Bull. Fish. Res. Stn., Ceylon. Vol. 16, No. 2, pp. 11–18, December, 1963

Survival of Staphylococci on Frozen Fish

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ABSTRACT

USING *Staphylococcus aureus* as the test culture it has been shown that cell injury occurs in two phases during freezing and storage at temperatures below freezing. Certain constituents of fish muscle appear to protect the cells during both phases of injury. The survival of bacteria on fish muscle is not influenced by the rate at which the fish muscle was frozen prior to inoculation.

There was no significant difference between growth of bacteria on quick frozen and slow frozen fish muscle after thawing. However there appeared to be a slight tendency for the lag phase of growth to be extended on thawed quick frozen fish muscle when compared with thawed slow frozen muscle.

Introduction

Much interest has been shown in recent years in the bacteriology of frozen fish particularly with reference to the growth and survival on them of food poisoning bacteria. However few such studies have been made of their incidence and survival on frozen fish. Attention to the importance of such studies was recently focussed by Raj and Liston (1961 *c*) by their observation that certain components of sea-foods protect some groups of bacteria from the lethal effects of freezing and low temperature storage.

During an investigation of a small but random sample of polythene packed frozen fish marketed by a local fish sales organization, it was possible to observe a significant incidence of coagulase positive staphylococci. This high incidence was no doubt associated with contamination during handling and the high rate of survival of this group on fish during freezing and storage. It was therefore decided to investigate certain aspects of the survival of this group on ' Tuna ' fish muscle during freezing and subsequent handling.

Methods

A coagulase positive *Staphylococcus aureus* isolated from a suppurating wound was used for the experiment. It was grown on Nutrient agar 7.5 per cent. sodium chloride. Inoculation of the fish muscle was made from a broth culture grown in nutrient broth and diluted appropriately with normal saline.

(i) *Freezing experiments*: Cells grown at 37°C. and in their log phase of growth were used. Acetone was cooled down to 30°C. and contained in a thermos flask. The stopper was so made as to hold a series of glass tubes ¼ in. in diameter and 5 ins. in length. The glass tubes were tapered at one end and sealed. Halfway down the tubes were placed pieces of Whatman No. 1 filter paper, ½ in. by 1½ ins. and rolled so as to fit tightly into the internal diameter of the tubes. These tubes were plugged with cotton wool at the open end and sterilized at 15 p.s.i. of steam pressure for 15 minutes. After cooling the filter paper inside the tubes were carefully inoculated with 0.1 ml. of the culture using a micro-pipette. The culture consisted washed cells of *Staph. aureus* (and in one experiment, of *Es. coli* Type I) which was held in the menstruum under investigation. After inoculation two of the tubes were used for control counts. The rest were inserted into the stopper of the thermos flask containing the cooled acetone and kept in a deep freezer. The stopper with the inoculated tubes was plunged into the flask of acetone and tightened. It was then closed with an outer cover containing insulating material. Two tubes were removed each time at various time intervals and allowed to thaw at 37°C. for 20 minutes. Counts were done on serial decimal dilutions of the contents of the

tubes carefully washed down with 10 ml. of Ringers solution which was also used as the diluent. The counts were done using the roll tube technique (Pretorius, 1961) using Nutrient agar with 7.5 per cent sodium chloride and the tubes being incubated at 37°C. for 48 hours.

The freezing menstrua investigated consisted of isotonic salt solution and fish extract which was collected during cyclic freezing and thawing of fish muscle.

(ii) *Storage experiments:* Fish muscle was finely minced and packed tightly on an aluminium tray to a thickness of $\frac{1}{4}$ in. taking as much precaution as possible to prevent undue contamination. The tray with the minced muscle was then frozen in a deep freeze maintained at —25° C. Using a cork-borer, $\frac{3}{4}$ in. diameter, discs of the frozen material were cut out and each disc was placed individually in sealed petri dishes for storage in the deep freezer for a further period of 48 hours. Some of the discs were sampled for the presence of phosphatase positive staphylococci. None were found.

Each disc was then heavily inoculated on one surface with the infective culture using a fine drop pipette delivering a constant volume each time. The inoculated surface was then closed over with an uninoculated disc. Two such pairs of discs were placed in polythene bags containing a marketable piece of Tuna steak and the bag sealed. These packets were then stored at −10° C. for varying lengths of time. On each day of sampling two packets of fish were withdrawn from the freezer chamber and from each the discs were carefully removed and bacterial counts done on them. Oxoid Nutrient agar with 10 per cent. salt was used as the medium.

(iii) *Effect of slow and quick frozen muscle on bacterial growth:* For these experiments slices of fresh bonito muscle were used. These slices were carefully cut taking the usual aseptic precautions and these discs of muscle were packed in polythene bags. Some of them were quick frozen in a blast freezer operating at an average temperature of −50° C. and the rest frozen at −10° C. for two hours. After thawing out for 1 hour at 37° C., they were inoculated as before and bacterial counts done on them.

Results

(i) It was observed that *Staph. aureus* is markedly proctected by fish muscle juice during freezing. The degree of this protection is compared with the resistence to freezing injury of staphylococci and *Es. coli* in Fig. 1 . It was observed that much of the damage to the cells occurs during the first 10 to 20 minutes when the surrounding medium freezes out. This was also confirmed during experiments where cells were frozen at lower temperatures when the time for maximal cell damage was found to be 5 to 10 minutes at —20° C. and 10 to 20 minutes at 10° C.

(ii) The protective effect of fish muscle both quick frozen and slow frozen, was observed in comparison to cells frozen in isotonic salt solution (Fig. 2). There was no significant difference between the slow frozen and the quick frozen muscle in their ability to protect the cells.

(iii) There was no significant difference between slow frozen and quick frozen fish muscle in their ability to favour bacterial multiplication after thawing except for a slight extension of the period of lag before active cell multiplication in the case of quick frozen muscle (Fig 3).

Discussion

There appear to be two phases of cell destruction during freezing and subsequent storage at low temperature. During the first phase there is a sharp decrease in numbers of cells while during the second phase of prolonged storage there occurs a gradual loss of cell viability. Our results have shown that during initial freezing of *Staph. aureus* in isotonic solution at −30° C. the decrease of the population is about 15 per cent. in the first 20 minutes of freezing after which it is about 4 per cent. in 20 days. These figures are no doubt highly variable depending on the conditions of freezing and the size of the initial inoculum: but in general it may be concluded that the initial loss of cell viability during cold injury reaches a maximum during the complete freezing out of the surrounding medium.

This first phase of cell destruction is no doubt associated with the physical damage to the cell wall caused by the crushing and spearing action of ice cystals. This is evident from the results and observations of the early experimenters on the freezing of cells in pure culture (Hess, 1934 ; Luyet & Gehenio, 1940; Wood, 1956; Lovelock, 1957). Recently Asahina (1962) using sea-urchin eggs provided experimental evidence that maximum cell damage occurs at or below the eutectic points of the salt solution in which the eggs were suspended. It was concluded that the formation of the ice crystals in the external environment damaged the cell wall. In addition it is possible that as the medium freezes and ice separates out as a pure substance, the cells and the solutes in the medium become gradually concentrated in the spaces among ice crystals. The concentration of the environment surrounding the cell can thus become potentially harmful, becoming most evident during slow freezing.

It is also well known that Gram positive cocci which possess a cell wall are more resistent to the hazards of freezing than Gram negative bacteria (Jones & Lochhead, 1939). This is also evident from the work of Raj and Liston (1961 a; b; c), who found that *S. typhimurium* and *Es. coli* more sensitive to freezing than *Staph. aureus* and *S. pyogenes*. Indeed *S. bovis* and *Cl. perfringens* were found to be even more resistent. The relative high incidence of staphylococci we had noticed during our survey was no doubt associated with this resistence to the harmful effects of cold.

But unfortunately much less is known about the mechanism of the protection conferred on these cells by the medium in which they are frozen. The degree of this protection has been shown to depend on a number of factors such as the nature of the substrate, freezing temperature, storage temperature and their duration (Keith, 1913; Haines, 1937; Weiser & Osterud, 1945). Increased survival rates of bacteria on frozen food has also been reported on by a number of workers (Gunderson & Rose, 1948; Proctor & Phillips, 1948; Squires & Hartsell, 1955; Larkin *et al*, 1955; Woodburn & Strong, 1960; Kereluk *et al*, 1961; Raj & Liston, 1961 a; b; c; White & White, 1962). Liston and Raj (1961 c) has provided further evidence that sea-foods protected microorganisms, including *Staph. aureus* from the lethal effects of cold injury.

But much of this work has failed to distinguish between the two phases of cell injury by cold. For instance the so called protective effect reported by Raj and Liston was evident during prolonged storage at low temperature; it is difficult to assess how much of this protection was provided by fish muscle during the first few critical minutes of freezing. The work now reported by us seems to indicate that even during this phase a significant degree of protection is provided to the bacterial cells by the water soluble fraction in fish muscle.

The conditions of freezing during the present series of experiments differed from those of previous workers. The use of the water soluble fraction of fish muscle impregnated on cellulose fibre, nevertheless, eliminates to some extent the errors such as those due to absorption effects that could occur during the freezing of a solution containing variable amounts of particulate matter. Another source of likely error is the effect of adhesion of bacterial clumps to protein fibres during serial decimal dilution thus causing a reduced number of colonies to develop from aliquots of such solutions during bacterial counts. The presence of a large but a constant amount of cellulose fibre in both the control and the test solution tends to equalise this error for purposes of comparative study. Even the amount of denatured protein fibres in the test solution will be smothered by the excessive numbers of cellulose fibres. The protective effect of fish muscle juice evident even under such conditions makes us believe that this effect is associated with phenomena occuring at the cell surface, related to the biochemical structure of that surface. Work now in progress on the protection of cells from freezing injury by certain chemical agents appears to confirm such a view and will be reported on later.

On the contrary Raj and Liston (1961 c) conjectured that the protection from cold injury was associated with the nature of the fish protein molecule, its colloidal properties interfering with crystallization of water in the food-stuff. This was thought to minimise the loss of water from the bacterial cell. But no evidence is available that such a loss does occur during freezing. It might be presumed that during freezing, the concentration of the salts in solution subjects the bacterial cells suspended in it to osmotic tension. But *Staph. aureus* is able to withstand high osmotic pressure since its own internal osmotic pressure has been reported to be between 20 to 25 atmospheres (Mitchell & Moyle, 1956). Their tolerance of salt concentrations upto 10 per cent.

or higher is evidence of their ability to resist osmotic loss of water in this way. In fact Scott (1953) thought that the high salt tolerance was a manifestation of their very low water requirements.

Further as pointed out by Raj and Liston themselves the extent of the protection they envisaged according to their hypothesis will depend on the extent of denaturation of the fish protein: that degree of damage to the protein resulting in a comparable reduction in their water binding capacity. However since these authors have used a homogenate of pre-cooked fish sticks where much of the protein is denatured to begin with, it is difficult to accept their explanation that multiple thawing and freezing exerts its exceptional lethality to bacterial cells by the reduction of the protective effect of undenatured protein in fish muscle during successive cycles leading to a corresponding loss of water binding capacity.

On the other hand the gradual loss of viability during prolonged storage at temperatures below freezing is probably associated more directly with the rate of dessiccation of the cells and the protective effect of the fish muscle during this phase probably takes place through a mechanism similar to the one postulated by Raj and Liston. Effect of fish muscle on cell viability reported by these workers was also confirmed during our investigations. In addition our results indicate that there was no significant effect of the rate of freeing of the fish muscle on its protective effects on bacteria.

Evidence regarding the growth of bacteria on thawed tissues is somewhat contradictory. Tissues which have been frozen and thawed are commonly thought to favour bacterial growth owing to the breakdown of cell structure during freezing and the consequent release of free fluids and nutrients. Evidence to this effect was presented by Gressel and Graie (1929) in the case of minced meat. Hartsell (1951) found that the multiplication of certain bacterial species on whole eggs was enhanced after the eggs had been frozen and thawed. It was however thought to be due to the physiological stimulation of the bacterial cells during freezing. When *Es. coli* was frozen in various menstrua, it has been found that the generation time during subsequent growth varied according to the medium (Squires and Hartsell, 1955). On the other hand Kallert (1928) found no bacteriological evidence that frozen meat was more perishable than fresh meat. In addition recent evidence has indicated that there was no reason to believe that liberated free fluids of thawed tissue favour bacterial multiplication. Sulzbacher (1952) working with frozen meat inoculated with a psychrophilic pseudomonad showed that the lag phase on frozen meat after thawing was longer than on fresh meat. Kitchell and Ingram (1956) found no change in the generation time of a pseudomonad inoculated into a nutrient medium and on to frozen and thawed meat, but they observed an increase in length of lag after freezing. White and White (1962) also found no change in the growth of *Strep. lactis* and *Leuc. mesenteroides* after freezing and thawing either in broth or on peas. But they found that the lag phase of the thawed frozen culture was prolonged in the case of broth.

Since the cell damage during slow freezing is greater than during quick freezing, we investigated the effects of these two processes in relation to subsequent bacterial multiplication on fish muscle so treated. Our results do not indicate a significant difference between them, except for a slight tendency for the lag phase to be prolonged in the case of bacteria growing on quick frozen muscle.

On the basis of available data it appears to be difficult to postulate with any confidence any single hypothesis to account for cell injury during freezing and the protective effect of tissues against it. More basic knowledge regarding the physical and chemical processes involved during freezing of cells and tissues is required before it will become possible to do so. Owing to the increasing importance of frozen fish in food distribution to consumers, the importance of such studies need hardly be stressed.

Conclusions

Cell damage during freezing appeared to proceed in two phases—first phase was marked by rapid destruction of the cells probably associated with the freezing out of the surrounding medium and the second phase characterised by the gradual loss of cell viability and probably associated with cell dessication. The protective effect of fish muscle was evident during both phases.

There was no significant difference between the rates of cell multiplication on quick frozen fish muscle compared to slow frozen muscle except for a slight lag at the initiation of cell multiplication on quick frozen muscle.

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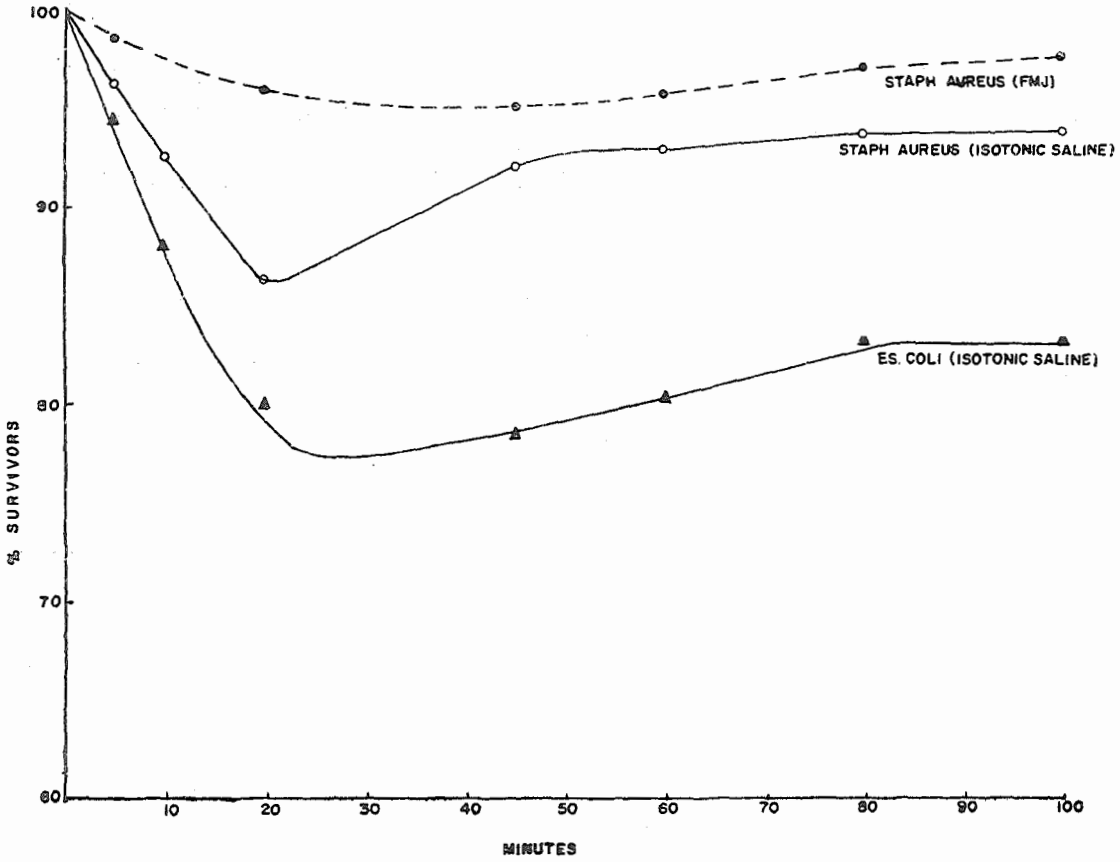

Fig. 1. Survival of bacteria during freezing at -30°C.

Note : FMJ = Fish muscle juice.

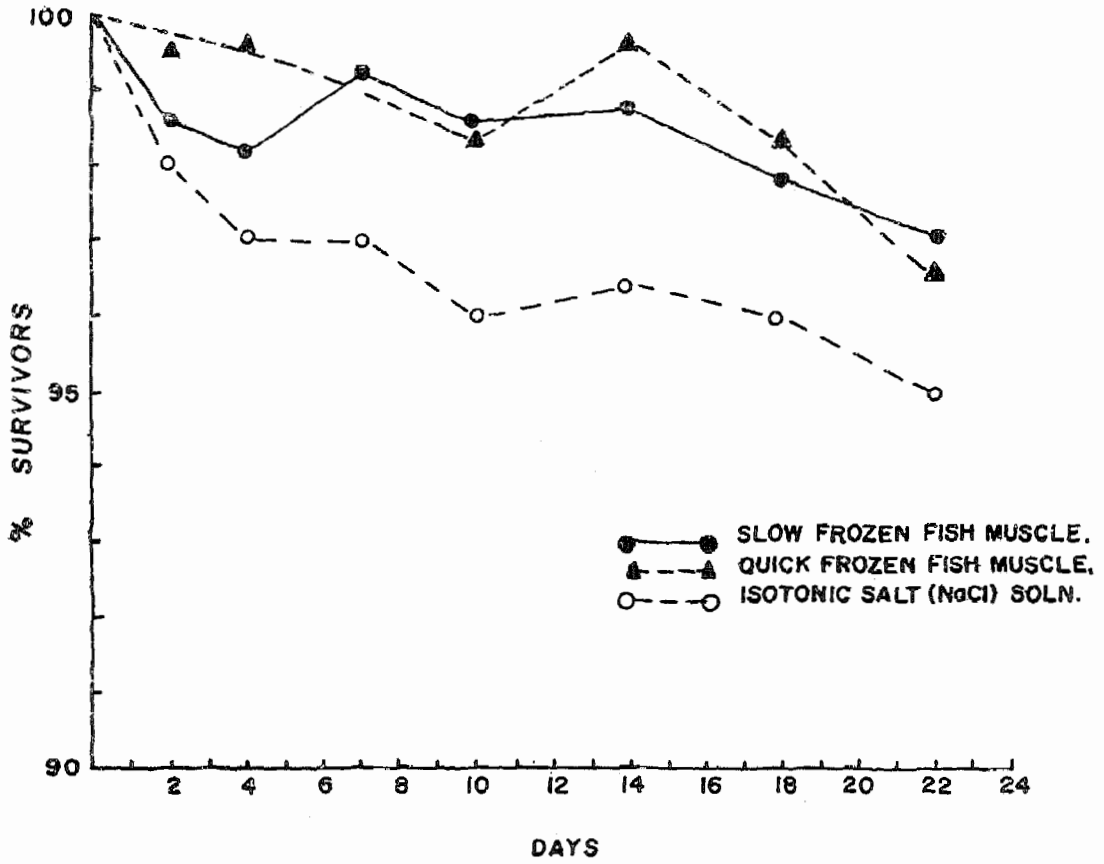

Fig. 2. Survival of *Staph. aureus* on slow frozen and quick frozen fish muscle and in isotonic solution during storage at –10°C.

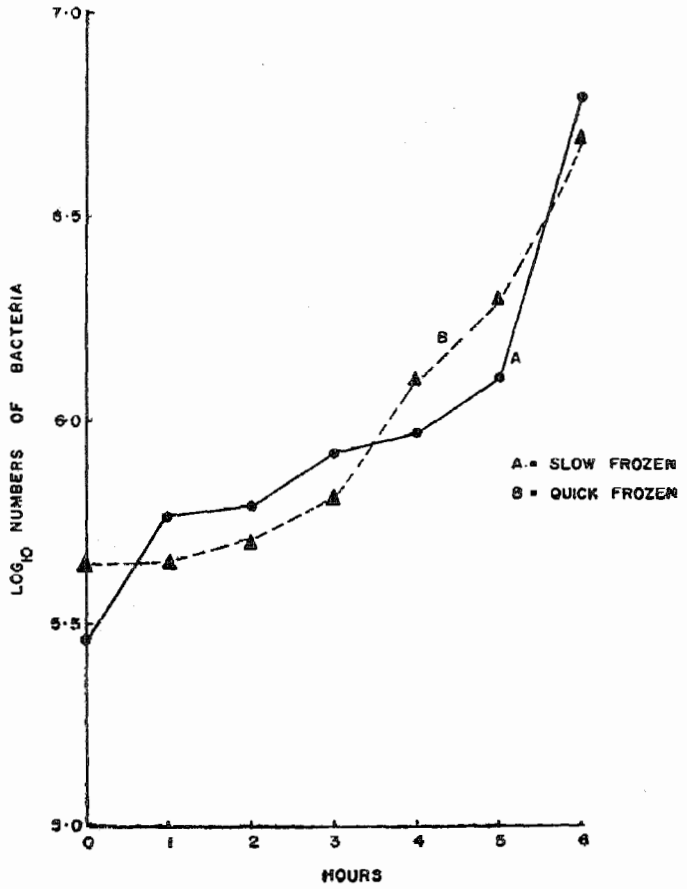

Fig. 3. Growth of *Staph. aureus* on fish muscle, slow frozen and quick frozen, and thawed out at 37°C.