76	2,540	31	2,300	28	492	6	68	1	14	1	10	1
10% "	1,200	1,130	94	864	72	575	48	230	19	145	12	48	4	13	1	6.1	1
20% "	530	580	100	371	70	592	100	360	68	223	42	180	34	95	18	29	1
30% "	860	1,200	100	241	28	611	71	533	62	310	36	120	14	60	7	28	3
40% "	1,600	1,600	100	1,375	86	1,375	86	1,470	92	688	43	704	44	496	31	128	8

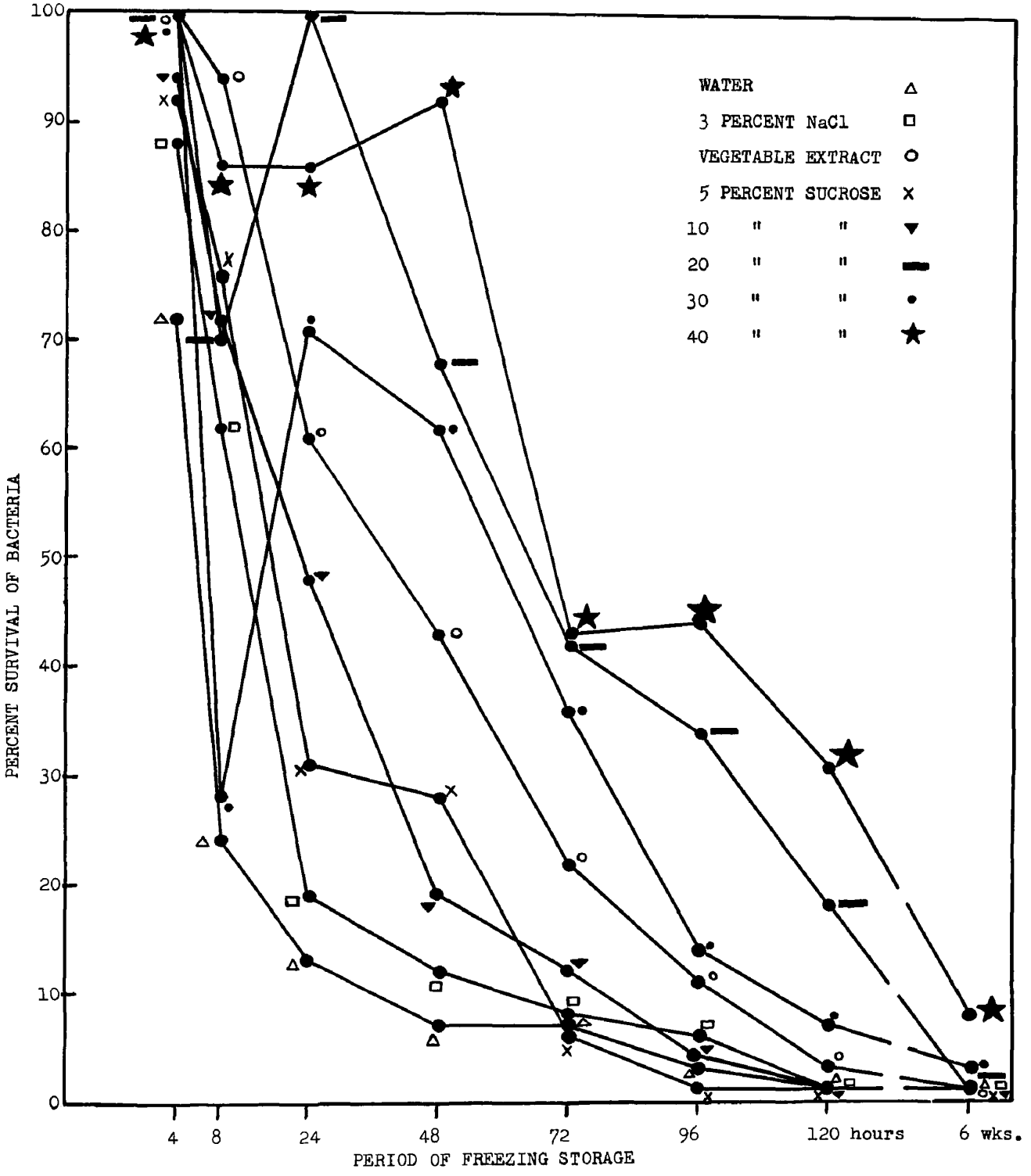

FIG. 13. SURVIVAL OF THERMOPHILIC BACTERIA (ORG. NO. 97 - BACILLUS SP.) FROZEN IN DIFFERENT SUBSTRATES AT 0°F.

Table No. 13

Survival of Bacteria Frozen in Different Substrates at 0°F.

Org. No. 100 -- Thermophile -- Bacillus sp.

Count per ml. Substrate in Thousands and Percentage Survival

Substrate	Initial Count	Period of Freezing: 4 hours Count	% Survival	8 hours Count	% survival	24 hours Count	% survival	48 hours Count	% survival	72 hours Count	% survival	96 hours Count	% survival	120 hours Count	% survival	6 weeks Count	% survival
Water	12,000	6,600	55	4,100	34	3,400	28	1,300	11	240	2	67	1	48	1	16	1
3% NaCl	9,400	2,000	21	3,570	38	2,915	31	1,500	16	280	3	76	1	72	1	21	1
Veg. Ext.	8,600	7,200	84	6,190	72	7,200	84	4,470	52	3,100	36	2,410	28	1,200	14	340	4
5% Sucrose	19,300	10,000	52	14,280	74	5,980	31	2,315	12	965	5	165	1	132	1	96	1
10% "	10,000	8,200	82	8,100	81	6,000	60	2,100	21	700	7	300	3	82	1	67	1
20% "	34,000	23,000	68	36,000	100	21,760	64	14,600	43	9,860	29	12,200	36	4,760	14	560	2
30% "	9,800	8,700	89	10,000	100	5,490	56	4,510	46	3,100	32	1,760	18	1,175	12	490	5
40% "	10,000	8,400	84	7,400	74	5,900	59	4,600	46	2,600	26	2,900	29	1,400	14	900	9

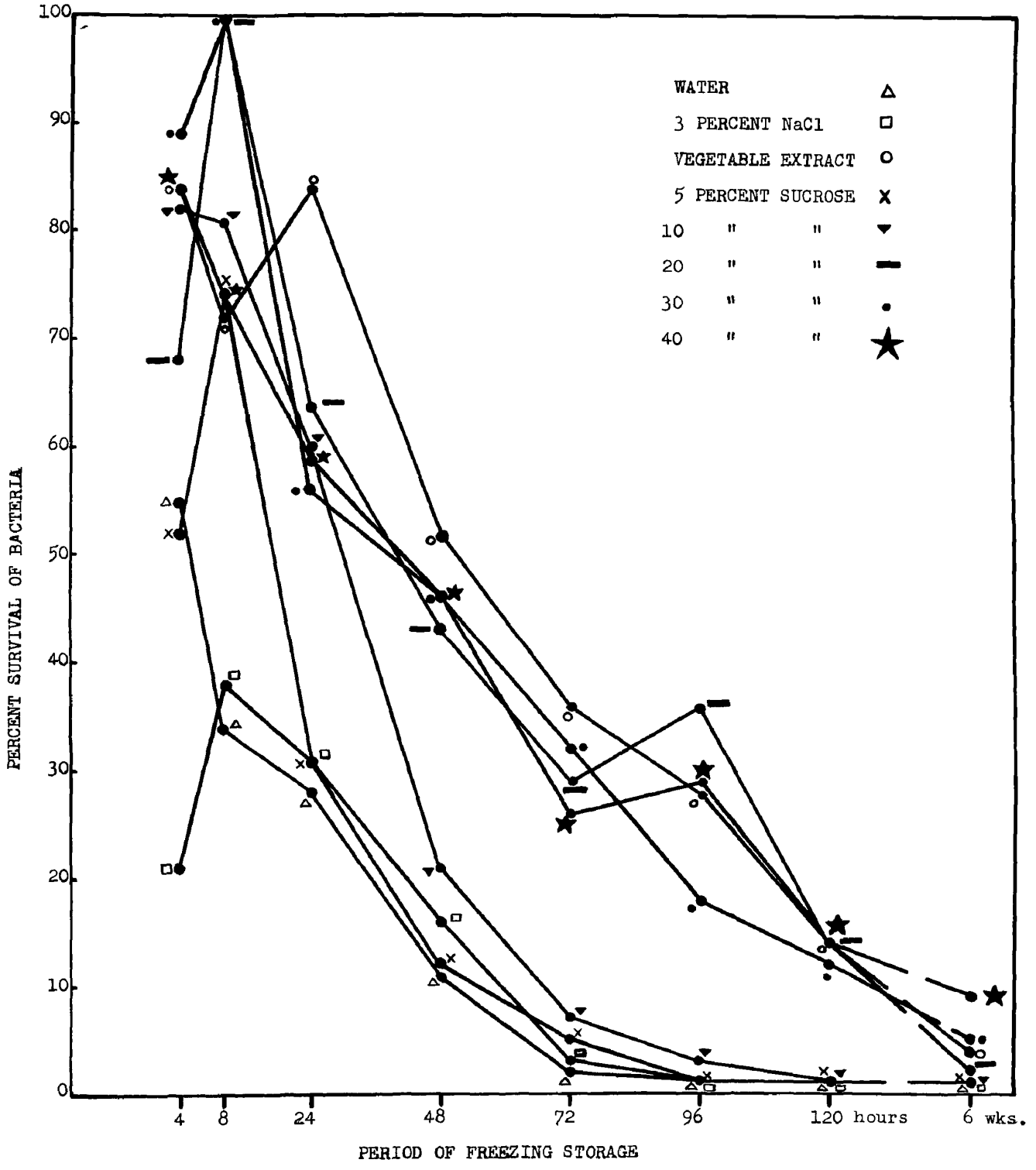

FIG.14. SURVIVAL OF THERMOPHILIC BACTERIA (ORG. NO. 100 - BACILLUS SP.) FROZEN IN DIFFERENT SUBSTRATES AT 0° F.

Table No. 14

Survival of Bacteria Frozen in Different Substrates at 0°F.

Org. No. 86 -- Thermophile -- Bacillus sp.

Count per ml. Substrate in Thousands and Percentage Survival

Substrate	Initial Count	Period of Freezing: 4 hours Count	% survival	8 hours Count	% survival	24 hours Count	% survival	48 hours Count	% survival	72 hours Count	% survival	96 hours Count	% survival	120 hours Count	% survival	6 weeks Count	% survival
Water	4,700	4,200	94	2,600	55	658	14	376	8	282	6	43	1	18	1	6.2	1
3% NaCl	5,000	5,000	100	3,200	64	1,300	26	600	12	450	9	150	3	38	1	12	1
Veg. Ext.	10,000	10,000	100	8,600	86	5,400	54	6,000	62	4,700	47	3,000	31	1,000	11	790	8
5% Sucrose	500	620	100	430	86	100	20	80	16	50	10	3.8	1	2.4	1	.8	1
10% "	10,000	16,000	100	8,200	82	3,900	39	3,600	36	2,800	28	1,600	16	800	8	200	2
20% "	6,300	7,800	100	6,200	98	2,390	38	2,900	46	2,330	37	1,200	19	440	7	56	1
30% "	5,300	4,200	80	2,100	40	2,970	56	954	18	1,270	24	1,100	22	1,700	32	740	14
40% "	200	180	90	130	65	76	38	60	30	54	27	82	41	36	18	14	7

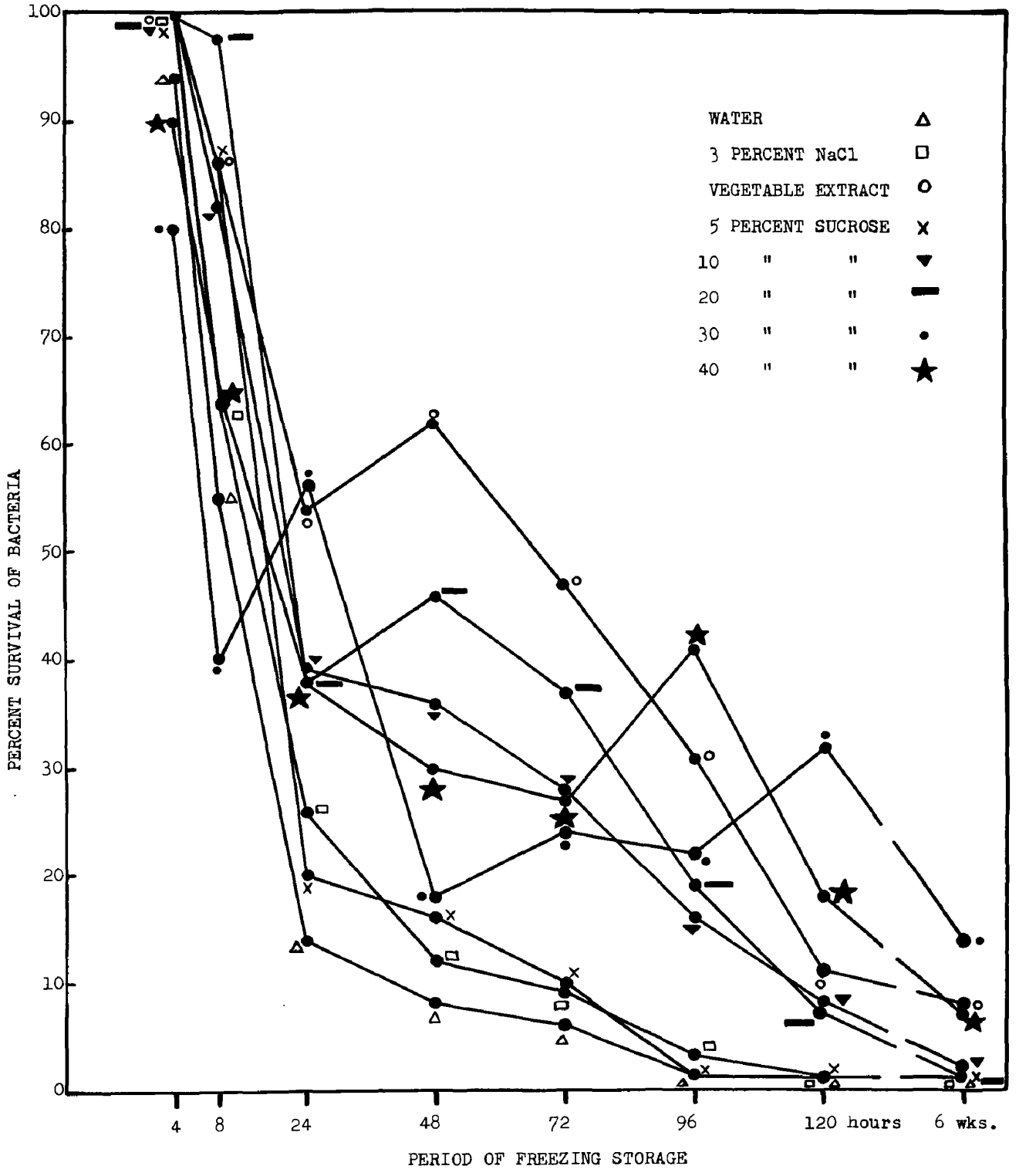

FIG. 15. SURVIVAL OF THERMOPHILIC BACTERIA (ORG. NO. 86 - BACILLUS SP.) FROZEN IN DIFFERENT SUBSTRATES AT 0° F.

Table No. 15

Survival of Bacteria Frozen in Different Substrates at 0°F.

(Average of All Organisms Studied -- 7 Organisms)

Percentage Survival

Substrate	Period of Freezing							
	4 hours	8 hours	24 hours	48 hours	72 hours	96 hours	120 hours	6 weeks
Water	53	38	29	24	24	17	14	9
3% NaCl	74	59	41	35	39	28	21	5
Veg. Ext.	87	81	68	58	52	48	35	26
5% Sucrose	93	64	30	30	29	15	12	6
10% "	78	68	52	41	32	32	18	20
20% "	76	77	63	57	50	39	27	20
30% "	84	66	67	58	49	33	28	20
40% "	86	81	66	59	48	50	33	19

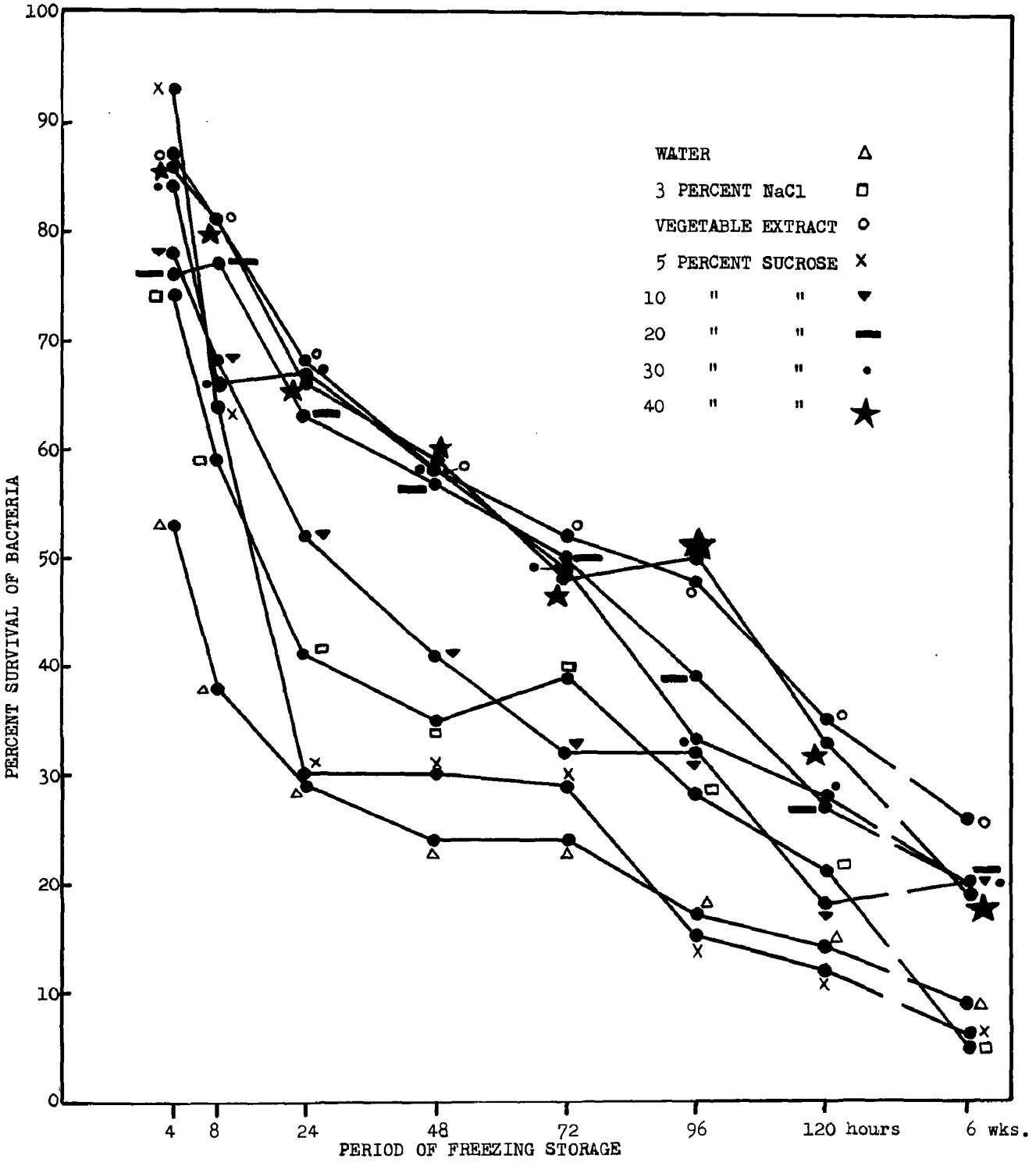

FIG. 16 . SURVIVAL OF BACTERIA FROZEN IN DIFFERENT SUBSTRATES AT 0° F.
(AVERAGE OF ALL ORGANISMS STUDIED)

there was a marked reduction during the first 4 hours of freezing followed by a gradual reduction with some variations throughout the 6 weeks of freezing storage. In all substrates there was a decided increase in counts at the 72 and 96 hour periods of freezing storage. The reason for the increased counts in this period is not known. Following the 96 hour period there was a decided decrease in numbers through to the end of freezing storage in the 3 per cent salt, vegetable extract, and 5 and 10 per cent sucrose. With water, and 20, 30 and 40 per cent sucrose substrates only slight decreases were noted following the 96 hour period. With the exception of the 5 per cent sucrose all of the higher sugar concentrations showed either similar or increased counts after 6 weeks' freezing storage compared with those obtained at the 4 hour period. After 6 weeks freezing storage the water, the 3 per cent salt and the 5 per cent sucrose appeared to give the least protection against freezing to this organism while 20 per cent sucrose and vegetable extract provided the greatest protection.

The species of Achromobacter exhibited even greater variability than the Micrococcus. No definite pattern was observed at any stage of the freezing process in any of the substrates used. Even after 6 weeks' freezing storage the results were considerably

different from those observed with other test organisms with the possible exception that in the water, salt and 5 per cent sucrose series this organism showed destructions greater than 80 per cent of the initial number. With this organism the 40 per cent sucrose solution appeared to provide less protection against freezing than any of the lower concentrations.

The results with Staphylococcus aureus (culture No. 490) in the various substrates are shown in Table 10 and Fig. 11. Although some fluctuations in counts from one period of analysis to another were noted these were not nearly as pronounced as with the two previous organisms. These data indicate that in the initial freezing process the water and 5 per cent sucrose provide the least protection to this organism against freezing. The low rate of survival with these two substrates continued throughout the freezing storage. The 3 per cent salt series showed no reduction in the first 4 hours of freezing but starting at the 8 hour period there is a steady decrease which continued through to the completion of freezing storage. The water, salt and 5 per cent sucrose substrates show about the same survival after 6 weeks' freezing storage. Of the remaining five substrates the vegetable extract and the 40 per cent sucrose provide the greatest protection throughout the freezing storage with the 10, 20 and

30 per cent sucrose being intermediate between these and the water, salt and 5 per cent sucrose substrates.

With Escherichia coli (culture No. 117) the vegetable extract and 40 per cent sucrose again provided the greatest protection throughout the freezing storage. In the initial freezing process the water, salt, 5 and 10 per cent sucrose showed the least survival. The water series with this organism showed considerable fluctuation with pronounced increases in counts at the 48 and 72-hour periods of analysis. The salt substrate showed the least protection with this organism. The 5 and 10 per cent sucrose followed a similar pattern throughout the storage period, ending with a low percentage survival, approximately the same as the salt suspension. The 20 per cent sucrose showed very little reduction in the first 4 hours of freezing but showed a marked reduction from then to the 48-hour period. This was followed by a levelling off up to the 96-hour period after which there was a steady decrease to the end of freezing storage.

With the three cultures of thermophilic spore formers (culture No's. 97, 100 and 86) one fact in particular is evident from the results presented in Tables 12 to 14 and Figs. 13 to 15. While there is

considerable difference in the rate of survival during the early period of freezing these organisms show a low percentage survival in all substrates after 6 weeks. In general, the three organisms in the different substrates follow a regular pattern throughout the freezing storage, showing a continual reduction in numbers from the initial count.

A summary of the survival of bacteria subjected to freezing in different substrates is shown in Table 15 and Fig. 16. These data indicate that after 4 hours' freezing the water substrate provides the least protection. The percentage survival with other substrates varies from 73 with the 3 per cent salt to 93 with 5 per cent sucrose. At the end of the freezing storage the vegetable extract shows the greatest survival with 26 per cent. Water, 3 per cent salt and 5 per cent sucrose show the least per cent survival indicating that the least protection from freezing was provided by these substrates. There was no difference in the rate of survival between the 10, 20 and 30 per cent sucrose substrates.

Discussion of Fluctuations

Since fluctuations in counts from one period of analysis to another were so prominent further studies were made to determine, if possible, the reason for

these variations. As previously noted in the review of the literature Pederson (23) noted these fluctuations in counts and attributed them to the breaking up of bacterial clumps during freezing. While this might be true during the initial freezing process it does not explain the fluctations which occur long after the initial freezing. Van Eseltine et al. (36) also noted increased counts in products during freezing storage and attributed them to growth of organisms before the product actually reached the freezing temperature. However, since these authors found increased counts after both 8 and 12 hours' freezing storage it would appear extremely unlikely that any growth would occur during this period since the temperature would be well below the range of growth for bacteria.

McFarlane (19) also records some fluctations in counts but makes no comment on the probable reason for the increases.

In the present investigation two organisms were studied, the Achromobacter species (No. 59) which had shown the greatest variability and the culture of Staphylococcus aureus (No. 490). This latter culture was chosen since microscopical examinations of this organism showed evidence of aggregations or clumps of cells.

Repeated tests with Staphylococcus aureus failed to indicate that the initial freezing process had any great influence on the number of colonies formed on agar plates. Taking the results of one of six suspensions frozen at 0 F. it was found that the counts per ml. were as follows: Initial, 10,500,000; 1 hour, 11,400,000; 2 hours, 10,400,000; 3 hours, 8,900,000; 8 hours, 9,800,000; 24 hours, 7,300,000; 48 hours, 9,400,000; 72 hours, 6,600,000; 96 hours, 3,500,000; 120 hours, 1,800,000; 168 hours, 1,200,000. From these data it is evident that no substantial increase in numbers occurred. Any variation in this particular series might easily be attributed to errors inherent in the plate count method since the difference in the colony count after the first two hours of freezing, during which period slightly increased counts were observed, varied from 104 to 124, and, as a point of interest, the 114 colony count was an average of duplicate plates.

Of considerably greater significance were the results of a series of studies made with the Achromobacter species. A suspension of this culture showed no evidence of clumping when examined under the microscope. However, when plate counts were made of different portions of the initial suspension which had been thoroughly shaken for a period of 15 minutes, variations in counts were noted from 3,600,000 to

12,400,000 per ml. Similar variations were observed in suspensions frozen at 0°F. for periods of 8 and 24 hours. At 8 hours the counts from different portions of a single suspension varied from 2,900,000 to 11,600,000; after 24 hours 3,500,000 to 14,000,000.

Similar tests with Staphylococcus aureus showed an initial variation in counts from 8,900,000 to 10,000,000; after 8 hrs. at 0°F., 9,800,000 to 10,800,000; after 24 hours 7,300,000 to 8,400,000.

These results suggest that fluctuations in counts from one period of freezing storage to another are associated with the uneven distribution of microorganisms. It is also suggested that some microorganisms show greater variability than others in suspensions. Prolonged shaking in a mechanical shaker failed to effect any important change in the degree of variation.

COMPARISON OF SURVIVAL OF SPORE AND VEGETATIVE STAGES OF BACTERIA SUBJECTED TO FREEZING.

There are few references in the literature relating to the freezing of spores. The only authentic work found was that reported by Haines (5) who froze aqueous suspensions of spores of Bacillus mesentericus

at a series of temperatures from -1°C. (30.2°F.) to -20°C. (-4°F.) for a period of 133 days. His results show that the spores of B. mesentericus suffered no change at any of the temperatures used.

In the present study five species of spore-forming bacteria isolated from either fresh beans or peas were used. Three cultures were thermophiles (Nos. 1, 4 and 5); the other two were mesophiles (Nos. 2 and 3). The vegetative stage of the organisms was obtained by frequent transfers and the culture for the test was used as soon as sufficient growth had appeared on the agar. The spore stage of the organisms resulted from leaving the cultures at their respective incubation temperatures for periods varying from 1 week for thermophiles to 2 weeks for mesophiles. To determine when the organisms were in the desired stage microscopical examinations were made. It was comparatively simple to obtain cultures in the vegetative stage, with the exception of organism No. 5 which apparently formed spores very rapidly. The spore stage of the organisms presented greater difficulty since vegetative forms were invariably present in small numbers.

The conventional method of heating the cultures to destroy vegetative cells was not used in these

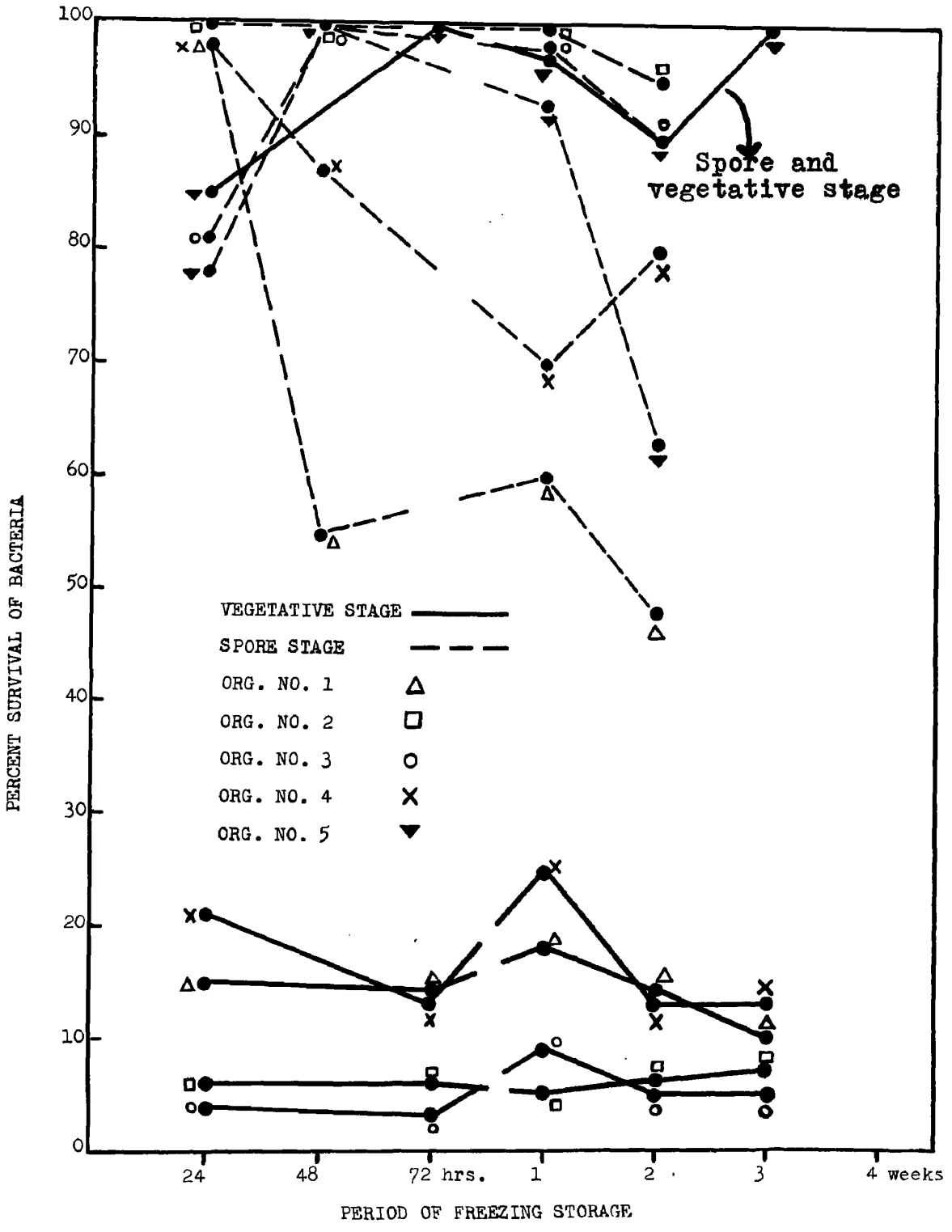

FIG.17. COMPARISON OF SURVIVAL OF SPORE AND VEGETATIVE FORMS OF FIVE SPECIES OF SPORE - FORMING BACTERIA.

studies since initial trials indicated that the susceptibility of the spores of the different cultures to heat varied considerably. To avoid delay together with the possibility that heating might affect the resistance of the spores to freezing the cultures were allowed to form spores under natural conditions. Since the spores were present greatly in excess of the vegetative forms it was felt that any differences as a result of freezing could be attributed to the spore stage.

The results presented in Fig. 17 demonstrate clearly that the vegetative stage is considerably less resistant to freezing than the spore stage. The data indicate that spores of thermophilic organisms are somewhat less resistant than those of mesophiles. The one culture out of line (organism No. 5) as indicated in this graph formed spores within the period of time in which growth appeared on the agar slant.

SURVIVAL OF BACTERIA FROZEN IN DIFFERENT PHASES OF GROWTH AT 0°F.

The interesting observation of Hegarty and Weeks (6) that cells of Escherichia coli in the logarithmic phase of growth were more susceptible to cold shock than mature cells led to a study of one series of cultures (Series A) and another of 8 cultures (Series B) of mesophilic bacteria both in the logarithmic stage of growth and in older cultures. The Series A were cultures which had been repeatedly transferred in the laboratory; Series B were cultures isolated from a fresh vegetable product.

The logarithmic stage was determined by growing the organisms in a vegetable extract and plating from this extract at various intervals. Since all cultures used in this study appeared to be growing actively after 24 hours incubation with no evidence of decrease in numbers it was considered that the cultures were in the logarithmic stage of growth at this period. The old cultures were tubes of inoculated vegetable extract which had been left at room temperature for approximately one week.

Results of this series of studies shown in Table 16 and 17 and Fig. 18 were disappointing. While

Table No. /6

Survival of Bacteria Frozen in Different Phases of Growth at 0°F. Series "A".

Count per ml. Substrate in Thousands and Percentage Survival

Cult. No.	Generic Classification	Initial Count	Period of Freezing: 4 hours Count	4 hours % survival	8 hours Count	8 hours % survival	24 hours Count	24 hours % survival	48 hours Count	48 hours % survival	72 hours Count	72 hours % survival	96 hours Count	96 hours % survival	168 hours Count	168 hours % survival	1 month Count	1 month % survival	Average
							Young Cultures												
41	Flavobacterium	2,000	1,200	60	925	46	1,000	50	1,100	55	800	40	720	36	950	48	820	41	47
58	"	5,800	3,900	67	5,800	100	5,000	86	4,400	76	3,800	66	3,700	64	6,600	100	6,100	100	82
62	Micrococcus	2,570	100	4	116	5	210	8	190	7	270	11	78	3	116	5	32	1	6
63	"	3,000	2,500	83	2,300	76	2,200	74	1,900	63	1,900	63	1,400	48	1,100	37	1,100	36	60
44	Achromobacter	4,000	1,500	38	2,000	50	1,600	40	400	10	800	20	720	18	480	12	37	1	24
69	"	16,000	8,000	50	10,000	62	8,900	56	10,000	62	9,000	56	7,400	46	10,000	62	6,100	38	54
72	Chromobacterium	33	22	67	33	100	13	40	13.5	42	9.4	28	10	30	1.4	4	6.9	11	40
73	Serratia	10,000	11,000	100	16,000	100	14,000	100	14,000	100	9,000	90	12,000	100	13,000	100	12,000	100	99
74	Sarcina	180	100	56	165	92	120	67	175	97	180	100	130	72	150	83	155	86	82
87	Bacillus	2,200	176	8	176	8	154	7	175	8	44	2	44	2	18.6	1	4	1	5
	Average			53		64		57		52		48		42		45		42	50
							Old Cultures												
41	Flavobacterium	190	70	37	110	58	90	47	130	68	27	14	28	15	87	46	18	10	37
58	"	110	70	64	35	32	33	30	80	73	71	65	33	30	30	27	62	56	47
62	Micrococcus	7,000	4,000	57	4,400	63	3,800	54	3,700	53	3,000	43	3,500	50	3,400	49	3,000	43	52
63	"	260	120	46	140	54	160	62	140	54	170	65	130	50	160	62	130	50	88
44	Achromobacter	1,200	1,100	88	984	82	900	75	1,100	88	960	80	1,100	92	768	64	516	43	77
69	"	870	770	11	960	100	870	100	800	92	600	69	400	46	750	86	670	77	73
72	Chromobacterium	70	25	36	6	9	4.8	7	.035	1	.01	1	.01	1	.01	1	.01	1	7
73	Serratia	1,120	750	67	830	74	860	77	770	69	910	81	840	75	660	59	770	69	71
74	Sarcina	120	70	58	110	91	100	83	93	77	130	100	88	73	79	66	57	52	75
87	Bacillus	1,000	680	68	720	72	530	53	460	46	680	68	850	85	380	38	160	16	56
	Average			53		64		59		62		59		52		50		42	55

Table No. 17

Survival of Bacteria Frozen in Different Phases of Growth at 0°F. Series "B".

Count per ml. Substrate in Thousands and Percentage Survival

Cult. No.	Generic Classification	Initial Count	Period of Freezing: 4 hours Count	% survival	8 hours Count	% survival	24 hours Count	% survival	48 hours Count	% survival	72 hours Count	% survival	96 hours Count	% survival	192 hours Count	% survival	1 month Count	% survival	Average
								Young Cultures											
125	Achromobacter	10,400	5,500	53	6,500	63	11,300	100	5,000	48	8,000	77	6,200	60	6,100	59	5,400	52	64
126	Flavobacterium	6,300	6,800	100	6,100	97	3,000	48	5,600	89	5,700	90	4,900	78	5,000	79	5,500	87	84
127	"	7,500	8,000	100	10,000	100	6,100	81	7,300	97	6,200	83	7,000	93	6,000	80	4,900	65	87
128	Achromobacter	14,600	4,700	32	5,100	35	7,900	54	7,300	50	4,400	30	6,500	45	4,200	29	5,600	38	39
129	Micrococcus	39,000	36,900	90	31,000	78	10,000	26	33,500	86	38,000	95	35,000	90	38,000	97	32,000	81	80
130	Achromobacter	11,000	4,800	44	4,800	44	6,200	56	4,900	45	4,200	38	6,000	55	4,600	42	4,000	36	45
131	"	3,700	2,300	62	2,100	57	1,900	51	2,100	57	2,300	62	2,400	65	2,200	59	2,100	57	59
132	Micrococcus	1,800	4,000	100	3,700	100	2,500	100	2,700	100	4,300	100	6,000	100	4,200	100	3,200	100	100
	Average			73		72		65		72		72		73		68		65	70
								Old Cultures											
125	Achromobacter	1,720	1,340	78	1,640	95	1,930	100	1,900	100	1,910	100	1,550	90	1,760	100	1,420	83	93
126	Flavobacterium	2,360	2,230	94	2,380	100	2,200	94	2,370	100	2,100	88	2,400	100	2,150	91	2,200	92	94
127	"	640	480	75	410	64	600	94	650	100	560	87	650	100	610	95	570	89	88
128	Achromobacter	170	50	29	54	32	40	24	50	29	75	44	95	56	54	32	25	15	33
129	Micrococcus	760	520	68	400	53	460	61	650	86	810	100	780	100	690	91	580	76	79
130	Achromobacter	246	203	86	158	64	175	71	194	79	171	70	225	91	191	78	201	82	78
131	"	670	660	99	600	94	690	100	710	100	840	100	700	100	720	100	650	97	99
132	Micrococcus	3,900	3,800	97	2,400	63	2,500	64	2,400	62	1,850	47	2,930	75	2,930	75	2,670	68	69
	Average			78		71		76		82		80		89		83		75	79

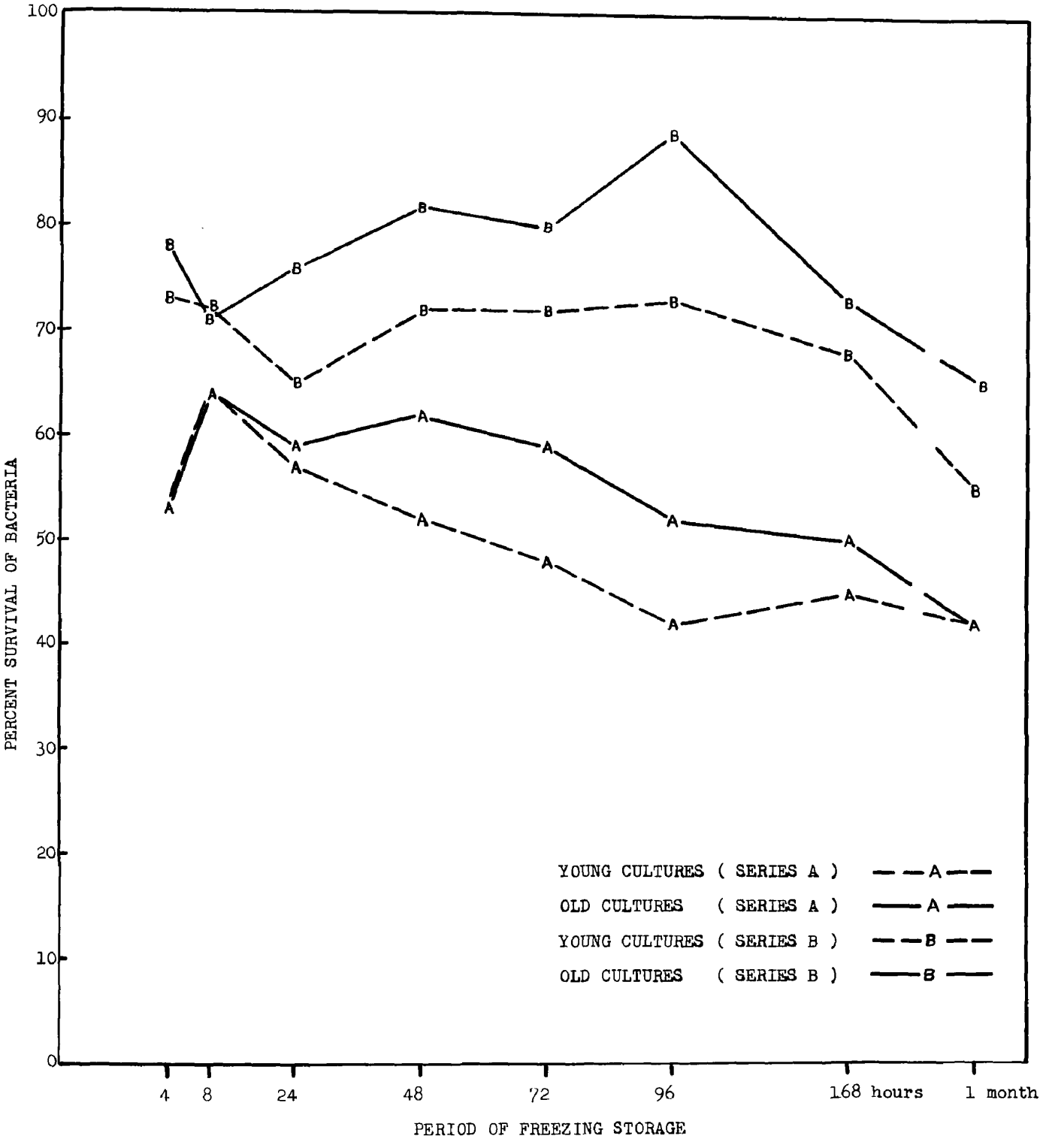

FIG.18. SURVIVAL OF BACTERIA FROZEN IN DIFFERENT PHASES OF GROWTH AT 0°F. SERIES A AND B.

some individual cultures did show less resistance to freezing in the logarithmic stage there were just as many that showed less or equal resistance to freezing in the old cultures. Of some interest are the results with members of the genus Achromobacter. Of the six cultures studied in the two series, five showed less resistance to freezing in the logarithmic phase of growth. None of the other genera represented showed any definite trend, some showing greater resistance to freezing in the logarithmic phase, others the opposite.

Attention should be drawn to the fact that the temperature of freezing used by Hegarty and Weeks in their studies was considerably higher than that used in this study. They were using a temperature of 0°C. (32°F.) compared with -17.8°C. (0°F.) in the present study. Also their results were based on findings with only one organism, Escherichia coli.

From the present studies it may be concluded that there are no important differences in the rate of survival between cultures of bacteria in the logarithmic phase of growth and of old cultures frozen at -17.8°C. (0°F.).

SURVIVAL OF MICROORGANISMS FROZEN CONTINUOUSLY AND INTERMITTENTLY IN WATER AT DIFFERENT TEMPERATURES

Previous studies by other workers have shown, as

indicated in the review of the literature, that intermittent freezing is more destructive to bacteria than continuous freezing. A study on this phase was conducted in the present investigation on four species of bacteria and three species of moulds isolated from fresh vegetable products. The three species of moulds used formed definite colonies on agar plates. Any spreading type was rejected since it would be difficult with the usual bacteriological technique to determine whether or not they were affected by freezing.

All cultures were frozen in water at 4 different temperatures, -28.89°C. (-20°F.), -17.8°C. (0°F.) -9.44°C. (15°F.) and 0°C. (32°F.). For each organism studied one suspension was used throughout for the intermittent freezing. This tube was removed at each period of analysis and after holding for 2 hours at room temperature following defrosting was returned to the freezer. For the continuous freezing the procedure was the same as for previous studies and separate suspensions were prepared simultaneously and frozen. One tube of suspension was removed from the freezer at each period of analysis and when the plating was complete was discarded. Analyses were made of each test organism initially and after 1, 2, 4, 7, 8, 9 and 10 days for bacteria and at intervals of 5 and 24 hours, 1, 2, 4, 5 and 6 weeks for moulds.

The results for bacteria shown in Table 18 and Figs. 19 to 22 give some interesting findings. With all four cultures studied the continuously frozen series showed considerable fluctuation from one period of analysis to another at all four temperatures. The series frozen intermittently showed no fluctuation and indicated pronounced reductions as compared to the continuously frozen series. It is of interest to note the results with two of the organisms held at 32°F. (culture No. 122 - Lactobacillus sp. and culture No. 123 - Micrococcus sp.). From the data presented it would appear that at 32°F. these two organisms showed a greater resistance to freezing than at any of the other temperatures used. However, it should be pointed out that at 32°F. the suspensions were not frozen and subsequent tests with these two organisms showed that growth occurred, particularly during the early hours of storage. There is little doubt from the results that, as a rule, intermittent freezing is more injurious to bacterial cells than continuous freezing at any of the four temperatures used.

Studies with the three cultures of moulds showed some interesting facts. (Table 19 and Figs. 23 to 25). The most noticeable feature is the pronounced resistance of moulds to both intermittent and continuous freezing as compared with the bacteria.

Table No. 18

Survival of Bacteria Frozen Continuously and Intermittently in Water at Different Temperatures

Count per ml. Substrate in Thousands and Percentage Survival

Org. No.	Generic Classification	Freezing Treatment	Freezing Temp.	Initial Count	1 day Count	1 day % survival	2 days Count	2 days % survival	4 days Count	4 days % survival	7 days Count	7 days % survival	8 days Count	8 days % survival	9 days Count	9 days % survival	10 days Count	10 days % survival
121	Flavobacterium	Continuous	-20°F.	6,800	5,600	82	5,600	82	4,500	66	6,000	88					5,200	76
			0°F.		5,400	80	4,900	72	3,800	56	4,400	65					4,700	69
			15°F.		4,000	59	6,100	90	4,300	63	3,400	50					4,600	68
			32°F.		4,900	72	6,700	99	5,700	84	3,300	49					3,100	46
		Intermittent	-20°F.				4,000	59	1,800	26	380	6	111	2	31	<1	8.5	<1
			0°F.				1,400	21	210	3	22	<1	7.3	<1	2.8	<1	1.6	<1
			15°F.				156	2	2.5	<1	.13	<1	.11	<1	.1	<1	.07	<1
			32°F.				3,400	50	274	4	3.1	<1	1	<1	.1	<1	.015	<1
122	Lactobacillus	Continuous	-20°F.	21,500	6,200	29	11,200	52	16,900	79	10,400	48					12,200	57
			0°F.		12,800	60	14,700	68	11,700	54	14,800	69					12,800	60
			15°F.		7,600	35	12,800	60	11,600	54	5,500	26					8,600	40
			32°F.		19,700	92	22,400	100	18,600	87	19,300	90					15,500	72
		Intermittent	-20°F.				560	3	54	<1	11	<1	4.9	<1	2.4	<1	1.2	<1
			0°F.				4,200	20	490	2	94	<1	20	<1	8.3	<1	4.8	<1
			15°F.				520	2	24	<1	4.2	<1	1	<1	.4	<1	.4	<1
			32°F.				18,700	87	18,000	84	12,200	57	13,400	62	9,800	46	6,600	31
123	Micrococcus	Continuous	-20°F.	7,600	5,400	71	6,000	78	5,300	70	5,700	75					5,400	71
			0°F.		5,900	78	6,400	84	4,700	62	6,600	87					5,400	71
			15°F.		3,800	50	4,600	61	3,900	51	4,400	58					5,400	71
			32°F.		6,900	91	8,300	100	6,600	87	7,900	100					7,100	93
		Intermittent	-20°F.				4,100	54	920	12	93	1	17	<1	2.3	<1	.23	<1
			0°F.				3,000	39	580	8	52	<1	4	<1	.5	<1	.02	<1
			15°F.				300	4	17	<1	.9	<1	.13	<1	.01	<1	.005	<1
			32°F.				6,900	91	7,000	92	7,200	95	6,000	79	5,900	78	5,000	66
124	Flavobacterium	Continuous	-20°F.	36,200	27,300	75	27,100	75	24,500	68	23,300	64					26,000	72
			0°F.		28,000	77	24,700	68	22,400	62	24,800	68					20,000	55
			15°F.		24,400	67	19,200	53	17,700	49	21,000	58					22,300	62
			32°F.		24,300	67	31,100	86	25,100	69	930	3					14,400	40
		Intermittent	-20°F.				17,200	48	7,000	19	2,700	7	33	<1	13	<1	.005	<1
			0°F.				14,900	41	3,500	10	420	1	3	<1	.1	<1	.005	<1
			15°F.				5,900	16	68	<1	.8	<1	.01	<1	.01	<1	.025	<1
			32°F.				20,900	58	2,000	6	76	<1	.12	<1	.01	<1	.01	<1

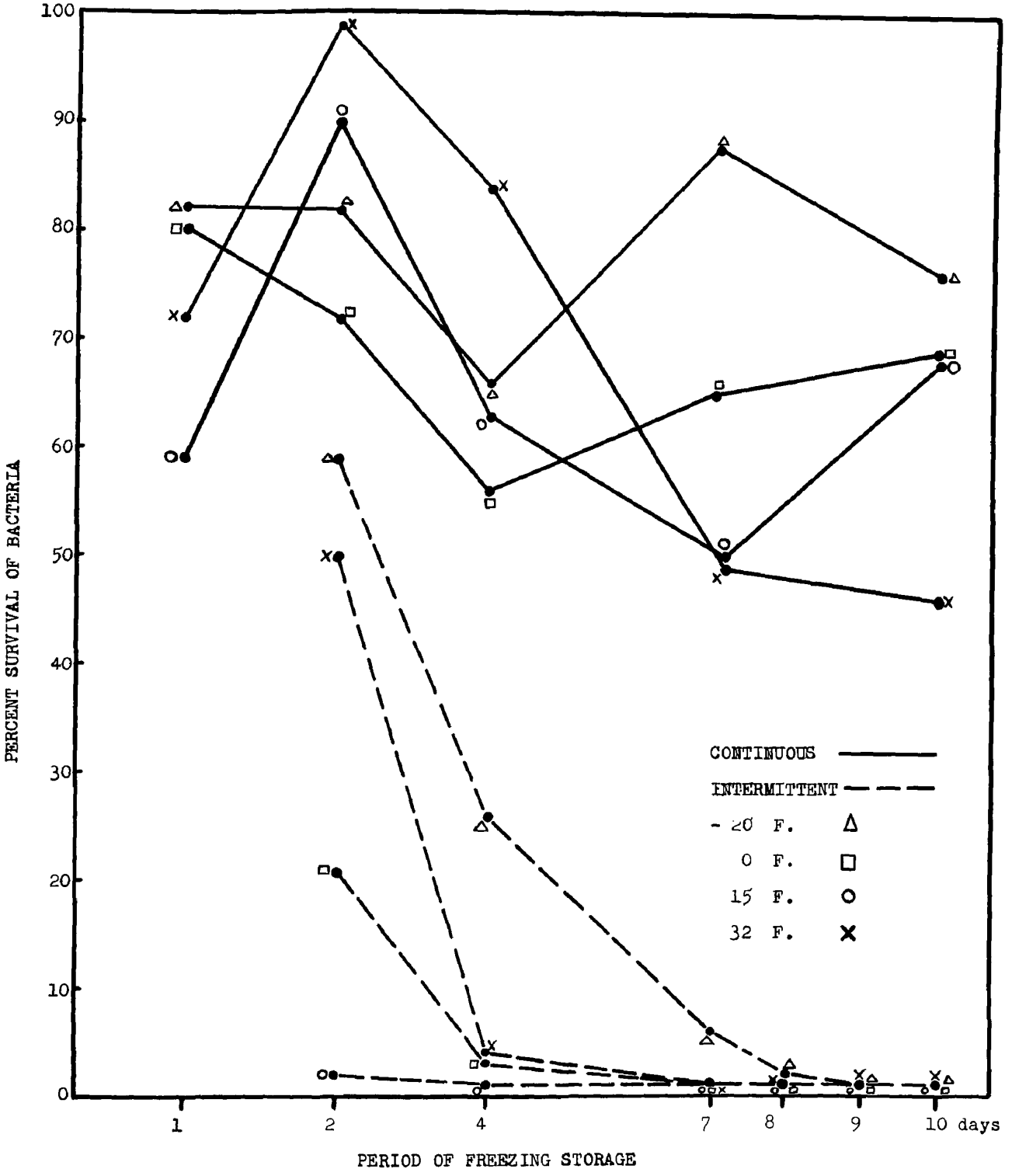

FIG.19. SURVIVAL OF MESOPHILIC BACTERIA (CULTURE NO. 121 - FLAVOBACTERIUM SP.) FROZEN CONTINUOUSLY AND INTERMITTENTLY IN WATER AT DIFFERENT TEMPERATURES.

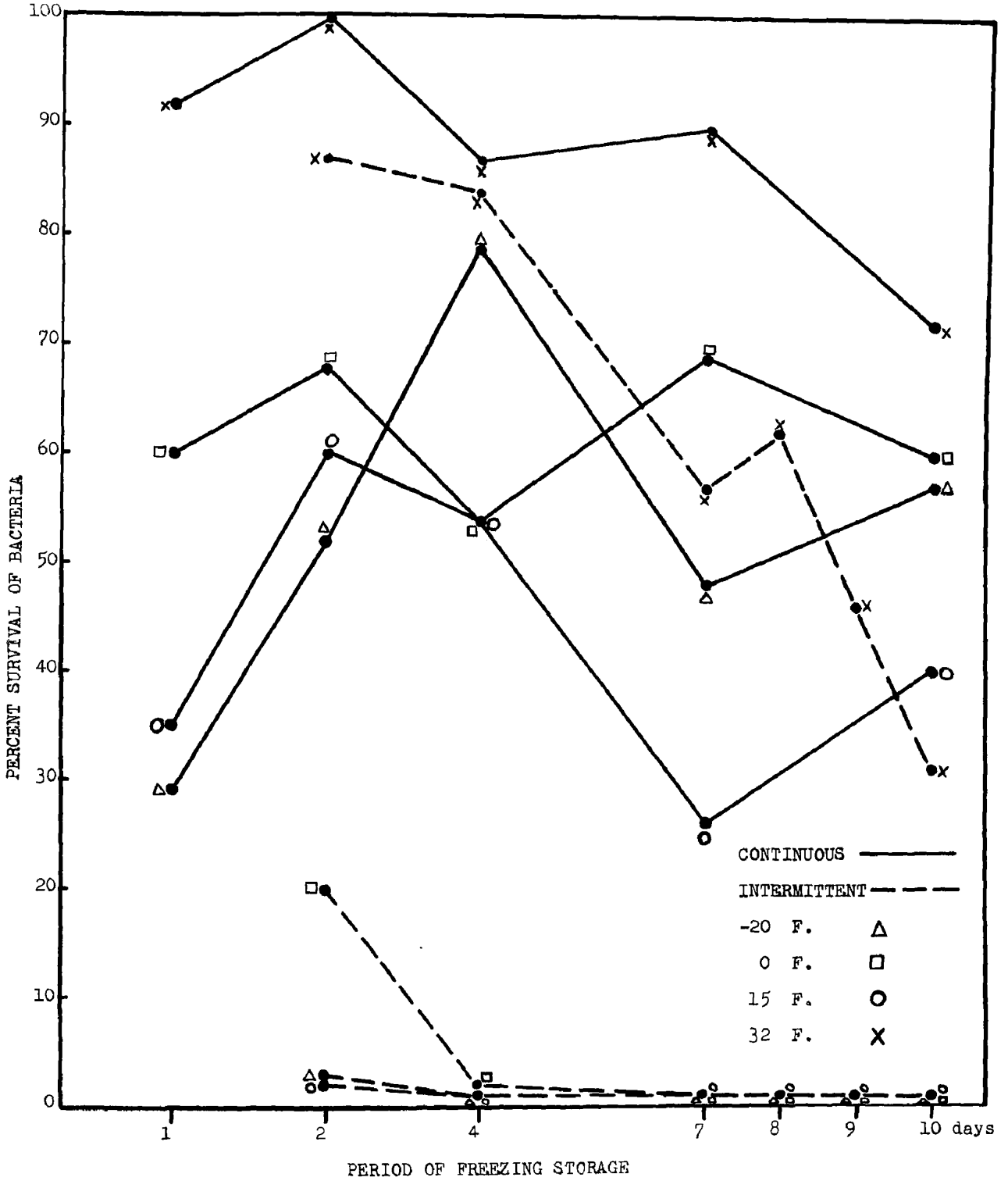

FIG.20. SURVIVAL OF MESOPHILIC BACTERIA (CULTURE NO. 122 - LACTOBACILLUS SP.) FROZEN CONTINUOUSLY AND INTERMITTENTLY IN WATER AT DIFFERENT TEMPERATURES.

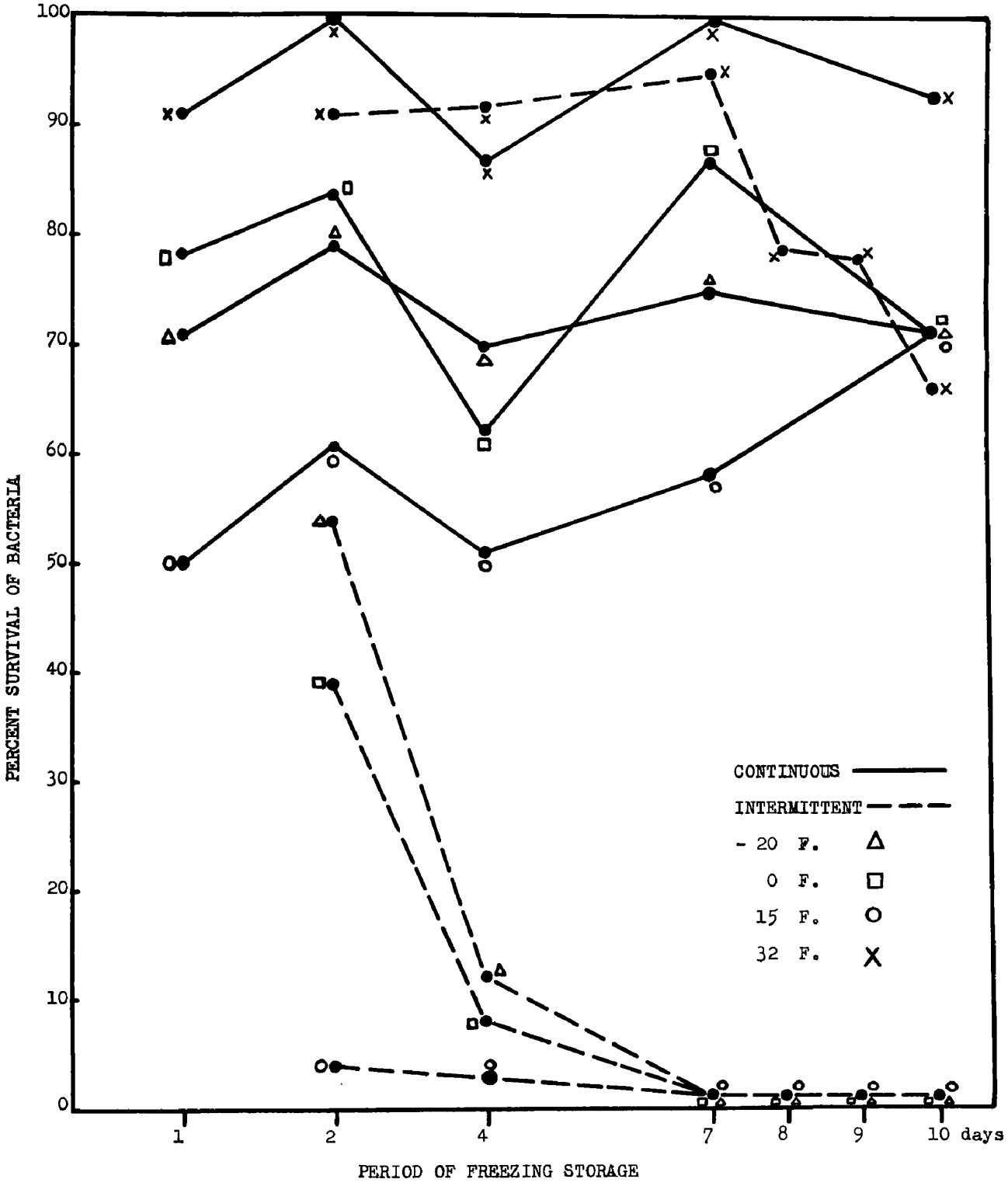

FIG.21. SURVIVAL OF MESOPHILIC BACTERIA (CULTURE NO. 123 - MICROCOCCUS SP.) FROZEN CONTINUOUSLY AND INTERMITTENTLY IN WATER AT DIFFERENT TEMPERATURES.

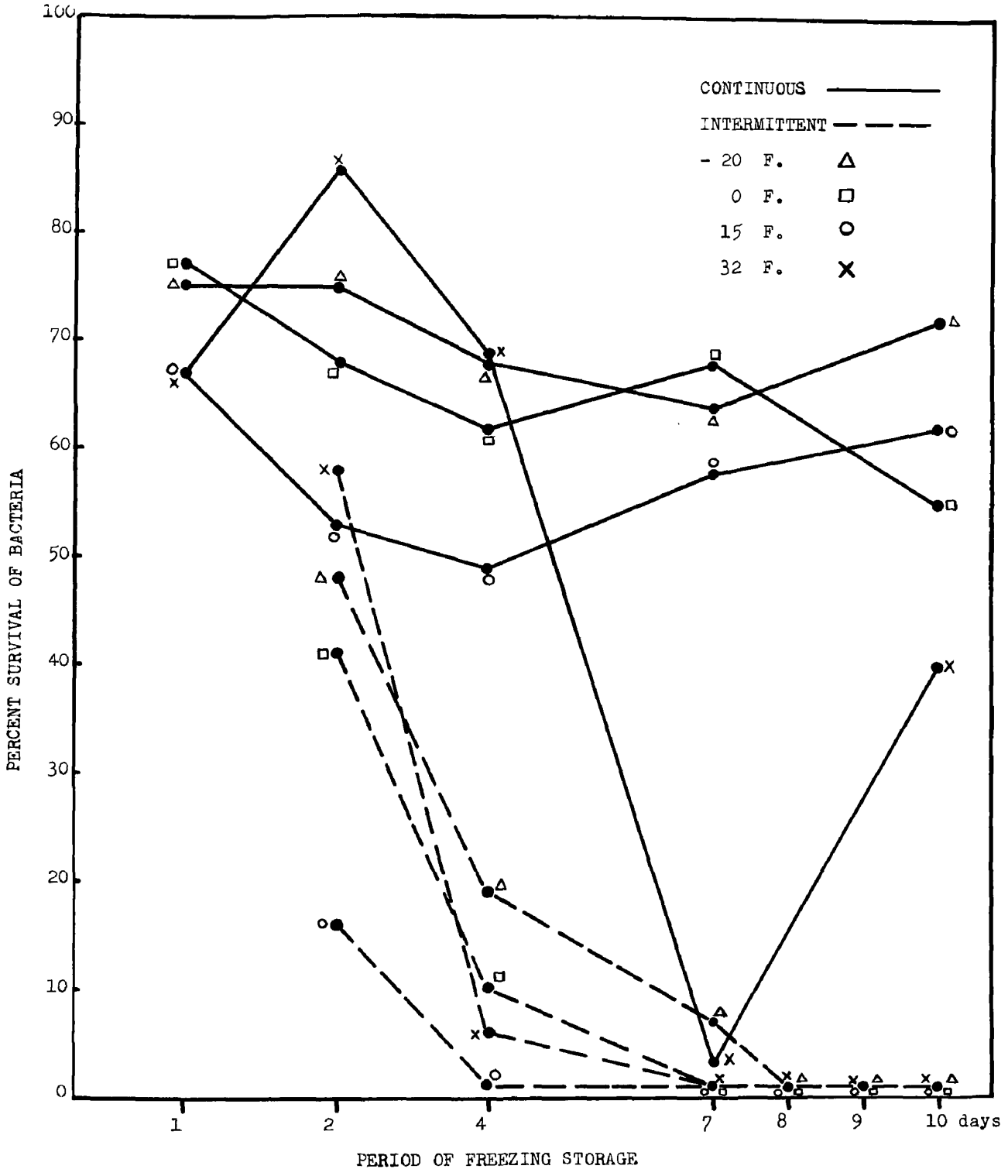

FIG.22. SURVIVAL OF MESOPHILIC BACTERIA (CULTURE NO. 124 - FLAVOBACTERIUM SP.) FROZEN CONTINUOUSLY AND INTERMITTENTLY IN WATER AT DIFFERENT TEMPERATURES

Table No. 19

Survival of Moulds Frozen Continuously and Intermittently in Water at Different Temperatures

Count per ml. Substrate in Thousands and Percentage Survival

Org. No.	Generic Classification	Freezing Treatment	Freezing Temp.	Initial Count	Period of Freezing: 5 hours Count	5 hours % survival	24 hours Count	24 hours % survival	1 week Count	1 week % survival	2 weeks Count	2 weeks % survival	4 weeks Count	4 weeks % survival	5 weeks Count	5 weeks % survival	6 weeks Count	6 weeks % survival
1	Penicillium	Continuous	-20°F.	63	76	100	65	100	56	89	62	98	69	100	59	94	59	94
			0°F.		64	100	64	100	60	95	55	86	58	92	52	83	48	76
			15°F.		69	100	65	100	53	84	50	79	48	76	41	65	43	69
			32°F.		54	86	80	100	74	100	69	100	98	100	66	100	50	79
		Intermittent	-20°F.				70	100	58	92	50	79	40	64	37	59	15	24
			0°F.				51	81	50	79	39	62	28	44	32	51	21	33
			15°F.				44	70	45	71	27	43	25	40	17	27	9.2	15
			32°F.				78	100	91	100	72	100	52	83	54	86	24	37
3	Aspergillus	Continuous	-20°F.	39	59	100	36	92	62	100	41	100	55	100	45	100	40	100
			0°F.		49	100	38	97	46	100	28	72	66	100	40	100	34	87
			15°F.		58	100	37	95	40	100	40	100	29	74	39	100	18	46
			32°F.		46	100	26	67	54	100	51	100	43	100	42	100	43	100
		Intermittent	-20°F.				33	85	39	100	27	69	22	56	14	36	9.8	25
			0°F.				39	100	32	82	15	38	32	82	13	33	7.8	20
			15°F.				26	67	28	72	25	64	18	46	11	29	7.6	19
			32°F.				35	90	62	100	53	100	20	51	20	51	25	64
4	Penicillium	Continuous	-20°F.	15	13	86	11	73	13	88	10	69	9.7	66	8	54	8.9	61
			0°F.		12	84	12	80	11	73	10	69	8.5	58	9.5	65	8.6	59
			15°F.		15	100	8.4	57	11	73	8.1	55	7.3	50	7.7	52	7.4	50
			32°F.		16	100	15	100	15	100	15	100	13	90	13	90	9.1	62
		Intermittent	-20°F.				9.4	64	7.8	53	4.9	33	2.3	16	3.5	24	1.9	13
			0°F.				8.3	56	5	34	4.6	31	4.6	31	3.9	27	1.9	13
			15°F.				9.3	63	6.9	47	3.6	24	1.5	10	1.2	8	.5	3
			32°F.				15	100	12	80	8.1	55	4.3	29	1.7	12	.5	3

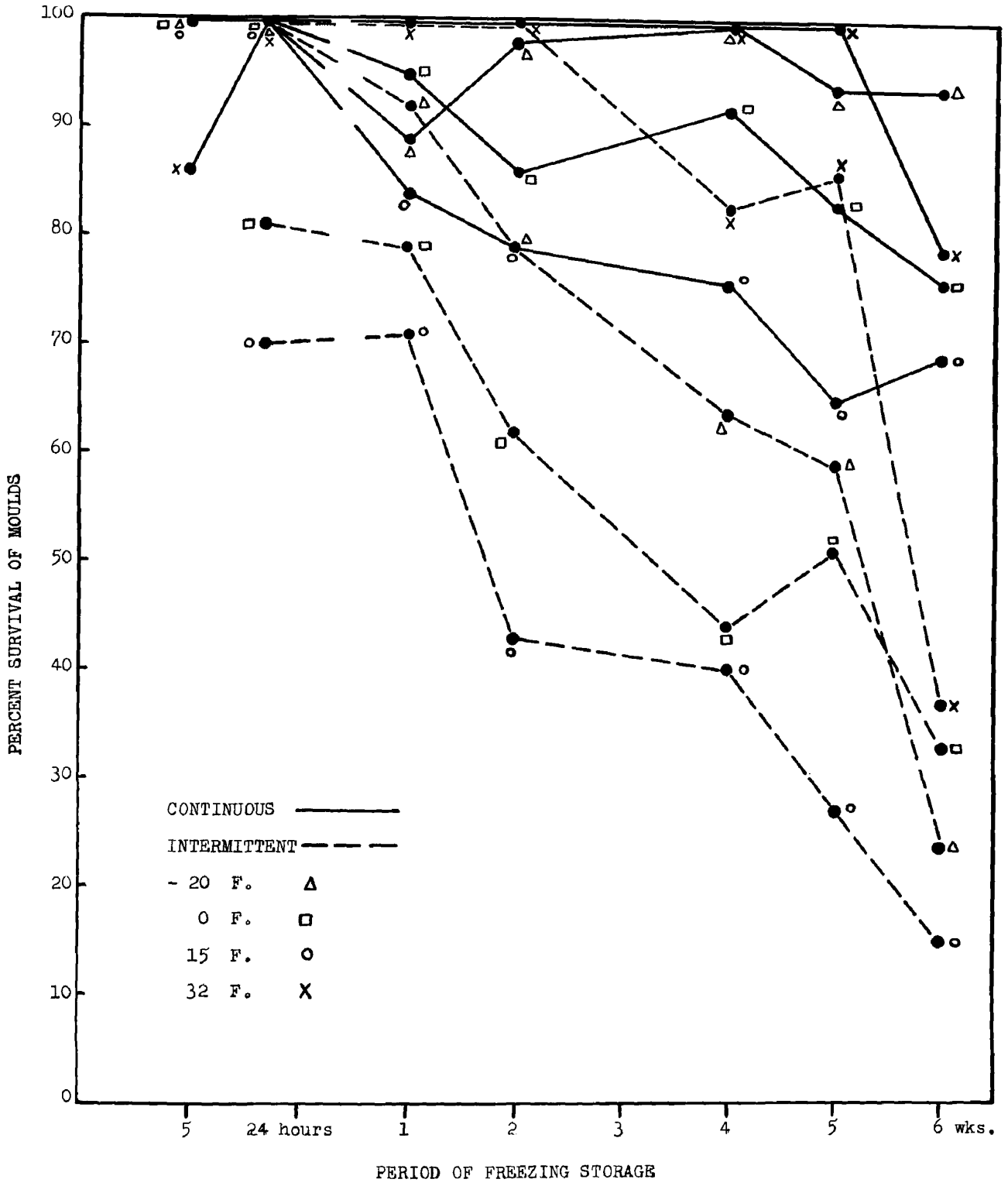

FIG.23. SURVIVAL OF MOULDS (PENECILLIUM SP. - ORG. NO. 1) FROZEN CONTINUOUSLY AND INTERMITTENTLY IN WATER AT DIFFERENT TEMPERATURES.

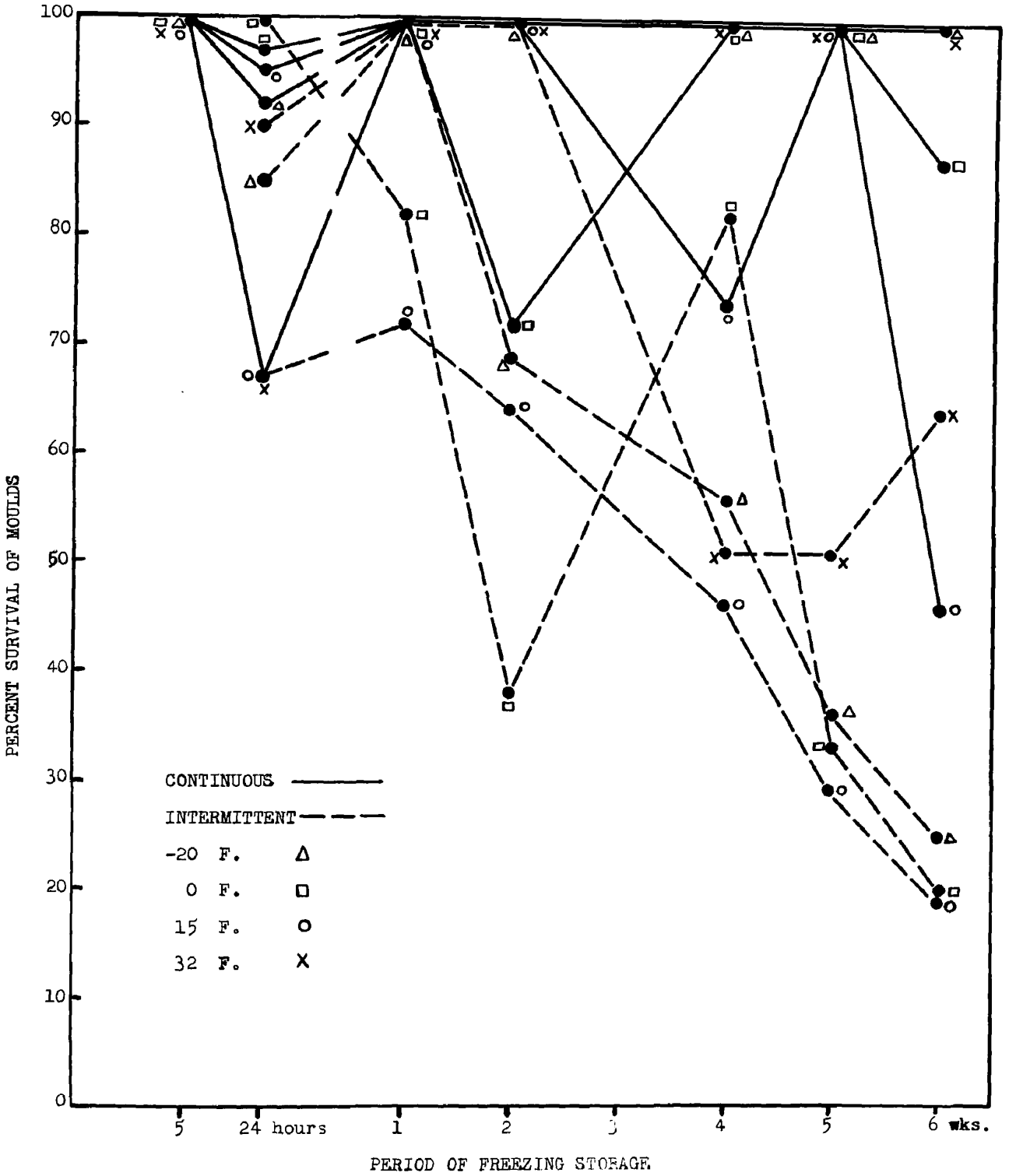

FIG.24. SURVIVAL OF MOULDS (ASPERGILLUS SP. - ORG. NO.3) FROZEN CONTINUOUSLY AND INTERMITTENTLY IN WATER AT DIFFERENT TEMPERATURES

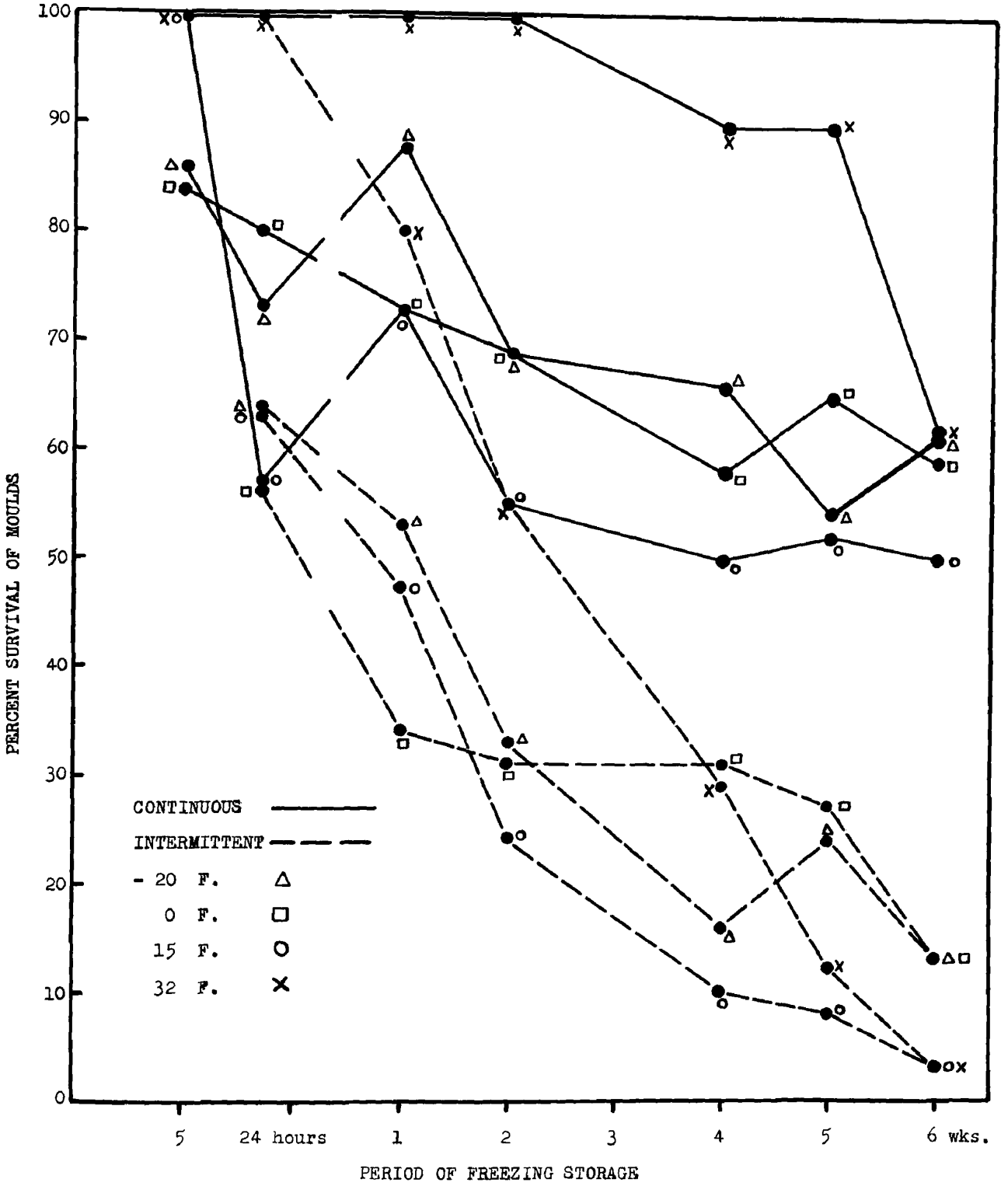

FIG.25. SURVIVAL OF MOULDS (PENECILLIUM SP. - ORG. NO. 4) FROZEN CONTINUOUSLY AND INTERMITTENTLY IN WATER AT DIFFERENT TEMPERATURES

This was particularly evident with the continuously frozen series during the early period of freezing storage and to some extent throughout the entire period of storage. With the intermittent series there was little doubt that moulds frozen intermittently were considerably more resistant than bacteria similarly treated.

From these results it may be concluded that intermittent freezing is more destructive than continuous freezing, both to bacteria and moulds. The data indicate that intermittent freezing is more destructive to bacteria than it is to moulds. Temperature generally is not an important factor in the range of -20°F. to 15°F. At 32°F. some of the test organisms showed growth.

THE EFFECT OF ANTIBIOTICS ON BACTERIA IN VEGETABLE PRODUCTS

Since the problem of natural antibiotics in vegetable products is so extensive in its scope no attempt was made in the present study to make a thorough investigation of the proportion of organisms producing substances which control the growth of other organisms. The only purpose in including the work here reported is to point out just one more complexity associated with studies on

the microbiology of frozen fruits and vegetables. The appearance of an antibiotic in the present study was accidental and came about when pure cultures of Serratia marcescens, Sarcina lutea, Staphylococcus aureus and Chromobacterium violaceum were inoculated into fresh and blanched peas. The purpose of the study was to determine the rate of growth of these test organisms in fresh and blanched peas both before and after freezing. The peas used for this study were not sterile, the rate of growth of the test organisms being determined by plate counts of only those colonies of organisms corresponding to the original test organism.

The results presented in Table 20 are for the fresh, unfrozen series. The results for the frozen series were quite similar to those of the fresh product with the exception of results with one test organism, Chromobacterium violaceum. Apparently this organism is very sensitive to freezing since in all tests it completely disappeared after 24 hours at 0°F. With Serratia marcescens in the fresh product it is of interest to note the more rapid rate of growth in the blanched product as compared with the counts in the unblanched product. This is likely due to the release of soluble solids in the blanch process which provides additional nutrients for the growth of this organism. Of particular interest were the results

with Sarcina lutea and Staphylococcus aureus in both blanched and unblanched peas. Held 24 hours at room temperature both of these test organisms had disappeared. Since the pH of the peas had dropped from a normal of 6.2 to 5.0 it was first thought that the increased acidity was responsible for the disappearance of the test organisms. However, the fact that Serratia marcescens showed only a slight decrease in numbers in the unblanched product and a normal rate of growth in the blanched product suggested that in addition to the acid an antibiotic might be the factor responsible for the peculiar results. Further studies in which the pH was held at 6.2 using a titrimeter indicated definitely the presence of an antibiotic which as subsequent plate tests showed was produced by a spore former and was active against Sarcina lutea and Staphylococcus aureus but not against Serratia marcescens or the Chromobacterium violaceum. The increased acidity was probably responsible for the disappearance of Chromobacterium violaceum in the unblanched product. Why it also disappeared in the blanched product in the Series A is not known unless an acid-producing type organism survived the blanch process and produced sufficient acid to inhibit this organism but not the others. Chromobacterium violaceum has been noted in other studies to be very sensitive to various agents.

Table No. 20

Rate of Growth of Pure Cultures of Bacteria in Unblanched and Blanched Peas

Series A - pH Unaltered - Dropped from Normal pH 6.2 to 5.0

	Count per Gram Product			
	Serratia Marcescens	Sarcina Lutea	Staphylococcus Aureus	Chromobacterium Violaceum
		Unblanched		
Initial	12,950,000	875,000	1,200,000	6,300,000
2 hours	14,700,000	700,000	900,000	9,450,000
4 hours	10,500,000	700,000	600,000	7,700,000
8 hours	5,600,000	0	3,500	3,500
12 hours	9,450,000	0	0	0
24 hours	3,500,000	0	0	0
		Blanched		
Initial	12,300,000	263,000	3,600,000	6,300,000
2 hours	22,000,000	455,000	1,800,000	14,350,000
4 hours	65,000,000	1,400,000	760,000	1,800,000
8 hours	149,000,000	1,400,000	35,000	350,000
12 hours	350,000,000	350,000	0	0
24 hours	2,100,000,000	<3,500	0	0

Series B - pH Adjusted - Held at Constant pH 6.2

	Count per Gram Product			
	Serratia Marcescens	Sarcina Lutea	Staphylococcus Aureus	Chromobacterium Violaceum
		Unblanched		
Initial	22,000,000	6,000,000	14,000,000	600,000
2 hours	36,000,000	7,200,000	12,600,000	840,000
4 hours	82,000,000	4,800,000	9,400,000	1,200,000
8 hours	96,000,000	960,000	6,200,000	3,100,000
12 hours	1,900,000,000	350,000	3,500,000	4,800,000
24 hours	3,600,000,000	<3,500	<3,500	12,000,000

DISCUSSION

A study of microorganisms associated with frozen fruits and vegetables presents many difficulties, as may be gathered from the work here reported. There are many variables to be considered which may have a definite influence on the rate of survival of microorganisms during freezing and subsequent freezing storage. Probably the most important factor involved is the type of organism present on the products. Results of the present study with almost 100 different species, isolated from fruit and vegetable products, clearly indicates that some species of bacteria show enormous reductions in numbers when stored at 0°F. for a period of 6 weeks while others show considerable resistance to freezing with a lesser reduction in numbers during the same period of freezing storage and under identical conditions. There are also tremendous differences in the rate of destruction of different species since many showed no reduction during the freezing process and early freezing storage; others showed a marked susceptibility to freezing with almost complete destruction during the first 4 hours of freezing. Differences were also noted within the species when cultures of Escherichia coli were studied. An important point is that all organisms studied in

this work did show reductions in numbers after 6 weeks freezing storage.

The substrate in which the organism is suspended for freezing has been considered an important factor by many workers. However, the results presented in this study, while indicating a definite protection to some organisms against freezing in substrates such as a vegetable extract and 40 per cent sucrose, show that other organisms are not appreciably protected by these substrates. The results suggest that the character of the substrate may not be nearly as important as was previously thought, since the results presented in a graph (Fig. 16) of the average of all organisms studied in the different substrates indicate that the difference between the percentage survival in vegetable extract and in water was 17 per cent. It is doubtful if this figure is significant since, if different cultures had been used, such as spore formers, then the difference in the rate of survival after 6 weeks freezing storage would be negligible.

The temperature of freezing has been thought by many to influence the effect on microorganisms. However, in this study, within the range of commercial freezing temperatures, -20°F. to 15°F., temperature did not appear to be an important factor, although some

individual cultures do show some difference in rate of survival at the different temperatures used. In these instances -20°F. appears to offer greater protection. Again the importance of this factor varies with the species of bacteria.

Definite evidence is presented in this and other studies on the effect of intermittent freezing as compared with continuous freezing. There is little doubt that intermittent freezing is more destructive to microorganisms than continuous freezing. From the point of view of evaluating the quality of frozen fruits and vegetables on the basis of the microbial content this is an unfortunate finding since a product, although showing a marked deterioration in quality due to defrosting and refreezing, will also show a relatively low microbial content. This means that in any analysis of frozen fruits and vegetables, particularly when the counts are low, the complete freezing history of the product must be available to the analyst. The results also suggest that the microbiological findings alone cannot be used in assessing the quality of frozen fruits and vegetables.

From the results presented it is obvious that it would be impossible to predict the effect of freezing on the microbial load of a fresh fruit or vegetable going into the freezer. However, attention should be

directed to newer methods in the commercial processing of frozen vegetables. Pre-processing procedures, in many plants, now employ a longer blanch to a peroxidase trace or negative reaction rather than the older procedure of using tests for the enzyme catalase which is inactivated at lower temperatures than peroxidase. The longer blanch results in a marked diminution in the initial microbial load and, perhaps of greater significance, a reduction in the number of types of microorganisms. This longer blanch should eliminate most bacteria other than spore formers and these, as indicated in the present study, show less resistance to freezing in the vegetative stage than many other types of bacteria. Thus, by reducing the opportunity for recontamination following the blanch process, and with continuous line operation, it is possible to produce frozen vegetables with a relatively low microbial content.

With fruits which are not subjected to blanching, the picture is entirely different. Up to the present no method has been devised to control the numbers of microorganisms on products going into the freezer. However, natural protection due to the lower pH of fruits will limit the types and growth of some of the microorganisms present on the product.

While no reference has been made in this paper

to the various theories which have been advanced concerning the death of microbial cells by freezing it might be well at this point to discuss some of these theories and present the views of the writer. Luyet and Gehenio (17) have dealt fully with these theories in their writings so no useful purpose would be served by going into detail in the present work. They list the theories which a number of workers have attributed the mechanism of death by freezing to as follows:

1. A withdrawal of energy
2. The attainment of a minimal temperature
3. Mechanical injury
4. Too rapid thawing
5. Dehydration
6. Various physiological, physical and chemical changes

Each one of these theories has some point in its favour but unfortunately many of the theories are based on observations with higher plants and animals and attempts have been made to apply these observations to microbial cells.

Many workers have subscribed to the theory of death of cells by mechanical injury. It was suggested that the production of ice crystals during the freezing process resulted in disintegration of microbial cells. However, Haines (5) was unable to find any change in

the microbial cells due to freezing. This finding was substantiated in the present study. Cells which survived freezing showed no apparent change in the metabolic processes of the organisms studied.

Rahn (26) states that "freezing involves several causes of death, and the most common cause, injury by ice crystals, is quantitatively unpredictable. Thus, no order of death can be expected and no order has been observed". Rahn does not present any data to substantiate his statements.

Based on observations in the present investigation it is the opinion of the writer that none of the first five theories listed are applicable to microbial cells since none account for the fact that some cells in an actively growing culture of bacteria are killed during the process of freezing while others apparently suffer no change. The writer suggests that "weak cells" in a bacterial suspension are the ones that are killed while the stronger cells survive. The reason for some cells being weaker than others in the same suspension is not known but it could be associated with some of the factors mentioned by Luyet and Gehenio (physiological, physical and chemical changes).

SUMMARY

Data are presented on the effect of freezing vegetables harvested from the same general area at various times showing wide differences in the rate of survival of bacteria. These data also indicate that freezing of vegetables effects a reduction in the number of types of bacteria present on the initial product. In the products studied, members of the genera Micrococcus and Flavobacterium were prominent in the frozen products.

Studies on the effect of rate of freezing on microorganisms in fruits and vegetables generally showed a higher percentage survival in vegetable products frozen by the contact method at -20°F. and stored at -20°F. as compared with products frozen by a static method and stored at either -20°F. or 0°F. With fruits, neither the temperature nor the rate of freezing had any important influence on the survival of microorganisms.

A study of pure cultures of five bacteria and one yeast substantiated earlier findings of Haines that microbial cells show no apparent change in appearance due to freezing. Further studies showed that the metabolic processes of cells which survived freezing were not affected since the rate of growth

and the ability to ferment a sugar broth in a given time were similar with both "fresh" and frozen cells.

The effect of freezing aqueous suspensions of 80 pure cultures of psychrophilic, mesophilic and thermophilic bacteria indicated that the latter group of bacteria were more susceptible to freezing with the psychrophiles and mesophiles showing greater resistance, both in the initial freezing process and throughout the period of freezing storage. Considerable fluctuations in counts in individual cultures were noted from one period of storage to another and these have been attributed to uneven distribution of the cells of organisms in the suspensions. These fluctuations were more prominent with some species of bacteria than with others.

Considerable differences in the rate of survival were also noted with different species of bacteria and, with one organism studied, within the species. Bacteria belonging to the genera Serratia, Sarcina, Micrococcus and Flavobacterium were more resistant to freezing than any of the other types studied.

Freezing seven pure cultures of bacteria in suspensions of water, 3 per cent salt, a vegetable

extract, and concentrations of sucrose varying from 5 to 40 per cent showed that the vegetable extract and 40 per cent sucrose generally provided the greatest protection to the organisms against freezing, while water, 3 per cent salt and 5 per cent sucrose showed the least protection.

Spores and vegetative forms of members of the genus Bacillus, subjected to freezing in aqueous suspensions, indicated that the spore stage of organisms is more resitant to freezing than the vegetative stage. Thermophilic spores showed less resistance to freezing than mesophilic spores.

Bacteria frozen at 0°F. in aqueous suspensions, in both the logarithmic phase of growth and in older cultures, showed that, although members of the genus Achromobacter were less resistant to freezing in the logarithmic phase, the over-all picture suggested that there was no important difference in the rate of survival between cultures in the logarithmic phase of growth and in older cultures.

Cultures of both bacteria and moulds frozen continuously and intermittently in aqueous suspensions at -20°F., 0°F., 15°F. and 32°F. showed that intermittent freezing was much more destructive to both bacteria and moulds. Moulds were generally more

resistant to freezing than bacteria both in the continuously and intermittently frozen suspensions. Temperature did not appear to be an important factor although there was a slight indication that -20°F. was less destructive to microorganisms in the continuously frozen series. However, with intermittent freezing temperature was definitely unimportant.

In a study in which pure cultures of chromogenic bacteria were inoculated into fresh and blanched peas it was found that with those organisms not inhibited by a natural antibiotic which was presnt, the rate of growth was greater in the blanched product, due possibly to the release of soluble solids in the blanch process. The growth of two organisms, Sarcina lutea and Staphylococcus sp., was inhibited by the antibiotic.

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