A STUDY OF CERTAIN FACTORS AFFECTING SURVIVAL OF VEGETATIVE CELLS OF *BACILLUS POPILLIAE* PRESERVED BY LYOPHILIZATION

by

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INTRODUCTION

Bacillus popilliae Dutky is the causative agent of milky disease in the larval stages of Popillia japonica Newman, the Japanese beetle. The bacterium, usually in the spore state, is ingested by the larvae and causes death in 2 to 3 weeks. During this period the number of spores in the haemolymph of the larvae increases to about 20 billion per ml. As the dead larvae decay the spores are released into the soil, ready to infect more larvae.

B. popilliae could be effectively used as a bacterial insecticide if spores could be produced efficiently in large numbers. This, however, is not the case. The organism, being fastidious in nature grows only moderately on artificial media and spore production on such media is nonexistant. Efforts to cause the organism to produce spores on an artificial medium have met with failure or only very limited success. The only commercial method of spore production to date is by larvae injection.

Although the usual method of larvae infection is injection or ingestion of spores, it is known that vegetative cells, being as virulent or more so than spores, also cause the disease. Therefore if large quantities of the vegetative cells can be grown efficiently and preserved for use at a later date, by a process such as lyophilization, vegetative cells could be used for the control of the Japanese beetle.

The purpose of this study is to determine the feasibility of lyophilizing vegetative cells of B. popilliae and of maintaining a high survival rate in the lyophilized product.

REVIEW OF LITERATURE

BACILLUS POPILLIAE

Bacillus popilliae, one of the organisms causing milky disease in larvae of the Japanese beetle, Popillia japonica Newman, was first described by Dutky (1940). Since that time the majority of work with B. popilliae has been directly of indirectly oriented towards use of this organism as a biological insecticide.

Dutky described the organism as a slender, nonmotile, spore forming, gram positive rod which preferred anaerobic conditions. The original isolation was on blood agar and unheated egg yolk media, however the characteristic spores were not observed in vitro. A method of grub inoculation was devised by Dutky in 1942 to provide sufficient numbers of spores for large scale dusting of Japanese beetle infested areas.

Both the vegetative cells and the spores of B. popilliae have been described as pathogenic for the Japanese beetle larvae (Dutky 1940, Dutky 1947, Haynes et al. 1961, St. Julian et al. 1963, Pridham et al. 1964). Dutky (1947) maintained B. popilliae on artificial media for over a year with consistent high yields of vegetative cells which produced milky disease and abundant spores when injected into Japanese beetle larvae. He also outlined some of the growth requirements of B. popilliae, indicating thiamine as being essential for growth. Other factors affecting the culture yields were carbohydrate content, pH, buffer capacity and reducing capacity of the medium.

Steinkraus (1957) succeeded in obtaining relatively good growth in an artificial medium comprised of tryptone, yeast extract and glucose, or other simple carbohydrates, although the number of cells per ml did not approach the number of cells per ml produced in the insect larvae. Cells grown on artificial media exhibited considerable variation in morphology and in reaction to changes in environment while cells grown parasitically were more stable and uniform in both respects.

Studying nutritional requirements, Sylvester and Costilow (1964), concluded that in synthetic media B. popilliae required biotin, eleven amino acids, thiamine (as did Dutky 1947), barbituric acid, glucose and K_2HPO_4. It was also established (Pepper and Costilow 1964) that glucose or similar carbohydrates were not catabolized when molecular oxygen was absent. This is in contrast to the conclusions of Dutky (1940) who stated that B. popilliae was preferentially anaerobic. Therefore B. popilliae was found to be strictly aerobic even though it is different from other aerobic microorganisms in that it does not produce catalase (Steinkraus 1957). This is thought to be a possible reason for the loss of cell viability soon after the organisms reach the stationary phase of growth on artificial media, since catalase degrades hydrogen peroxide. Some merit can be attributed to this theory since cells in the stationary phase retain viability longer when oxygen availability is reduced, and since a system for production of H_2O_2 was consistently found in older cells (Pepper and Costilow 1965).

LYOPHILIZATION

Methods

Shackell (1909) drew attention to the importance of freezing and drying from the frozen state as a means of preventing damage from high concentrations of dissolved solids during the drying of liver glycogen. Drying in vacuo since that time has expanded in many directions both in methodology and in usage.

Generally the methods used are divided into two general categories; freezing by evaporation (Campbell-Renton 1942, Rayner 1943, Greaves 1944) and prefreezing (Hammer 1911, Swift 1921, Elser et al. 1935, Roe 1936, Stillman 1941). In the former, small amounts (0.1-.02 ml) of the suspension are subjected to a relatively high vacuum. The suspension is frozen due to the loss of heat caused by evaporation of part of the water in the suspension. In the latter, the suspensions were first frozen and then subjected to a vacuum.

Flosdorf and Mudd (1935) were the first to give a detailed description of the method known today as the "lyophile" process. They prefroze cultures in a Methyl Cellosolve - Dry Ice bath and attached the tubes to a manifold which contained cold condensers for the removal of the water vapor. A variation of this method was later described (Flosdorf and Mudd 1938) in which the cold condensers were replaced by Drierite. Both methods were successful for drying bacteria although no quantitative results were given. Many other workers have reported results using both the "lyophile" process and the variation of Flosdorf and Mudd

(1938) which is called the "Cryochem process" (Naylor and Smith 1946, Glover 1946, Stamp 1947, Hornibrook 1949).

Since seldom did any two workers use the exact same techniques there are many modifications using both the evaporation freeze and the prefreeze methods. Presently the prefreeze method is generally considered more acceptable since freezing by evaporation necessitates the use of much smaller amounts of material and also because of the increased concentration of salts due to the loss of water which occurs in the evaporation process. In most cases, the method of desiccation varies either as to the chemical used or to the type and temperature of condenser used to trap the water vapor (Greaves 1954). The "lyophile" procedure is the most widely used and in the words of Greaves (1954); "apparatus of this nature will be found in many laboratories in all parts of the world".

Stabilizing Medium

Prior to 1940 the choice of suspending medium for lyophilization seemed to have been among saline, water, serum, blood and some type of meat extract. Exceptions to this were skim milk used for drying lactic acid bacteria (Rogers 1914) and a heavy suspension of killed staphylococci (Otten 1930) which greatly improved survival of Haemophilus pertussis, Pasteurella pestis and Neisseria meningitidis.

Otten (1932), using Vibrio cholerae was one of the first workers to compare different suspending media. His work showed an increase in survival of V. cholerae when distilled water was

used instead of saline and even higher survival when meat extract was used. Elser et al. (1935) found serum better than either saline or broth when drying N. meningitidis and Neisseria gonorrhoeae. Leifson (1936) dried a variety of organisms in water, 0.3% beef extract, 1.0% peptone, pork infusion, and pork infusion and blood, finding that the organisms normally thought of as more sensitive to drying (V. cholerae and N. gonorrhoeae) died off quickly in all but the pork infusion with blood. The more resistant organism (Salmonella typhi) survived in all five suspending media for at least 64 days.

Heller (1941) used 1% solutions of glucose, sucrose, salicin and tryptophan and solutions of the colloids: starch, gum tragacanth, gastric mucin and peptone also in 1% solution to dry Streptococcus pyogenes and Escherichia coli. With the crystalline compounds, the lower death rates were observed with the compounds which the organisms could dissimilate. With the colloid compounds tried, the death rates decreased as the hydrophilic property of the colloid increased. Highest viability was obtained with a mixture of dissimilable crystalline compound and a hydrophilic colloid.

Stamp (1947) conducted experiments using 10% gelatin and 0.25 to 0.5% ascorbic acid to dry ten different organisms. The more resistant organisms showed good survival rates up to 4 years but the species more sensitive to drying gave less than 0.2% survival after 3 days and none after 2 years.

A suspending medium containing 2.0% dextrin, 0.5% thiourea, 0.5% ammonium chloride was worked out by Naylor and Smith (1946)

with viable counts taken after each constituent was added. They obtained good survival when drying Serratia marcescens which is fairly resistant to drying. No information was given on the effect of their suspending medium on organisms more sensitive to drying.

Fry and Greaves (1951) carried out a series of experiments in which both resistant and sensitive organisms were dried using a variety of substances as suspending media. They found that the addition of 5 to 10% glucose or lactose to other protective suspensions greatly increased the survival rate. In one experiment the survival of V. cholerae was 0.6% after 5 years when dried in "Mist desiccans" which is a mixture of three parts serum and one part broth containing 7.5% glucose.

Over 60 different compounds were used by Miller and Goodner (1953) to dry BCG. They found that the best survival (10% for 10 months) was obtained by using 0.1% to 0.25% sodium glutamate or aspartate. Haynes et al. (1961) lyophilized 12 strains of B. popilliae using sterile bovine serum as a stabilizing medium and found viable cells after 5½ to 20 months.

Several theories of the protective action of the suspending medium have been set forth. Among these is that of Heller (1941) who suggested that the protective effect is concerned with metabolic processes of the cell. He based this theory on the fact that in his experiments E. coli was protected by xylose and tryptophan which it dissimilates and not by salicin which it does not use, whereas S. pyogenes was protected by salicin which it dissimilates, but not by xylose and tryptophan which it does not

dissimilate.

Miller and Goodner (1953) proposed a theory that a substance could protect a cell by rendering the cell wall impermeable. However results obtained by Benedict et al. (1961) indicated that this was not the case since efforts to dry S. marcescens using gastric mucin and dextran gave lower survival either alone or when incorporated in their control which was 75% cell supernatant plus 25% water, than did their control alone.

Scott (1958) postulated that a fundamental cause of death in dried organisms is the alteration of cell protein by reactions between carbonyl containing compounds in the cells and the amino groups of cell protein which results in removal of amino side chains. He found that carbonyl containing substances such as glucose and particularly ribose caused loss of viability whereas sucrose did not.

Cho and Obayashi (1956) found that 1% Na glutamate gave a much more stable product when used to dry BCG. Greaves (1960a) found that the optimum concentration of Na glutamate to be between 5 and 10% which lead him to believe that the Na glutamate acts by neutralizing the carbonyl groups and by buffering the final water content at 1%. Muggleton (1960) found that the beneficial effects of Na glutamate were abolished by the carbonyl containing glucose but not by sucrose. Annear (1964) obtained 54 -57% survival of Salmonella ndolo after 24 months by soaking cellulose tufts with a suspension of the organisms in 20% Na glutamate and drying under vacuum with P_2O_5 as a desiccant. No prefreezing was performed and it was thought that evaporative

freezing did not occur. Greaves (1960a) states: "In the present state of our knowledge a drying medium should be compounded as follows: 1) A support material so that a good, solid cake is formed. Use 5% dextran or 5% bovine albumin. 2) A buffer to hold the final water content at 1%. Use 5 to 10% sucrose or 5% Na glutamate. 3) A neutralizer of carbonyl groups. Use 1% Na glutamate if sucrose is omitted. I find this drying medium very satisfactory for all bacteria and viruses."

Greaves, as so many other workers have not done, did not define the meaning of satisfactory in terms of per cent survival.

Method of Freezing
And Other Factors Affecting Survival

The rate of freezing and temperature reached can have a profound effect on survival of dried cells since the cells killed during freezing cannot be successfully lyophilized. Proom and Hemmons (1949), Luyet (1951) and others believe that cells are killed by penetration of the cell wall by ice crystals. Luyet (1960a, b) established that the faster the rate of cooling the smaller the size of ice crystals formed and at a high freezing rate (several hundred degrees per second) there are no crystals formed. Few cells would then be killed by ultrarapid freezing.

Many who have studied freezing rates (Mazur et al. 1957a, b; Meryman 1956; Heckly et al. 1958) have concluded that a high percentage of cells survive when frozen slowly.

Essentially the controversy between rapid and slow freezing

is one between the theory of physical damage and chemical damage as a cause of cell death during freezing. Meryman (1960a) states that much of the confusion concerning freezing rates may be due to the additives since the mechanisms of protection during slow freezing may be quite different from rapid freezing. Therefore a substance affording protection during slow freezing may be quite ineffective during rapid freezing and vice versa. Heckly (1961) states that disagreement among authors as to superiority of rapid freezing over slow freezing is probably a result of the differences in organisms, and/or suspending medium. Heckly (1961) found that varying the method and rate of freezing without additives had no significant effect on survival of either S. marcescens or Klebsiella pneumoniae. Meryman (1960b) states that a freezing rate should be in the order of 1 C per minute on the basis of purely physical evidence. There is a great amount of disagreement on the rate of freezing but almost all workers agree that the temperature should be lowered below the eutectic point of the suspending medium.

Cell Concentration

Otten (1930) dried S. typhi, Shigella shigae, and V. cholerae in concentrated cell suspensions and the same suspensions diluted tenfold. In each case the more concentrated suspension gave higher survival. Stamp (1947) found that a dimunition in cell concentration gave an increase in survival. Fry and Greaves (1951) concluded that there were no differences in survival between three different suspensions of the same organism having

a dilution factor of 10,000 between the highest and lowest. Benedict et al. (1958) working with S. marcescens and Naylor-Smith solution found that with 10% NS solution the survival decreased with a decrease in cell concentration whereas with 100% NS solution the reverse was true.

Nutrition and Age of Cells

Usually the organisms to be lyophilized were grown on the medium that was most convenient. Van Drimmelen (1956) compared survival rate of Brucella abortus grown on agar with B. abortus grown in broth and found no differences either immediately after drying or after 1 month of storage. Muggleton (1960) dried BCG that had been grown on four different media and obtained essentially the same survival rate for all four.

Heckly (1961) states: "There is general agreement that mature cells are the most resistant to lyophilization". However Proom and Hemmons (1949) found that 7 hr cultures of S. dysenteriae are more resistant to drying than either 12 hr or 3 to 6 day cultures. They indicated similar results with E. coli. Lemcke (1959) showed a direct correlation between age of culture and per cent survival. The survival increased from 0.02% for 1.25 hr cultures to 12.9% for 18 hr cultures. Naylor and Smith (1946) found that S. marcescens grown at 30 to 34 C for 18 to 24 hr survived better than cells grown for either a longer or shorter time. Benedict's (1958) experiments established that cells grown under constant aeration at 29 or 30 C survived significantly better than those grown at 21 or 25 C. Fry (1954) states: "It is

wise not to attempt to dry very young cultures".

Haynes, et al. (1961) lyophilized cells of B. popilliae 3 to 4 days of age (the time of maximum growth). No survival percentages were given nor were comparisons made with younger cells.

Nature of the Organism

All workers agree that some organisms are much more resistant to drying than others. On the upper end of the scale of non-spore formers (Fisher 1950, Fry and Greaves 1951, Fry 1954) streptococci, especially S. pyogenes, seem to be the most resistant to drying. Sporeformers survive drying so well that they are seldom used in assays of lyophilization work. Haynes et al. (1961) lyophilized vegetative cells of B. popilliae and found them to be viable after 20 months. An exception is anaerobic sporeformers, which in the work of Haynes et al. (1955) did not survive lyophilization. The majority of genera studied are only moderately resistant to lyophilization. These include Salmonella, Shigella, Brucella, Pasteurella, Mycobacterium, Serratia, Lactobacillus.

The least resistant genera seem to be Vibrio, Neisseria and Leptospira. Stamp (1947) lyophilized a large variety of bacteria and reported unsatisfactory survival for only two, Vibrio and Neisseria. Similarly, Proom and Hemmons (1949) showed that Vibrio and Neisseria were the most sensitive of a large variety of organisms. Proom and Hemmons (1949) stated that all organisms in their collection survived drying to some degree with the exception

of Leptospira.

Degree of Vacuum and Drying Temperature

Heckly (1961) stated that 20 to 100 u Hg is considered to be a good vacuum and quite satisfactory for most lyophilizing. However, if the temperature of the suspension is below -40 C the vapor pressure is approximately 100 u Hg and the vacuum cannot be lower than the vapor pressure. The vacuum necessary depends on the temperature at which the substance to be lyophilized is held, in other words, the vapor pressure of the material (Heckly 1961). Early workers lyophilized their preparations using relatively high pressures, for instance Hammer (1911) evacuated to 18 mm Hg and Swift (1921) used a pressure of 2 to 3 mm Hg, but both workers kept the cultures frozen by external cooling. Stamp (1947) dried at pressures of 100 to 300 mm Hg. Most other workers have used the best vacuum system available. Reasons for this are based on principles and theories as outlined by Meryman (1960a, b), Rowe (1960), and Greaves (1960b).

Storage Atmosphere and Temperature

Rogers (1914) stored dried cultures of bacteria in vacuum, air, oxygen, nitrogen, hydrogen, and carbon dioxide. He found the highest survival with vacuum storage and the lowest with storage in air and oxygen. All the gases used with the exception of air were carefully dried. Stark and Harrington (1931) state: "Tests showed that exposure of extremely dry bacteria to free oxygen gas causes a pronounced killing of the bacteria."

Naylor and Smith (1946) working with S. marcescens found that survival in air after 49 days was only 9% compared to 99% survival in vacuum. With nitrogen treated to remove residual oxygen, carbon dioxide and moisture or untreated nitrogen, survival was only 26 to 28%. Benedict et al. (1961) subjected lyophilized S. marcescens to wet and dry oxygen and nitrogen and concluded that oxygen, not moisture, was the cause of death. They found loss of viability to be directly related to exposure time. The oxygen of air was found to kill 95% of dried S. marcescens in 10 minutes. Christian and Stockton (1956) found that survival of S. marcescens and S. aureus dropped sharply when sealed under a pressure of 100 to 150 u Hg as compared to a pressure of 60 to 70 u Hg. However losses of survival were only slightly greater at 700 to 750 u Hg than those at 100 to 150 u Hg. Heckly et al. (1960) found that B. melitensis survived storage equally as well in nitrogen as under vacuum.

Rogers (1914) reported that lactic acid bacteria dried in milk gave a decreasing survival as the storage temperature was raised. Thirty to 60 day storage at 30 or 37 C gave very little survival. Weiser and Hennum (1947) found that lyophilized cultures of E. coli gave a higher survival rate when stored in a refrigerator than at room temperature. Proom and Hemmons (1947) obtained similar results with E. coli and N. meningitidis. Heckly et al. (1960) found that the number of B. melitensis cells surviving when stored at 0 C was 1000 times greater than those stored at 20 C after 6 months.

Residual Moisture

Fry and Greaves (1951) put forth the hypothesis that glucose is protective because it acts to regulate the final moisture content and prevents the suspension from becoming too dry. Workers before that time with the exception of Fisher (1950) strived to obtain maximum dryness. Fry (1954) states that the literature of bacterial drying abounds with phrases such as "the cultures must be as dry as possible". Scott (1960) reported that at temperatures of 0 to 30 C cultures of Salmonella newport survived better at 0.00a_w (a_w = thermodynamic activity of water in a solution which is in equilibrium with the dried culture). Muggleton (1960) referring to dextran states: "Dextran is singularly devoid of any water-retaining properties in itself, and suspensions of BCG and other bacteria (e.g. S. aureus, E. coli, H. pertussis) are readily 'over dried' with a simple freeze-drying apparatus and these show complete loss of viability. Such freeze-dried products have less than 0.1% water, measured by vapor pressure methods (Edward's apparatus), and demonstrate that complete removal of water is incompatible with life." He also stated that when drying BCG, residual moisture of 0.8 to 1.2% is optimum and removal of more water than this causes excessive loss of viability during freeze-drying whereas more residual moisture (e.g. more than about 1.8%) causes excessive loss of viability during storage.

MATERIALS AND METHODS

Growth of B. popilliae

B. popilliae NRRL B-2309, obtained from the Northern Utilization Research and Development Division, Agricultural Research Service, U.S. Department of Agriculture, Peoria, Ill., was used in all studies.

Cells were grown in 300 ml Erlenmeyer flasks containing 100 ml of broth medium. The medium (Rhodes et al. 1966) consisted of 1.5% yeast extract (Difco), 0.5% tryptone (Difco), 0.3% K_2HPO_4, and 0.2% glucose. A 20% solution of glucose was sterilized separately and 1.0 ml of this solution was added to 99 ml of the broth medium giving a final concentration of 0.2%. Constituents of the medium were autoclaved at 121 C for 15 minutes. The pH was checked by potentiometer and adjusted to 7.0-7.2. Inoculated cultures were shaken on a New Brunswick rotary shaker at 200 rev./min. and incubated at 25 C. Actively growing cultures were maintained by 24 hr transfer.

Preparation of Cells for Lyophilization

Five ml of a 24 hr culture were used to inoculate each flask of growth medium. After 9-12 hr or 24 hr the cells were harvested by centrifugation at 1800 rpm for 15 minutes in an International swing head centrifuge. Supernatant was poured off and cells were resuspended in the stabilizing medium to a final concentration of approximately 1.0×10^9 to 2.0×10^{10}. One-half ml of this suspension was then placed in 10 ml glass

ampoules (American Instrument Company, Inc.) or in 3 ml. glass ampoules (made in the lab.).

Prefreezing and Lyophilization

Cells, as prepared above, were prefrozen in one of two ways. Cells were either shell frozen in an ethylene glycol monomethyl ether-Dry Ice bath at -65 C for 2 minutes or they were plug frozin in liquid nitrogen vapor. Cells were frozen in the latter method by placing ampoules in a tube leading to an otherwise enclosed reservoir of liquid nitrogen. A wire holder located approximately 2 feet above the surface of the liquid nitrogen held the ampoules in place. Liquid nitrogen entering the reservoir from a pressurized tank forced the vapor up through the tube giving a temperature drop from ambient to approximately -165 C in 15 minutes. Rate of temperature drop was regulated by increasing or decreasing the amount of liquid nitrogen entering the reservoir.

The temperature was determined by using an iron-constantan thermocouple. Voltages were determined by using a Leeds and Northrup potentiometer.

After prefreezing, vials were immediately placed on an American Instrument Company Lab Model Freeze Dry Apparatus, unless otherwise noted. No vials were placed on the lyophilizer until a pressure of less than 150 u Hg was reached. Vials were lyophilized at ambient temperature, unless otherwise noted, for 4 to 5 hr at approximately 10 u Hg. A jeweler's torch was used to seal the ampoules so that vacuum was maintained.

Plate Counts

Plate counts were made on growth medium solidified with 2.0% agar. Control counts were established by making triplicate serial dilutions of 1.0 ml of the unfrozen suspension in 0.1% tryptone water. Five replications of each appropriate dilution were plated. One tenth ml was spread uniformly over the surface of the medium with a glass spreader.

Experimental counts were made in the same manner after the dried cells had been reconstituted with 2.0 ml of growth medium. This gave an initial dilution of 1:4 which was corrected for when making the plate counts. After 5 to 7 days' incubation at 25 C, colonies were counted using a Quebec colony counter. All counts reported are log averages of the triplicate dilutions.

Stabilizing Media

Sixty five stabilizers including proteins, polysaccharides, colloids, reducing agents and a variety of other miscellaneous materials such as surface active agents and dimethyl sulfoxide or combinations of these were screened. Four of these stabilizers were selected for further work because of their protective action based on viability of cells immediately after lyophilization. These stabilizers were 0.5% amylomaize high amylose plus 5.0% L monosodium glutamate, 0.5% gum tragacanth plus 5.0% L monosodium glutamate, 1.0% gelatin, and horse serum plus 5.0% L monosodium glutamate.

Yeast Extract Study

Certain lots of yeast extract failed to support growth of B. popilliae. A total of 20 different lots of yeast extract from five different manufacturers was screened for amount of growth produced in growth medium. Growth medium was made, inoculated and incubated as described earlier. Optical density readings were taken at 525 mu at 2 hour intervals for 24 hours.

Study on Survival of Cells Held at Different Ampoule Temperatures during Lyophilization

Twenty-four hr cells were prepared and prefrozen in an ethylene glycol monomethyl ether -Dry Ice bath at -65 C and lyophilized as described earlier. Ampoules were held at one of three temperatures: -17 C in an ice-salt bath; at 0 C in ice alone or at ambient temperature. The stabilizing medium was 0.5% gum tragacanth plus 5.0% L monosodium glutamate.

Tempering Studies

Twenty-four hr cells suspended in 0.5% gum tragacanth and 5.0% L monosodium glutamate were prefrozen at -65 C and then stored at -17 C and -65 C for 1 hr prior to being placed on the lyophilizer. Ampoules were held at -17 C during lyophilization.

Young Cell Studies

Nine to 12 hr cells (log phase) were prepared, prefrozen at -65 C, and lyophilized as described earlier. Ampoules

were held at -17 C during lyophilization. Stabilizing media used were 0.5% gum tragacanth plus 5.0% L monosodium glutamate and horse serum plus 5.0% L monosodium glutamate.

Study on Effect of Concentration of L Monosodium Glutamate

Nine to 12 hr cells, suspended in different concentrations of L monosodium glutamate were prefrozen in liquid nitrogen vapor and lyophilized with ampoules held at ambient temperature. Concentrations of L monosodium glutamate used were 0.25%, 0.5%, 1.0%, 3.0%, 5.0%, 7.0% and 10.0%.

Survival Study - Method I

Twenty-four hr cells were prefrozen at -65 C and lyophilized as described earlier. Ampoules were held at -17 C during lyophilization and were stored at 7 C in a refrigerator. Viability was checked weekly up to 1 month. Stabilizing media used were 0.5% amylomaize high amylose plus 5.0% L monosodium glutamate, 0.5% gum tragacanth plus 5.0% L monosodium glutamate and 1.0% gelatin.

Survival Study - Method II

Nine to 12 hr cells, prepared as described earlier, were prefrozen in liquid nitrogen vapor. Ampoules were held at ambient temperature during lyophilization. Storage was at room temperature in the dark. Vials were opened immediately after

drying and at six months. The stabilizing medium was 0.5% gum tragacanth plus 5.0% L monosodium glutamate.

RESULTS AND DISCUSSION

Yeast Extract Study

Early in the work, great variation was found in the amount of growth produced in the media. Day to day transfers of the culture would suddenly stop producing optimum growth and colonies on solidified medium were nonexistant or almost microscopic after the regular 5 to 7 day incubation. The colonies failed to appear or increase in size, if present, upon further incubation.

Suspicions as to the cause of this problem were focused on the yeast extract used in the growth medium when it was noted that certain lots of yeast extract required a greater amount of NaOH to achieve the required pH of 7.2.

Twenty lots of yeast extract from five different companies were screened for their effect on growth of B. popilliae. All growth media were made in exactly the same way. Optical density readings ranged from 0.00 to 0.74 after 24 hr as shown in Table 1. Of the 20 lots tested only three gave growth considered to be maximum (Company A-5, 9, 12). Two others (Company A-6, Company E-1) gave growth which was considered to be good but less than maximum. The remaining 15 lots gave growth which was considered to be unsatisfactory.

Growth curves are shown in Plate I for a typical "good"

TABLE 1. Effect of different lots of yeast extract on growth of *Bacillus popilliae*

Brand of yeast extract	Lot number	O.D. after 24 hr
A	1	.41
A	2	.25
A	3	.17
A	4	.14
A	5	.72
A	6	.66
A	7	.28
A	8	.28
A	9	.74
A	10	.25
A	11	.29
A	12	.74
A	13	.10
B	1	.00
C	1	.40
C	2	.00
C	3	.00
D	1	.00
E	1	.59
E	2	.45

and "poor" yeast extract.

Complete amino acid (except L glutamine) and vitamin arrays were added to samples of the "poor" yeast extract. No increase in growth was noted. Growth in medium made with "good" yeast extract could be inhibited by adding as little as 0.5% "poor" yeast. Therefore, it is thought that the lack of growth was caused by an inhibitory substance in the yeast extract rather than a lack of nutritional requirements. Irie et al (1962) found that different lots of yeast extract gave great variation in growth of Lactobacillus bulgaricus. He considered the cause to be lack of nutritional requirements since addition of pantethine and "Tween 80" improved growth.

Effect on Survival of Cells Held at Different Ampoule Temperatures During Lyophilization

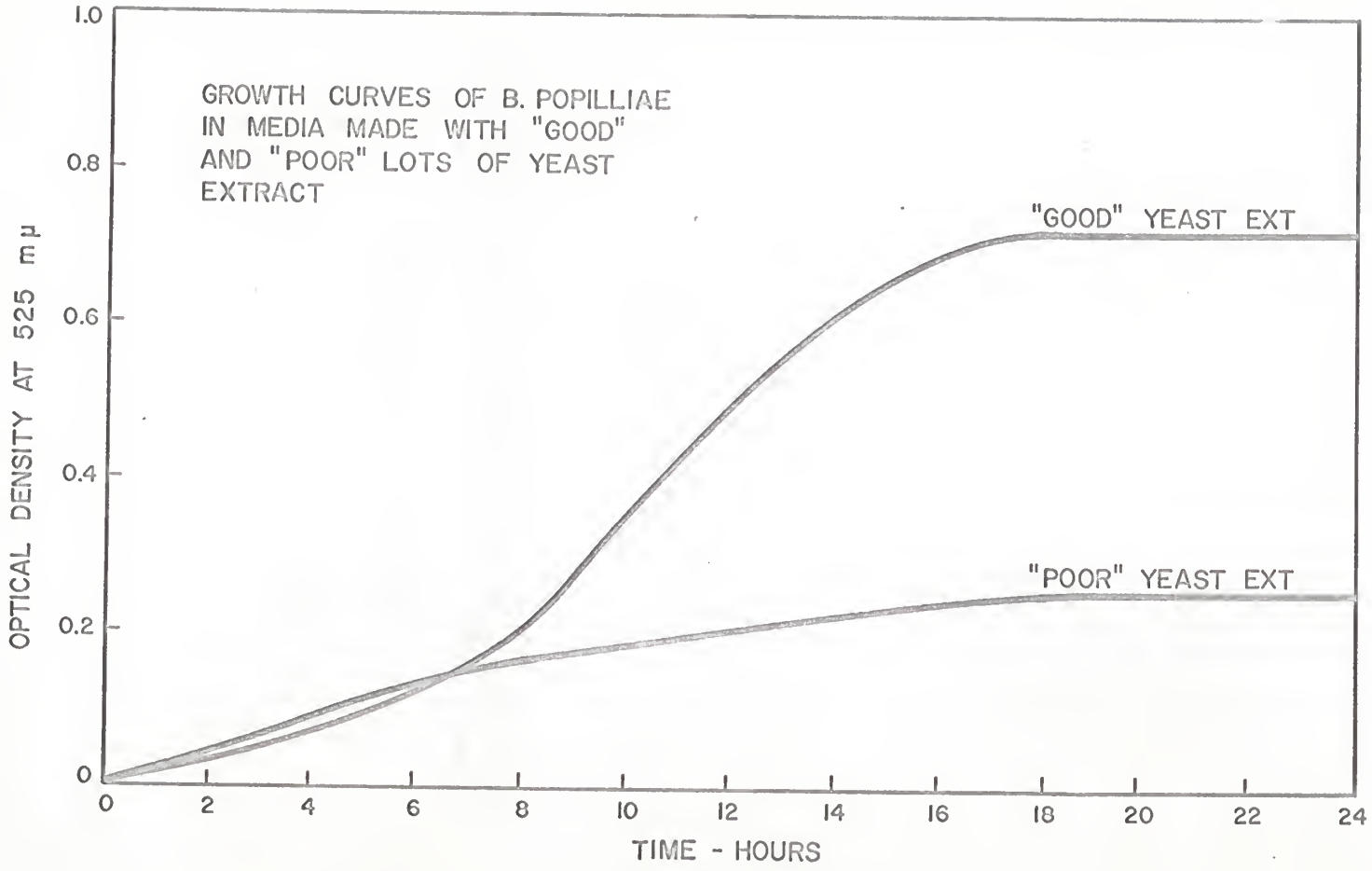
Ampoules held at ambient temperature during lyophilization exhibited considerably higher viability immediately after lyophilization than did those held at -17 C or 0 C. This higher viability rate (22.2% for vials held at ambient temperature compared to approximately 3% for those held at -17 C and 0 C) may have been due to the more efficient removal of the water.

Since the rate of water removal depends on heat added to the system and on the straightness of the path which the water vapor must travel, it is possible that efficiency of water removal could affect survival of the cells. The drying process starts on the surface facing the path of exit of the water vapor

PLATE I

Growth curves of Bacillus popilliae in media made with "good" and "poor" lots of yeast extract

PLATE I


GROWTH CURVES OF B. POPILLIAE
IN MEDIA MADE WITH "GOOD"
AND "POOR" LOTS OF YEAST
EXTRACT
"GOOD" YEAST EXT
"POOR" YEAST EXT
OPTICAL DENSITY AT 525 mμ
1.0
0.8
0.6
0.4
0.2
0
0
2
4
6
8
10
12
14
16
18
20
22
24
TIME - HOURS

and continues toward the opposite boundary. If evaporation of the water in the center of the specimen is not fast enough, thawing could occur causing death.

Samples lyophilized at ambient temperature were satisfactorily dried on an average of 1 hr faster than those held at -17 C and 0 C.

Effect of Tempering On Survival of B. popilliae

It was thought that more cells might survive lyophilization if cells were given a chance to "adjust" to the initial shock of freezing. However, this was not the case. No differences in survival were noted between cells held for 1 hr at -17 C and cells held at -65 C or between cells frozen similarly and immediately lyophilized.

Effect of Age of Cells on Survival

It was found that young cells of B. popilliae (9-12hr) survived lyophilization better than cells in the stationary phase (24 hr). The average per cent survival of young cells was 12.65 compared to 2.55% for 24 hr cells.

This, with a few exceptions, is contrary to findings of most workers as cited in the review of literature. It is possible that certain species of bacteria could survive lyophilization better at a younger age since waste materials produced by the cells could be concentrated to toxic strength by the freezing process.

In the case of B. popilliae this could be hydrogen peroxide which the organism produces but is unable to degrade (Pepper and Costilow 1965).

Effect of Concentration Of L Monosodium Glutamate on Survival

Since there is controversy concerning the concentration of L monosodium glutamate which gives the best survival (Miller and Goodner 1953, Cho and Obayashi 1956, Greaves 1960a), a study was made to determine the optimum concentration to use for lyophilization of B. popilliae. Results of this study are shown in Table 2.

TABLE 2. Effect of different concentrations of L monosodium glutamate on survival of Bacillus popilliae

Concentration %	Control, cells/ml	Viable immediately after lyophilization - %
0.25	6.23×10^9	2.04
0.5	3.72×10^9	1.71
1.0	5.61×10^9	2.23
3.0	8.47×10^9	5.0
5.0	4.06×10^9	42.80
7.0	--*	--*
10.0	--*	--*

* High concentrations caused bubbling during drying and no cells were recovered in dilutions plated

It can be seen from these data that the best survival was given by a concentration of 5.0% L monosodium glutamate. This is

in agreement with the work done by Greaves (1960a) and contrary to the results of Miller and Goodner (1953) and Cho and Obayashi (1956). It must be remembered that different organisms were used by these workers and variations in results are possible due to the different characteristics of the organisms.

Survival Study - Method I

Highest survival was obtained with 0.5% gum tragacanth plus 5.0% L monosodium glutamate. More than a tenfold increase in survival of cells was noted initially and maintained up to 1 month with this stabilizer. Results of this study are shown in Table 3. The 1.0% gelatin and the 0.5% amylomaize high amylose plus 5.0% L monosodium glutamate were about equal in their protective action, although the viability of cells in 1.0% gelatin seemed to drop over the 1 month period. The number of cells surviving after

TABLE 3. Survival of cells of Bacillus popilliae - Method I

0.5% amylomaize high amylose plus 5.0% Na glutamate as stabilizer

Time	Viable cells	% viable
Control	1.0×10^{10}	--
Immediately after drying	2.91×10^{7}	0.29
1 week	3.10×10^{7}	0.31
2 weeks	4.92×10^{6}	0.04
3 weeks	2.20×10^{7}	0.22
4 weeks	2.77×10^{7}	0.29

TABLE 3. (cont.)

0.5% gum tragacanth plus 5.0% Na glutamate as stabilizer		
Time	Viable cells	% viable
Control	9.60×10^9	--
Immediately after drying	3.55×10^8	3.70
1 week	3.02×10^8	3.14
2 weeks	4.10×10^8	4.28
3 weeks	1.96×10^8	2.08
4 weeks	1.84×10^8	1.91
1.0% gelatin as stabilizer		
Time	Viable Cells	% viable
Control	1.60×10^{10}	--
Immediately after drying	4.62×10^7	0.29
1 week	4.55×10^7	0.28
2 weeks	2.84×10^7	0.17
3 weeks	1.04×10^7	0.06
4 weeks	2.34×10^7	0.15

1 month in the 0.5% gum tragacanth plus 5.0% L monosodium glutamate was lower than immediately after drying. However, it was felt that this drop would be insignificant if a much higher initial survival could be obtained.

Survival Study - Method II

B. popilliae, frozen in liquid nitrogen vapor at -195 C and thawed slowly in ice water retained virtually 100% viability. However cells frozen and thawed in a similar manner at -65 C rarely gave more than 1.0 or 2.0% viability (C.E. Herzmann, personal communication).

Viability of cells prefrozen in liquid nitrogen vapor, based on counts immediately after lyophilization, is shown in Table 4. Survival of cells lyophilized in this manner averaged 59.3% immediately after drying compared to 3.7% survival of cells lyophilized as in Survival Study - Method I.

TABLE 4. Survival of cells of Bacillus popilliae - Method II

Exp.	Control cells/ml	Cells/ml viable immediately after drying	Viable immediately after drying - %
1	2.67×10^9	1.29×10^9	47.3
2	4.06×10^9	1.74×10^9	42.8
3	1.83×10^9	1.07×10^9	58.4
4	7.75×10^9	4.42×10^9	57.0
5	1.91×10^{10}	9.13×10^9	47.8
6	1.43×10^{10}	6.60×10^9	46.0
7	4.63×10^9	3.23×10^9	69.9
8	6.76×10^9	5.64×10^8	83.3
9	1.20×10^9	9.46×10^8	78.7
10	3.14×10^9	1.95×10^9	62.1

From these data it is concluded that lyophilization of B. popilliae using Method II is greatly superior to Method I, at least on the basis of survival immediately after drying.

Table 5 shows the percentage of cells lyophilized by Method II surviving after 6 months' storage. The average viability after this period was 10.5% for the six experiments.

TABLE 5. Survival of cells of Bacillus popilliae (Method II) after six months' storage

Exp.	Control cells/ml	Viable immediately after drying - %	Viable after 6 months' storage - %
1	1.91×10^{10}	47.8	2.8*
2	1.43×10^{10}	46.0	1.6*
3	4.63×10^{9}	69.9	25.8
4	6.76×10^{9}	83.3	15.6*
5	1.20×10^{9}	78.7	14.5
6	3.14×10^{9}	62.1	2.4

* only one sample

It must be remembered that these cells were stored at room temperature which fluctuated between 25 and 30 C. It has been reported that survival of cells of B. melitensis stored at 0 C was 1000 times greater than those stored at 37 C (Heckly et al. 1960) Other workers (Rogers 1914, Weiser and Hennum 1947, Proom and Hemmons 1947) have shown that lyophilized cells stored at temperatures slightly above freezing survived many times better

than those at room temperature or above. If the number of cells surviving in this experiment were increased only six times by reducing the storage temperature, the survival would then be virtually 100% of the cells viable immediately after drying. The fact that cells stored in the refrigerator, in Method I, showed very little decrease in viability after storage for one month also leads to this conclusion.

It has been shown that cells of B. popilliae can be lyophilized and stored, maintaining a relatively high number of viable cells for at least 6 months. It is thought that a combination of factors, including use of young cells, prefreezing in liquid nitrogen vapor and the use of 0.5% gum tragacanth plus 5.0% L monosodium glutamate, and the temperature at which cells are held during lyophilization, rather than any one of these, is responsible for the high survival obtained in Method II. It is felt that this combination plus storage of the dried product at a temperature slightly above freezing provides a means for preserving and maintaining large numbers of viable cells of B. popilliae for use as a bacterial insecticide.

SUMMARY

When growing B. popilliae in preparation for lyophilization it was found that different lots of yeast extract used in the medium greatly affected growth.

B. popilliae was lyophilized to determine the effect of different stabilizing media, tempering of cells, use of young

cells, temperature at which cells are held during lyophilization, and different concentrations of L monosodium glutamate on survival.

Two general methods of lyophilization and maintenance were used to retain viability. In Method I cells were suspended in three different stabilizers, prefrozen in an ethylene glycol monomethyl-Dry Ice bath at -65 C and lyophilized at -17 C. The dried product was stored at 7 C in a refrigerator.

In Method II cells were suspended in 0.5% gum tragacanth plus 5.0% L monosodium glutamate, prefrozen in liquid nitrogen vapor and lyophilized at ambient temperature. The dried product was stored at room temperature.

Of all stabilizers screened, 0.5% gum tragacanth plus 5.0% L monosodium glutamate gave the best survival. Tempering of cells had no effect on survival. Young cells survived better than mature cells. The optimum concentration of L monosodium glutamate was 5.0%. Cells held at ambient temperature survived better than those held at lower temperatures.

Cells lyophilized and maintained by Method II gave a much higher survival than cells lyophilized and maintained by Method I.

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A STUDY OF CERTAIN FACTORS AFFECTING SURVIVAL OF VEGETATIVE CELLS OF *BACILLUS POPILLIAE* PRESERVED BY LYOPHILIZATION

by

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ABSTRACT

Vegetative cells of Bacillus popilliae Dutky were lyophilized to determine the effect of certain factors on the survival of this organism.

Sixty-five materials or combinations of materials were screened for their protective effect during lyophilization. The best protection of B. popilliae was given by 0.5% gum tragacanth plus 5.0% L monosodium glutamate.

It was found that "tempering" of cells after prefreezing had no effect on survival after lyophilization. Also cells lyophilized at ambient temperature survived better than cells lyophilized at lower temperatures.

Cells in the early log phase survived lyophilization better than cells in the stationary phase of growth.

The optimum concentration of L monosodium glutamate in the stabilizer was 5.0%.

Two general methods of lyophilization were used. In Method I, stationary phase cells were prefrozen at -65 C and lyophilized. Cells were held at -17 C during lyophilization and the dried product was stored at 7 C.

In Method II, early log phase cells were prefrozen in liquid nitrogen vapor at -165 C and lyophilized. Cells were held at ambient temperature during lyophilization and the dried product was stored at room temperature. Method II gave a much higher survival both immediately after lyophilization and after storage.