

THE PATHOGENICITY OF BACILLUS POPILLIAE FOR MICE. EFFECTS OF
CERTAIN ANTIMICROBIAL AGENTS ON VIABILITY
OF BACILLUS POPILLIAE.

by 4589

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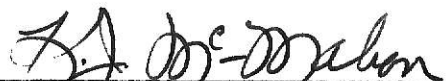
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INTRODUCTION

Recent controversy concerning the safety of chemical insecticides has stimulated interest in the possibility of using pathogenic bacteria for effective control of insect parasites. Biological control of insects is advantageous, because microorganisms employed are usually quite specific for insects and are not known to be injurious to plants, man, or animals. The harmful effects of many chemical insecticides to the animate population have reduced their reliability. Also, it has become apparent that insects surviving insecticide applications have given rise to progeny quite resistant to these insecticides, requiring higher dosages to give control as well as development of new insecticides to replace those no longer effective (Dutky 1959).

The problem of residues and insecticide resistance, together with the toxicity of many insecticides for a wide range of wildlife, may cause serious consequences in the future due to unwanted destruction of birds, predators, and parasites that play a part in natural control. Consequently, workers in many parts of the world have renewed the search for disease-producing organisms that can be used effectively in insect control. Appropriation bills to increase research designed to discover and introduce nonchemical methods of pest control have recently been introduced.

Recognition of the dangers of wide-spread, indiscriminant use of pesticides has encouraged legislation in some states and

countries for elimination or limitation of certain pesticides within their boundaries. The Federal Insecticide, Fungicide and Rodenticide Act now covers all formulations moving in interstate commerce for controlling, mitigating or repelling pests, as well as products used as plant regulators, dessicants, and defoliants (Martin et al. 1965).

The bacterial sporeformers causing insect diseases include some of the agents used most successfully in microbial control of insects. One such agent, Bacillus thuringensis, is used to control certain vegetable and garden pests and, in addition, has some large scale applications for certain types of beetle control. Another bacterium that has reached the goal of commercial production and application in this country is Bacillus popilliae Dutky (Dutky 1940), the causative agent of type A milky disease of the Japanese beetle. However, this bacterium is pathogenic only to closely related species of beetles belonging to the family Scarabaeidae.

Milky disease gives a lasting control for the Japanese beetle larvae, since the spores produced in the infected insects become residual in the soil. Therefore, the accumulation of spores in the soil will produce a reasonably permanent control.

Use of B. popilliae as a control agent has been delayed because the organism does not grow on usual bacteriological media, and, in spite of much effort, there is not yet available a technique for mass production of spores outside the insect

body. Problems with cultivation and methods of production of B. popilliae for insect control are being elucidated. Eventually, the wide-scale use of this organism in areas of beetle infestation is anticipated. Even today, this pathogen is being employed in the eastern states for control of the Japanese beetle. This field of research, involving the use of various pathogenic microorganisms for biological control of insect pests, will undoubtedly expand if more efficient and economical methods for growth, commercial preparation and field application of B. popilliae are discovered. Many insect pathogens exist, but the commercial use of these pathogens in the future will depend upon the discovery of an efficient method for the production and application of B. popilliae.

Since B. popilliae is being employed commercially for the control of the Japanese beetle, its public health significance is also of interest. In light of this fact, it was considered desirable to definitively determine the potentiality of this organism for infecting warm-blooded animals. It was the purpose of this study to determine the pathogenicity of B. popilliae for mice.

In addition, the effects of certain antimicrobial agents on the growth of B. popilliae were investigated. Interest in this phase of the work was mainly concerned with using such antimicrobial agents to control contamination of laboratory cultures.

REVIEW OF LITERATURE

Japanese Beetle

The Japanese beetle, Popillia japonica Newman, was first found in the United States in 1916 near Riverton, New Jersey. It spread rapidly through western countries, causing an alarming amount of damage to economic and ornamental plants. The failure of early eradication efforts was followed by a Federal quarantine action in 1919. Currently, most states east of the Mississippi are infested; 275 kinds of plants are attacked, and the estimated annual damage is 25 million dollars (Sharpe 1966).

Only about a dozen beetles were found in 1916. These beetles measured about one-half inch in length with metallic green body and legs (Dickerson and Weiss 1918). From this small beginning the beetle multiplied rapidly and spread by natural means at a rate of 5 to 10 miles a year until by 1933 infestations were known to occur in 17 states (Hadley and Hawley 1934). By 1962 it had invaded over 100,000 square miles in the eastern part of the United States, and it had become established in several isolated areas beyond this region of natural distribution. However, a study of the climatological data indicates that the beetle can spread westward naturally to about the 100th Meridian into North Dakota, South Dakota, Nebraska, Kansas, Oklahoma, and Texas (Fleming 1962).

Normally the beetles begin to emerge from the soil during the month of June and they are present until October.

After mating the female beetle digs into the soil and deposits a few eggs. The grub forms a cell in the soil slightly larger than its body and feeds on small roots. The grub passes through three stages, or instars, before reaching maturity in the spring. Metamorphosis to the pupae stage is rapidly followed by transformation to the adult beetle. These leave the soil, having passed through a complete life cycle in one year.

The adult beetle feeds on a wide variety of plants. The fruit and foliage of many berry and small fruit plants are destroyed by the beetle and feeding is often extensive on many fruit trees. Corn is one of the preferred plants of the beetle and its destruction creates a great economic loss each year. The beetle also attacks a wide variety of ornamental herbaceous garden plants, ornamental shrubs and vines, shade trees, non-economic plants and weeds. The grubs feed on the roots of a wide variety of garden and field crops, ornamental plants and grasses. They are most abundant in well-kept lawns, and in pastures and golf courses (Smith 1930).

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, B. popilliae has been studied extensively to determine the possibility of using this organism commercially as an effective biological control agent.

Dormant spores are present in the soil from areas where milky disease is prevalent. Ingestion of such spores by the insect larvae causes the fatal disease. Beard (1945) demonstrated that the normal method of invasion was by the mouth, rather than by penetration of the integument or the tracheal walls. Grubs, with their mouths sealed with a synthetic resin, were able to live in inoculated soil without becoming infected. Germination of the spores occur and the vegetative bacteria proliferate in the hemolymph of the insect larvae. The first evidence of infection is the presence of vegetative rods, not spores, in the blood, indicating that the spores germinate in the alimentary tract and the bacteria reach the hemolymph in the vegetative form. The bacterium sporulates and large numbers of spores form during the terminal stages of the disease. The maximum number of spores is reached in 7 to 10 days after ingestion giving the hemolymph a distinctive milky appearance. Dutky (1940) estimated that the average number of spores produced in a grub is about 5 billion, whereas Beard (1945) and Langford et al. (1942) estimated about 2 billion.

The vegetative form of the bacterium is a nonmotile Gram-positive rod measuring about 0.9 by 5.2 microns (Dutky 1940). The rods become swollen with a centrally located endospore associated with a smaller refractile paraspore. The spores are very resistant to adverse conditions and may remain viable for many years, but the vegetative stage of the bacterium is killed by exposure to sunlight, heat, or cold. Slight or no sporula-

tion occurs in vitro (Rhodes et al. 1965, Rhodes et al. 1966, Sharpe 1966, Steinkraus and Prouvidenti 1958, Steinkraus and Tashiro 1955).

A method of grub inoculation was devised by Dutky in 1942 to provide sufficient numbers of spores for dusting of Japanese beetle infested areas. However, the production of large quantities of spores necessary for wide scale commercial use was inefficient using this grub inoculation procedure. Haynes and Rhodes (1966) discovered that sporulation would occur in tryptone-glucose-yeast extract broth shaken cultures if activated carbon (charcoal) was present during growth. Other investigations reported 0.1 to 0.3% spore formation in cells of colonies grown on a solid medium under strictly defined conditions (Rhodes et al. 1965). Sharpe et al. (1970) isolated a new strain of B. popilliae, designated NRRL B-2309 M, which sporulates regularly in laboratory culture with the production of 20% spores in 10 to 12 days.

However, use of this pathogen for control of the Japanese beetle requires a more effective method of producing large numbers of spores. The production of infective spores requires the efficient cultivation of virulent vegetative cells. B. popilliae is considered fastidious, and cultivation of it led to a complex medium composed of tryptone, yeast extract and glucose (Steinkraus 1957), prolonged incubation periods (Dutky 1963), or other techniques unsuited to large-scale production.

A small amount of fermentable carbohydrate is required for appreciable growth of this organism in liquid media. Sylvester and Costilow (1964), concluded that in synthetic media B. popilliae required biotin, eleven amino acids, thiamine, 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, but required aerobic conditions. Rhodes et al. (1965) studied the aerobic characteristics of B. popilliae and obtained maximal populations between 5×10^8 and 2×10^9 viable cells per milliliter of culture medium during incubation periods of 18 to 24 hr at 30 C.

Death of B. popilliae occurs soon after it reaches the stationary phase of growth on artificial media. Rhodes et al. (1965) discovered that maximal populations persisted in a culture for periods of only 1 to 4 hr followed immediately by rapid death of cultures, so that less than 1% of the cells remained viable after 48 hr, and viability often was lost entirely by the end of 72 hr of incubation. Death was not associated with lysis of the cells and continuous neutralization, augmented buffering, various techniques of dialysis, or slow feeding of the carbohydrate did not markedly alleviate the characteristic death of the cultures.

Some investigators (Costilow et al. 1966, Steinkraus 1957) believe that the rapid death of this organism is the result of an accumulation of hydrogen peroxide. The accumu-

lation occurs because vegetative cells of B. popilliae do not produce catalase (Steinkraus 1957), which degrades hydrogen peroxide. This theory is supported by the return of viability in the stationary phase when oxygen availability is reduced. Also, a system for production of H_2O_2 was consistently found in older cells (Pepper and Costilow 1965). Accumulation of toxic peroxides may prevent spore formation. Another possibility is that cells committed to spore formation may be unable to again grow vegetatively. These cells then die because they are unable to complete sporogenesis.

Alternative methods for the control of Popillia japonica have been considered since sporulation of B. popilliae in artificial culture has not been encouraging. Vegetative cells could be used as a control agent due to their virulence when injected into beetle larvae. Another factor to consider is the stability of the vegetative cells. Haynes et al. (1961) showed that lyophilized vegetative cells remain viable for at least 20 months. Lingg (1966) and Lingg et al. (1967) reported the development of a lyophilization procedure by which survival rates of over 65% could be achieved. Storage survival of the cells remained above 10% for at least 6 months at room temperature. This method of lyophilization consisted of suspending the organisms in a stabilizer containing sodium glutamate and gum tragacanth followed by controlled freezing in liquid nitrogen vapor.

If stabilized vegetative cells of B. popilliae are to be used to control the Japanese beetle, the cells must survive

in soil and be infective upon ingestion. Accordingly, lyophilized B. popilliae survived for at least one year in soil exposed to dry air (Lingg and McMahon 1969). These cells were infective when injected into Japanese beetle larvae. Therefore, it would appear that the lyophilized cells could be dispersed in a mixture of calcium carbonate and talc dust to use for insect control. However, the lyophilized cells do not remain viable in moist soil and methods to treat the cells for retention of their viability in atmospheres of high relative humidity have not been discovered.

Neither excessively wet nor extremely dry soils impair the effectiveness of the spores. Spores remained viable in air-dry soil for more than 10 years (White 1940). The spores can survive cold temperatures and are resistant to heat, but are reduced in potency by exposure to sunlight for more than 8 hr (White 1946). However, when spores were mixed with talc and chalk and exposed in a layer a thirty-second inch or more in depth, sunlight did not reduce their potency significantly during an exposure of 72 hr (White 1946).

Although injection of 10^5 to 10^6 spores per larvae is required to produce greater than 50% infection, injection of the same number of vegetative cells kills the larvae (Pridham et al. 1964, St. Julian and Hall 1968). This level of dead vegetative cells also kills the larvae (St. Julian, unpublished data). Other data regarding artificial infection of larvae with spores support the contention that they germinate poorly.

When 300 to 1000 viable vegetative cells are injected into the hemolymph of larvae, 50 to 80% of the larvae become grossly infected. By comparison, 10,000 to 1 million spores must be injected into larvae to gain a comparable percentage of infection; viable counts based on outgrowth show that about 300 to 1000 germinable spores are involved (St. Julian et al. 1967). A relatively large number of spores, compared to vegetative cells, is needed for optimum infectivity because only a small number of spores germinate in the larvae hemolymph. Therefore, the use of vegetative cells as a control agent is again supported since, more spores than vegetative cells are required to cause infection of the larvae.

The effects of B. popilliae on the grubs appear to be physiological. Beard (1945) found no necrosis of any organ system or tissues, and prior to the moribund condition, no difference in the activity of infected and healthy grub was apparent. A mechanical block of the blood by the spores was not observed. This block appeared to be remote because of the open circulatory system.

Considerable variation in infectivity occurs between different strains of the bacteria. A strain developed in grubs of one species may have a high or low virulence to grubs of other species (Fleming 1968). These strains can be differentiated from closely related aerobic sporeformers serologically, as well as by conventional morphological and cultural tests (Steinkraus 1967). B. popilliae and B. lentimorbus have related pathogenic

properties for the larvae of the Japanese beetle, but the two species are immunologically distinct, since whole cell antigens do not cross-react. Spores of B. popilliae appear to be better antigens than the vegetative cells but also show no cross reaction. The intact spore has at least one antigen in common with the vegetative cell.

Commercial Use

Methods of study to produce sufficient material for field application were developed that side-stepped the difficulty of culture. Following field studies that established the practicability of its use in control (Dutky 1937, White and Dutky 1940), methods were worked out for large-scale application and production. A program of distribution throughout the beetle infested area in 14 eastern states and the District of Columbia was begun in 1939 and largely completed by 1952. The progress of the program has been outlined rather thoroughly (White and Dutky 1942, White and McCabe 1951). Since the start of the Federal-State program, 184,000 pounds of spore dust were produced and applied to 140,973 sites totaling 109,119 acres in the 220 countries treated (Report of the Chief of the Bureau of Entomology and Plant Quarantine 1953). Three patents (Dutky 1941, 1942, Dutky and Fest 1942), were issued covering the major features of the techniques, and several licenses have been issued for the commercial production of the material.

Dutky's (1941) procedure involved the inoculation of live larvae with spores or vegetative cells and incubating until the disease developed and billions of spores appeared in the hemolymph. The infected larvae were dried, ground, and mixed with an extender in proportions calculated to yield 100 million spores per gram of compounded dust. This method yielded infective spores that, in field tests, have proven effective as a biological control agent. However, there are obvious limitations to the production of the volumes necessary for large acreage applications. The development of a practical method for producing enormous numbers of infective spores in plant-size fermentation equipment is of major concern. Two problems exist in the development of this method: (1) propagation of maximum cell populations and (2) sporulation in vitro. Sharpe (1966) developed conditions for the proliferation of vegetative cells in shaken flasks and aerated fermentors, but satisfactory sporulation in vitro has not yet been accomplished.

Lyophilization

Lyophilization has proved to be very successful in the preservation of a large variety of microorganisms, including more than a thousand strains of aerobic sporeformers as well as many fastidious types (Haynes et al. 1955). B. popilliae Dutky likewise survives lyophilization and thereby can be preserved in pure culture for long periods of time (Haynes et al. 1961).

Generally, methods used for lyophilization 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.

Organisms are usually suspended in a stabilizing medium selected to increase the per cent survival. 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.

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 crystalline compounds, the lower death rates were observed with those compounds which the organisms could dissimilate. When colloid compounds were 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.

Haynes et al. (1961) lyophilized 12 strains of B. popilliae using sterile bovine serum as a stabilizing medium and found viable cells after 5 1/2 to 20 months. Lingg et al. (1967) discovered that a suspending solution consisting of 5% sodium glutamate plus 0.5% gum tragacanth produced the highest per cent survival of lyophilized B. popilliae.

Several theories have been suggested to elucidate the protective action of the suspending medium. Heller (1941) suggested that the protective effect is concerned with metabolic processes of the cell. Another theory proposed that a substance could protect a cell by rendering the cell wall impermeable (Miller and Goodner 1953).

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.

The rate of freezing and temperature reached have an effect on survival of dried cells. Proom and Hemmons (1949), Luyet (1951) and others believe that cells are killed by penetration of the cell wall by ice crystals. Luyet (1960 a,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. However, many who have studied freezing rates (Mazur et al. 1957 a,b; Meryman 1956; Heckly et al. 1958) have concluded that a high percentage of cells survive when frozen slowly.

Another factor affecting survival rate is the concentration of the cell suspension being lyophilized. Some investigators have found that more concentrated suspensions gave higher survival (Otten 1930) while others found that a diminution in cell concentration gave an increase in survival (Stamp 1947). 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 concentration. Therefore, it appears that the concentration of cells used to obtain high survival rates is dependent upon the type of organism being lyophilized and the suspending solution.

Age of the cells affects survival differently with various organisms. Some experimenters have stated that mature cells are the most resistant to lyophilization (Heckly 1961) while others have found younger cells to be more resistant (Proom and Hemmons 1949). Lingg et al. (1967) viability studies indicate that 9 to 12 hr cells of B. popilliae survived freeze-drying better than 24 hr cultures.

Degree of vacuum and drying temperature also have a profound effect upon survival of lyophilized cells. Heckly (1961) stated that 20 to 100 μ Hg is considered to be a good

vacuum and quite satisfactory for most lyophilizing. Lingg et al. (1967) maintained a vacuum of approximately 10 μ Hg throughout the 4 to 5 hr drying period of B. popilliae.

Storage atmosphere and temperature is usually the same regardless of the organisms being lyophilized. Rogers (1914) found the highest survival with vacuum storage and the lowest with storage in air and oxygen. Many workers agree that oxygen has a destructive action upon microorganisms preserved by freeze-drying (Benedict et al. 1961, Stark and Harrington 1931, Naylor and Smith 1946). The amount of residual moisture influences viability during storage. However, disagreement exists concerning the optimum water activity. Usually the cultures should be almost dry (Fry 1954), but complete removal of water is incompatible with life (Muggleton 1960).

LD₅₀

When a group of animals is exposed to different concentrations of microorganisms capable of causing death, the percentage mortality plotted against the dose usually gives an S-shaped curve. Brooks (1918) revealed that an inherent variability among individuals of a population in their susceptibility is considered to be responsible for the S-shaped character of the curve.

The literature cites two methods often used to determine the lethality of a microorganism or toxin, the method of probits and the 50% end-point of Reed and Muench. The method

of probits has been actively promoted by Bliss (1934, 1935) and studied by Gaddum (1933). This method uses arbitrary probability units, termed "probits" plotted against a log scale of doses to increase the symmetry and transform a dosage-mortality curve to a straight line. Practical aspects of this method have been used to determine the action of hot water upon Japanese beetle pupae (Fleming and Baker 1932).

The 50% end-point of Reed and Muench was developed from an earlier suggestion (Gaddum 1933, Bliss 1935, Reed and Muench 1938). Trevan (1929) sponsored the use of the median lethal dose (LD_{50}), which is defined as the dose which will make half of the population react to the stimulus tested. The LD_{50} is often used today because it accounts for the indefinitely large errors of estimation in the extreme doses.

The procedure most often used for estimating LD_{50} and its standard error is the cumulative sum method (Pizzi 1950, Carpenter 1965). This method assumes that the animals which react to a given dose would also have reacted to bigger ones, and those which did not respond at a certain level would not have reacted at still lower levels. Large numbers of animals at dilutions near the value for 50% reaction are used and the correct value is interpolated by the procedures described by Gaddum (1933). Using large numbers of test animals at different dilutions is generally necessary to cover a wide range of possible variations in the end-point.

The method of Reed and Muench (1938) utilizes the large, total number involved. It is essentially the one given by Reed (1936), somewhat simplified and adapted to rapid use. The effect is that of using, at the two critical dilutions between which the end-point lies, larger groups of animals than were actually included at these dilutions. By inclining to equalize chance variations, the method tends to define the point more nearly than would be possible if it were simply interpolated between the two bracketing results.

Another method uses survival time as a quantitative measure of toxicity in biological assays (Roth et al. 1956). The organism is serially diluted, a group of subjects is inoculated with each dilution and, in the case of quantal response, the numbers of subjects responding to each dilution are recorded. The proportion of subjects responding is usually related to the logarithm of dose by a sigmoid curve which is roughly symmetrical (Meynell 1957). The length of survival after infection is recorded and used to calculate the lethality of the substance injected. However, use of survival time is limited to certain organisms because of the variability in modes of action of these organisms.

There are errors in technique regardless of the method used. However, most of these errors will not drastically affect the results if large numbers of test animals are used in each dilution. Steward et al. (1968) found a 14% error in the placement of intraperitoneal injections of mice. They consider

this error inherent in the technique and not easily corrected. Minor et al. (1969) reported that 10-20% of intraperitoneal injections are placed somewhere other than the peritoneal cavity. Misplacements were in the stomach, lumen of the intestines, uterine horn, and subcutaneously. None of the technical modifications they used would consistently eliminate or reduce the error of placement.

Effects of Antimicrobial Agents

Research was conducted by Pridham et al. (1965) to determine whether antimicrobial agents could be added to culture media to prevent the development of contaminants during routine culture of B. popilliae. The shaken-tube sensitivity test and the sensitivity-disc test, based on general procedures outlined in Part 147 of the Federal Register (Food and Drug Administration 1963), were used. The use of antimicrobial agents was considered, because of the fastidious nature of B. popilliae and because of the organisms marked susceptibility to contamination during routine culture.

Results obtained from experiments with many agents indicate that vancomycin, ristocetin, amphotericin B, nystatin, and possibly some of the sulfa compounds could be included in growth media at concentrations which did not inhibit milky disease bacteria but did inhibit some other organisms. It was concluded from investigations with these various antimicrobial agents that the milky disease bacteria exhibit the

same sensitivities to antibiotics as do other gram-positive bacteria, and further suggested that species and strains of the bacteria differed quantitatively in their response to these antibiotics.

Dutky (1963) stated that growth and development of B. popilliae in the blood of Japanese beetle larvae could be prevented by the injection of 20 ug of dihydrostreptomycin per larva. He further stated that penicillin G, chlortetracycline hydrochloride, and sodium sulfadiazine inhibited the bacteria on artificial media but not when injected into infected larvae.

Examination of some of the liquid cultures of B. popilliae NRRL B-2309 which contained sublethal concentrations (0.1 to 1 $\mu\text{g/ml}$) of vancomycin hydrochloride and ristocetin A and B sulfates showed that morphological changes had occurred in some of the cells (Pridham et al. 1965). The central area of the cell was swollen and some cells contained partially refractile material. Rounding-off of one tip and narrowing of the other tip of the cell was also observed. No parasporal bodies, characteristic of B. popilliae, were noted. Marked elongation of cells was seen with higher concentrations of the antibiotics.

MATERIALS AND METHODS

Routine 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 (Lingg and McMahon, personal communication), which will be referred to as JB medium, contained 1.5% tryptone (Difco), 0.5% yeast extract (Difco), 0.6% K_2HPO_4 and 0.2% glucose (sterilized separately). 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 of a 5% inoculum. Cultures were also maintained for longer periods of time by placing the flasks at 4° C immediately after inoculation. These cells resumed growth when the flasks were transferred to 25° C.

Lyophilization

Cells were grown for 9 to 12 hr and centrifuged at 1800 rpm for 15 min. The supernatant fluid was poured off

and cells were resuspended in a stabilizing medium consisting of 5% sodium glutamate plus 0.5% gum tragacanth. Enough stabilizer was added to obtain a control count of approximately 1.0×10^9 to 2.0×10^{10} cells per milliliter. The suspension was placed in 10 ml glass ampoules (American Instrument Company, Inc.) in 0.5 ml amounts. Cells were lyophilized according to the method of Lingg et al. (1967).

Three ampoules were chosen at random and viable counts were obtained to determine the percentage viability immediately after lyophilization. Each lyophilized pellet was reconstituted in 2 ml of JB medium to give an initial dilution of 1:4 and plated in the same manner as the control cells.

Viable Counts

Viable counts were made on JB medium solidified with 1.5% agar. Counts were established by making triplicate serial dilutions of 1.0 ml of the cell suspension in 0.1% tryptone. Five replications of each appropriate dilution were plated. Volumes of 0.1 ml were spread uniformly over the surface of the medium with a glass spreader.

After 5 to 7 days' incubation at 25 C, colonies were counted using a Quebec colony counter. All counts were reported as log averages of the triplicate dilutions. Viable counts recorded in all experiments were obtained by the procedure explained, unless otherwise noted.

Preparation of Cells for Injection

Vegetative B. popilliae used for mouse injection was grown in shaken cultures of JB medium incubated at 25° C. After 14 hr, the cells were harvested by centrifugation at 1800 rpm for 15 min. Cells were washed twice to remove soluble products of growth and to eliminate the possibility that deaths could be due to extracellular toxin. For harvesting, washing, and suspension of injectable materials, 0.1% tryptone (pH adjusted to 7.0-7.2) was employed. One-tenth percent tryptone is relatively innocuous to B. popilliae (Pridham et al. 1964, St. Julian et al. 1963). Pridham et al. (1964) investigated the effects of various diluents on both Japanese beetle larvae and on milky disease bacteria and concluded that distilled water was not a suitable diluent for milky disease bacteria.

A large concentration of cells was obtained by resuspending the pellets in 0.1% tryptone to a count of approximately 5.0×10^9 to 5.0×10^{10} cells per ml. The actual number of cells to inject, for determination of the LD₅₀, was estimated by injecting ten-fold dilutions of a concentrated suspension into small groups of mice. The numbers of viable organisms injected into mice were estimated from plate counts of each dilution.

Ampoules of lyophilized cells used for injection were stored at 4° C for 1 month before opening and resuspending in 0.1% tryptone to a concentration of approximately 1.0×10^{10} .

B. subtilis was injected into mice to give a comparison of pathogenicity. Growth was accomplished by inoculating JB medium and incubating at 25 C for 24 hr. The method of preparation and injection was similar to that used for B. popilliae.

Method of LD₅₀ Determinations

Five to seven week-old white mice, both males and females, of uniform stock, weighing from 18 to 35 g, were used throughout this study. Intraperitoneal injections were given in 0.5 ml volumes using a 1 cc tuberculin syringe with a 1/2 inch, 27-gauge needle. Injections were made using a two-man procedure. Arioli and Rossi (1970) compared the two-man with the one-man procedure and discovered a consistent reduction in error of placement, from 12% using the one-man to 1.2% using the two-man procedure.

For each determination, a two-fold dilution series of a suspension of organisms was injected into 35 mice, 7 mice per dilution. Noninjected mice and mice receiving sterile 0.1% tryptone solution by intraperitoneal injection were used as controls throughout. A solution of 5% sodium glutamate and 0.5% gum tragacanth was injected into mice to determine if the stabilizer used for lyophilization was innocuous to mice.

LD₅₀ Calculations

Calculation of LD₅₀ was by the method of Reed and Muench (1938). It should be kept in mind, that the LD₅₀ is the dose

of organisms required to kill 50% of a group of animals within a specified period. Since experimental results often do not contain a group in which exactly half the animals die, the dosage that would be expected to produce this result must be estimated or calculated.

On the assumption that mice surviving the largest dose or organisms injected will also survive smaller doses, the survivors after 48 hr were cumulated successively, beginning with the largest dose. Likewise, based on the opposite assumption, deaths were cumulated successively, beginning with the smallest dose. Percentage mortality was then determined by dividing the total cumulation of dead mice by the total cumulation of both alive and dead mice of each dilution.

Since a logarithmic series of organisms was injected, it was appropriate to use a logarithmic calculation. The formula employed for calculation of LD₅₀ was based on the Reed and Meunch Method of calculation (Reed and Meunch 1938).

$$\text{Log LD}_{50} = \log \left(\begin{array}{c} \text{No. injected} \\ \text{at \% mortality} \\ \text{below 50\%} \end{array} \right) + \left(\frac{50 - \% \text{ mortality below 50\%}}{\% \text{ mortality above 50\%} - \% \text{ mortality below 50\%}} \right) \times \log 2$$

The factor log 2 was employed because each dilution of organisms injected was double the one before. The middle term is actually the proportionate distance of the end-point above the dilution giving next below 50% mortality.

The standard error of LD₅₀ for all experiments was derived by using the formula given by Gaddum (1933) and modified by Pizzi (1950):

$$S. E. LD_{50} = \sqrt{\frac{0.79 h R}{n}}$$

where h is the interval between dosages, R is the interquartile range and n is the number of animals per dose. It should be noted, that this method is somewhat biased because the doses are not symmetrically spaced about the LD₅₀ with equal intervals between dosages.

Isolation and Growth in Pure Culture

Mice which succumbed to the infection, with very few exceptions, were dead within 48 hr; and the majority died within 24 hr following intraperitoneal injection. The survivors, nevertheless, were kept under observation for 5 days.

In the initial stages of the work, peritoneotomy was performed on all dead mice. Cultures of B. popilliae were isolated by inoculating JB broth or JB agar medium containing antimicrobial agents, with peritoneal exudate and heart blood. Peritoneal exudate was removed aseptically with a cotton swab and heart blood was obtained by searing the heart and removing a few drops of blood with a sterile syringe and needle. Since cultures were usually positive for the B. popilliae injected, the laborious process of isolation and identification was later discontinued.

Preliminary experiments indicated that the combination of antimicrobial agents cholistimethate, nystatin and vancomycin (Difco) or ristocetin and polymyxin B (Difco), referred to

as CNV and RP respectively, were satisfactory for isolation of B. popilliae from infected mice. Varying concentrations of the antimicrobial agents were incorporated into JB medium to give selective inhibition of contaminating organisms.

Reinjection of Isolated Organisms

Organisms isolated and identified as B. popilliae were reinjected to determine if death could be reproduced in the experimental animal. B. popilliae was isolated from the peritoneal exudate by streaking plates of JB agar medium containing antimicrobial agents. A typical colony was picked and inoculated into JB broth. After 14 hr, incubation at 25° C, the growth was washed, centrifuged and resuspended in 0.1% tryptone. A concentrated suspension of approximately 3.0×10^{10} cells was injected into 6 mice, 0.5 ml per mouse. A two-fold dilution was made and 6 more mice were injected with this suspension.

Gram stains were made from smears of the peritoneal cavity, pleural cavity, heart blood, liver and spleen. Gram stains were prepared from random animals in all experiments for the purpose of identifying B. popilliae.

Subcutaneous Injection

B. popilliae was injected subcutaneously to determine if lethality for mice was dependent upon the route of injection. A concentration of approximately 4.0×10^{10} cells per ml was prepared in the same manner as those prepared for the intra-

peritoneal injections. Subcutaneous injections were given in 0.25 ml volumes to 6 mice. Mice were observed frequently over a period of 7 days for the presence of superficial lesions.

Injection of Heat-Killed Cells

B. popilliae was grown for 14 hr in JB medium and centrifuged at 1800 rpm for 15 min. Cells were washed twice and resuspended in 0.1% tryptone to a concentration of approximately 4.0×10^{10} cells per ml. A portion of the suspension was heated in a water bath at 60° C for 2 hr. Another portion was heated at 100° C for 30 min. Viable counts were obtained before heat-killing. An aliquot of the heated cells was added to JB medium to determine if all organisms had been killed.

Groups of 12 mice were injected intraperitoneally with 0.5 ml amounts of the heat-killed suspensions. Control mice were injected with 0.1% tryptone.

Study on Effects of Antimicrobial Agents

Studies indicated that Bacto-antimicrobial mixture CNV (Difco), and Bacto-antimicrobial mixture RP (Difco) were efficient in reducing contamination while allowing growth of B. popilliae. Dessicated mixtures CNV and RP were obtained from Difco in sterile vials and rehydrated by aseptically adding sterile distilled water. Antimicrobial vials CNV contained 750 mcg cholistimethate, 1,250 units nystatin and 300 mcg

vancomycin per ml, and antimicrobial vials RP contained 1,000 mcg ristocetin and 2,500 units polymyxin B per ml.

Growth of B. popilliae in JB broth medium containing antimicrobial agents was studied to determine the optimum concentration of the agents to inhibit contaminating organisms. Varying quantities of CNV and RP were aseptically added to 100 ml of JB broth contained in 300 ml Erlenmyer flasks. Each quantity from 0.25 to 3.0 ml was added to triplicate 100 ml samples. Broth medium containing the antibiotics were inoculated with a 3% inoculum of a 9-10 hr culture and incubated at 25° C. Controls without any antimicrobial agents were also inoculated.

After 24 hr, 1 ml of each sample was removed and plated for viable count. Growth was immediately halted by adding 0.5 ml formalin 1:3 to each flask. Triplicate samples were removed and optical density was determined by reading the absorbance at 600 nm on a Spectronic-20 colorimeter/spectrophotometer model no. 33-29-61-62 (Bausch and Lomb, Rochester, N.Y.).

Antimicrobial agents were incorporated into JB agar medium to determine the highest concentration that would allow luxuriant growth of B. popilliae. Varying amounts from 0.25 to 2.0 ml were added to 100 ml of agar medium and 14 hr actively growing cultures of B. popilliae were plated. Control counts were obtained on JB medium without any antimicrobial agents. Colony sizes were measured by means of a calibrated

ocular micrometer and plates containing colonies smaller than 0.5 mm were not counted. B. subtilis was plated on medium containing antimicrobial agents to establish if this organism could be inhibited.

Study on Effects of CNV and RP
on Lyophilized B. popilliae

Effects of antimicrobial agents on lyophilized cells were studied by resuspending the pellet in 2 ml of JB medium and plating on medium containing CNV and RP in concentrations of 0.5 ml per 100 ml medium. Preliminary experiments indicated that this was the highest concentration of these antibiotics that allowed luxuriant growth of vegetative B. popilliae. Cell count immediately after lyophilization was 1.21×10^{10} cells per ml. Lyophilized cells used were stored at 4° C for 6 weeks before using.

Study on Effects of CNV and RP
on Lyophilized B. popilliae in Soils

Preparation and inoculation of soils was by the method of Lingg and McMahon (1969). Prior to inoculation, 50 g of loam soil was placed in a pint (0.55 liter) jar, autoclaved for 1 hr at 121° C, and dried over CaSO_4 for 3 days. Cells were lyophilized and stored at 4° C for 1 month or longer before soil inoculation.

Lyophilized pellets were pulverized in the unopened ampoule on a vortex mixer immediately before use. The con-

tents of the vial were added to the soil and mixed by vigorous shaking for several min. Jars of soil with loosened screw-cap lids, to allow free exchange of air, were then placed in desiccators containing CaSO_4 and stored at 25°C .

Immediately after being inoculated, 1 g-soil samples were removed with a sterile spatula, weighed on sterile paper and diluted in 0.1% tryptone. Viable counts were then obtained using the method described earlier. Soil dilutions were also plated on JB medium containing 0.5 ml CNV and RP per 100 ml medium.

RESULTS AND DISCUSSIONS

LD₅₀ of Vegetative B. popilliae

Pathogenicity is dose-dependent. Therefore, the possible dose dependence for infection of mice with the insect pathogen B. popilliae was studied. The dose causing death was determined using the method of Reed and Meunch (1937), since the LD₅₀ end-point obtained by this method is less affected by small chance variations than any other method. The advantages of using the dilution by which half the animals are affected have been set forth by Gaddum (1933) on the basis of pharmacological tests.

Pathogenicity determinations, using such small numbers of mice for each test, are obviously inaccurate and results from repeat tests could vary markedly. The best method of determining the end-point would involve the use of large numbers of mice at dilutions near the 50% mortality, but this is scarcely practical since the end-point may vary between wide limits. Also, the use of highly refined statistical procedures were hardly justified in view of the uncontrolled variables involved.

The data obtained are summarized in Table 1. It is evident that B. popilliae can kill mice if a sufficient number of microorganisms are injected. B. popilliae is even much less lethal to mice than the commonly referred to nonpathogenic B. subtilis. Therefore, the large number of bacilli required to cause death appears to make this microorganism nonpathogenic to mice.

TABLE 1. CALCULATION OF LD₅₀ OF B. POPILLIAE

Dilution	Dose ^a	Results in 5 Days		Total		Per Cent Mortality ^b
		Alive	Dead	Alive	Dead	
1:1	4.26 x 10 ⁹	0	7	0	9	100
1:2	2.52 x 10 ⁹	5	2	5	2	28.6
1:4	1.06 x 10 ⁹	7	0	12	0	0
1:8	4.92 x 10 ⁸	7	0	19	0	0
1:16	2.74 x 10 ⁸	7	0	26	0	0

^a actual number of cells injected per mouse

$$\text{b\% mortality} = \frac{\text{total dead mice}}{\text{total dead} + \text{total alive}} \times 100$$

$$\log \text{LD}_{50} = \log (2.52 \times 10^9) + \left(\frac{50-28.6}{100-28.6} \right) \times \log 2$$

$$= 9.4014 + (0.2997)(0.3010)$$

$$= 9.4014 + 0.0902 = 9.4916$$

$$\text{LD}_{50} = 3.10 \times 10^9$$

$$\text{S.E. LD}_{50} = 5.46 \times 10^8$$

Lamanna and Jones (1963) in their studies with B. cereus and B. cereus-like insect pathogens described the insect pathogenic spore formers as pathogenic but nonvirulent. Comparison of the LD₅₀ of the insect pathogens with B. cereus showed their pathogenic similarities, but the LD₅₀ of B. anthracis was approximately 10^7 times less than the number of B. cereus or insect-pathogens required to kill mice. By using this analogy, B. popilliae would be described as pathogenic but nonvirulent to mice.

The LD₅₀ of the vegetative B. popilliae was calculated by the method of Reed and Meunch as previously described. Observing Table 1, it is evident that the LD₅₀ lies between 2.52×10^9 and 4.26×10^9 , but nearer the former. It is assumed that, since the mortality at 4.26×10^9 is 71.4% above that at 2.52×10^9 , while the 50% point is 21.4% above, the LD₅₀ or end-point is $21.4/71.4$ distance from 2.52×10^9 to 4.26×10^9 . This is actually the proportionate distance used in the formula, below Table 1, to calculate an LD₅₀ of 3.10×10^9 .

Subcutaneous Injection of B. popilliae

Doses required to kill mice are dependent upon the route of injection. Deaths occurred upon intraperitoneal injection but not upon subcutaneous injection of even ten-fold greater numbers of organisms. Approximately 1.0×10^{10} cells were injected subcutaneously. Although mice survived subcutaneous injection, the site of injection sometimes became indurated.

Approximately 5 to 7 days later, virulent B. popilliae were isolated from the open necrotic lesions. Isolation and identification of the organisms was accomplished using the same technique as that used in the isolation from the peritoneal exudate. Thus, the subcutaneous route cannot be viewed as one preventing either the in vivo proliferation of the bacteria or the elaboration of a toxin. Rather, the skin would appear to offer a barrier to development of the infection.

The results imply that localized growth in skin, and any accompanying production of toxin, are insufficient to cause death. Death appears to depend upon the widespread diffusion and proliferation of the organism or toxin in the host body.

Lamanna and Jones (1963) introduced three hypotheses concerning the cause of death due to injection of B. cereus-like insect pathogens. These hypotheses might also be true for B. popilliae. Their first suggestion was that fatal effects were not the result of extra-cellular toxin production, since toxin might be expected to diffuse from skin lesions to harm essential organs for life. Another possibility was that toxin production per bacillus was low and the limited number of bacteria concentrated in a walled-off skin lesion was insufficient to produce a fatal dose of toxin. A third possibility was that local absorption by skin prevented the escape of toxin into the general circulation. None of these hypotheses were further explored. However, the presence or absence of a heat-stable extra-cellular toxin of B. popilliae was determined by injecting heat-killed cells.

Injection of Heat-Killed Cells

Heat-killing of B. popilliae eliminated this organism's lethality for mice. Death did not occur with the intraperitoneal injection of as many as 2.0×10^{10} heat-killed cells, which is almost ten-fold more organisms than the number of viable cells required to cause death. Since, symptoms of infection were not observed in any of the mice injected, it would appear that the lethal doses of killed organisms is much greater than the limits of experimentation. This would seem to rule out the production of a heat-stable toxin.

Deaths observed could be due to a true infection rather than the presence of toxin initially present in the injected material. Mechanical obstruction of some vital organ was another possible explanation of death that was considered. This possibility was rejected, because the heat-killed cells did not cause death when lethal doses were injected. It was further observed, that the cells were not disrupted after the heating process. Therefore, the same quantity of foreign material was injected regardless of the viability of the cells.

It is also possible that virulence might be due to the elaboration of an extra-cellular, heat-labile toxin. The latter is believed to be the cause of death, because of Dutky's (1963) discovery of a heat-labile toxic substance of B. popilliae that was lethal to the grub of the Japanese beetle. Development of these toxic products in the blood is probably the cause of death in mice.

Pathogenicity of B. popilliae
Isolated from Infected Mice

Organisms were isolated from mice that had succumbed to infection with B. popilliae. Identification of the isolated organisms was accomplished by observation of colonies containing the typical satellite halo of B. popilliae. The colonies picked and inoculated into JB broth were further observed for the characteristic birefringent turbidity of this organism and identified by gram stain and microscopic examination.

Death was reproduced in mice reinjected with the isolated organisms. Six mice were injected with a concentrated suspension of 3.0×10^{10} organisms and 100% mortality occurred. However, none of the 6 mice injected with a suspension of 1.4×10^{10} cells succumbed to infection. This number is greater than the LD_{50} of vegetative B. popilliae that has been growing in vitro for a long period of time. Therefore, passage of this organism through animals does not enhance its virulence, rather it seems to slightly decrease its pathogenic ability.

LD_{50} of Lyophilized B. popilliae

It has been suggested that lyophilized cells might be used for biological control in the field. If this is the case, the LD_{50} of this state of the organism should be determined to establish its relative pathogenicity. Accordingly, the LD_{50} of the lyophilized cells was calculated by the Reed and Meunch Method to be 3.39×10^8 . The difference in the LD_{50}

of lyophilized cells compared to actively growing vegetative organisms establish the fact that pathogenicity of B. popilliae is dependent upon its physical state, i.e., vegetative or lyophilized. The LD₅₀ of lyophilized cells is shown in Table 2.

This LD₅₀ is approximately ten-fold less than that calculated for the broth culture of B. popilliae, but is still large enough to be considered nonpathogenic. It is possible that the smaller LD₅₀ value of the lyophilized organisms is due to an elimination of the more sensitive cells by the freezing and drying processes. Pathogenicity would be increased if lyophilization selected for more resistant organisms, because the more resistant strains would tend to survive longer in the host. Another possible explanation for the increased virulence of the lyophilized cells is the fact that gum tragacanth increases virulence when injected with bacteria (Olitski 1948). This possibility exists since gum tragacanth was a constituent of the stabilizer used for lyophilization.

LD₅₀ of Bacillus subtilis

The LD₅₀ of B. subtilis was studied for comparative purposes. With this relatively avirulent organism, the LD₅₀ was calculated to be 4.23×10^8 . It should be noted immediately, that there is no appreciable difference in the numbers of B. subtilis and B. popilliae required to kill mice. Death time and signs of infection were very similar for both organisms.

TABLE 2. CALCULATION OF LD₅₀ OF LYOPHILIZED B. POPILLIAE

Dilution	Dose	Results in 5 Days		Total		Per Cent Mortality
		Alive	Dead	Alive	Dead	
1:1	5.42 x 10 ⁹	0	7	0	28	100
1:2	2.32 x 10 ⁹	0	7	0	21	100
1:4	8.95 x 10 ⁸	0	7	0	14	100
1:8	6.20 x 10 ⁸	1	6	1	7	87.5
1:16	2.40 x 10 ⁸	6	1	7	1	12.5

$$\log LD_{50} = \log (2.40 \times 10^8) + \left(\frac{50-12.5}{87.5-12.5} \right) \times \log 2$$

$$= 8.3802 + (0.50)(0.3010)$$

$$= 8.3802 + 0.1505 = 8.5307$$

$$LD_{50} = 3.39 \times 10^8$$

$$S.E. LD_{50} = 7.52 \times 10^7$$

Thus, there appear to be remarkable similarities in the nature of pathogenesis of B. subtilis and B. popilliae.

The calculation of the LD₅₀ of B. subtilis can be observed in Table 3. Results show that the LD₅₀ of B. subtilis is less than the LD₅₀ calculated for B. popilliae. Therefore, if B. subtilis is considered avirulent to mice, then B. popilliae must also be described as avirulent.

Effects of Antimicrobial Agents on Vegetative Cells

Results obtained with CNV and RP suggest that it might be possible to include such agents in growth media for B. popilliae at concentrations which did not inhibit this organism but did inhibit some other organisms. The resistance of B. popilliae to CNV and RP is established in Fig. 1 and 2 on pages 43 and 44. The amount of growth was measured in terms of the optical density of the culture. This method of measuring growth should be quite representative of the actual multiplication of the organisms, since the original inoculum was only 3% of the total volume. However, viable counts were simultaneously obtained to determine the actual number of viable cells.

Results in Fig. 1 and 2 indicate that the growth of B. popilliae is influenced very little by the addition of small amounts (approximately 0.5 ml/100 ml medium) of CNV and RP. Resistance to such concentrations suggests that these

TABLE 3. CALCULATION OF LD₅₀ OF B. SUBTILIS

Dilution	Dose	Results in 5 Days		Total		Per Cent Mortality
		Alive	Dead	Alive	Dead	
1:1	1.25 x 10 ⁹	0	7	0	15	100
1:2	8.30 x 10 ⁸	2	5	2	8	80.0
1:4	3.30 x 10 ⁸	4	3	6	3	33.3
1:8	1.97 x 10 ⁸	7	0	13	0	0
1:16	1.14 x 10 ⁸	7	0	20	0	0

$$\log LD_{50} = \log (3.30 \times 10^8) + \left(\frac{50-33.3}{80-33.3} \right) \times \log 2$$

$$= 8.5185 + (0.3576)(0.3010)$$

$$= 8.5185 + 0.1076 = 8.6261$$

$$LD_{50} = 4.23 \times 10^8$$

$$S.E. LD_{50} = 1.19 \times 10^8$$

Figure 1.

Effects of CNV on 9-10 hr cells of B. popilliae. Cells were grown in JB broth medium containing varying concentrations of CNV. Each point represents average from 3 samples read in triplicate.

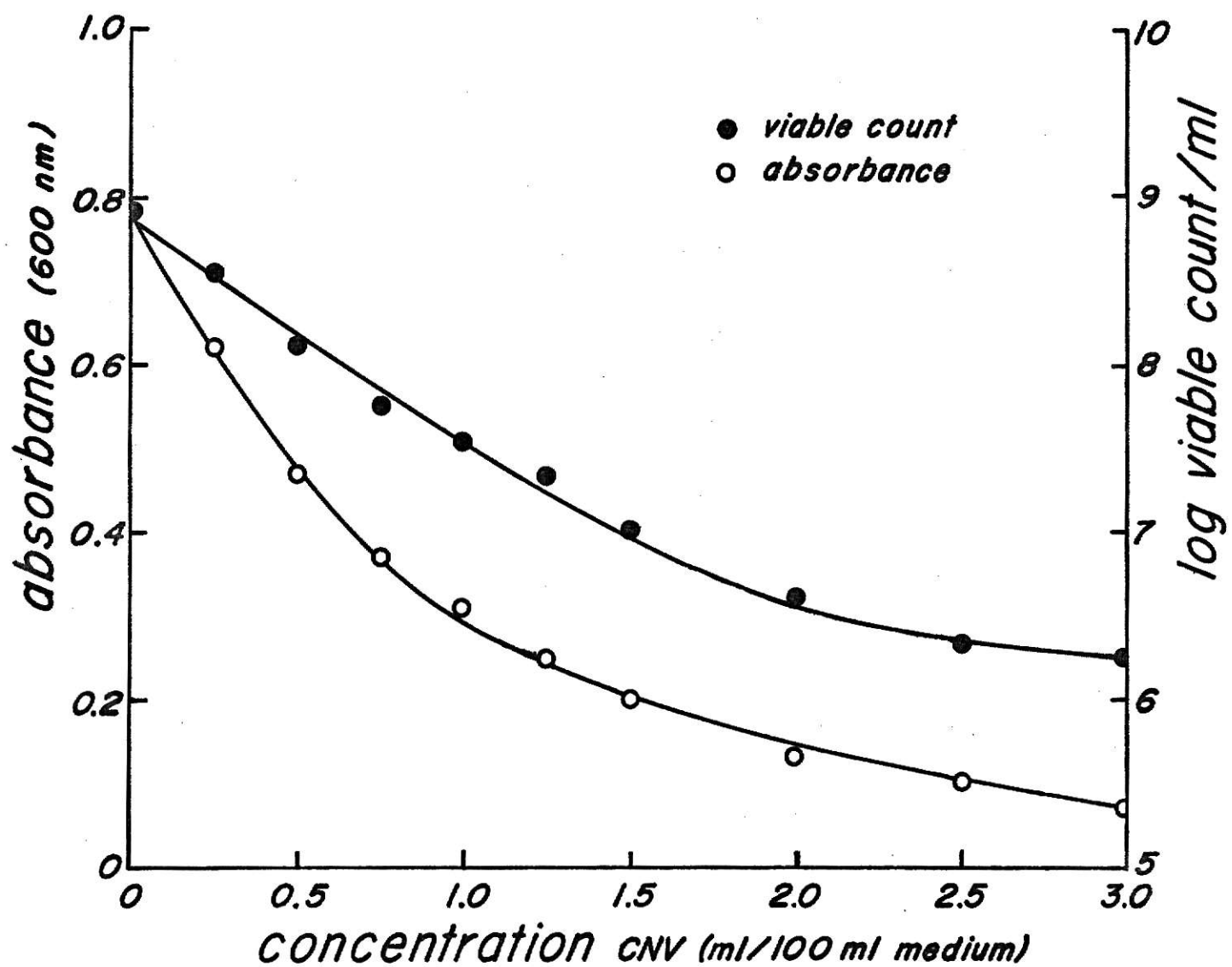
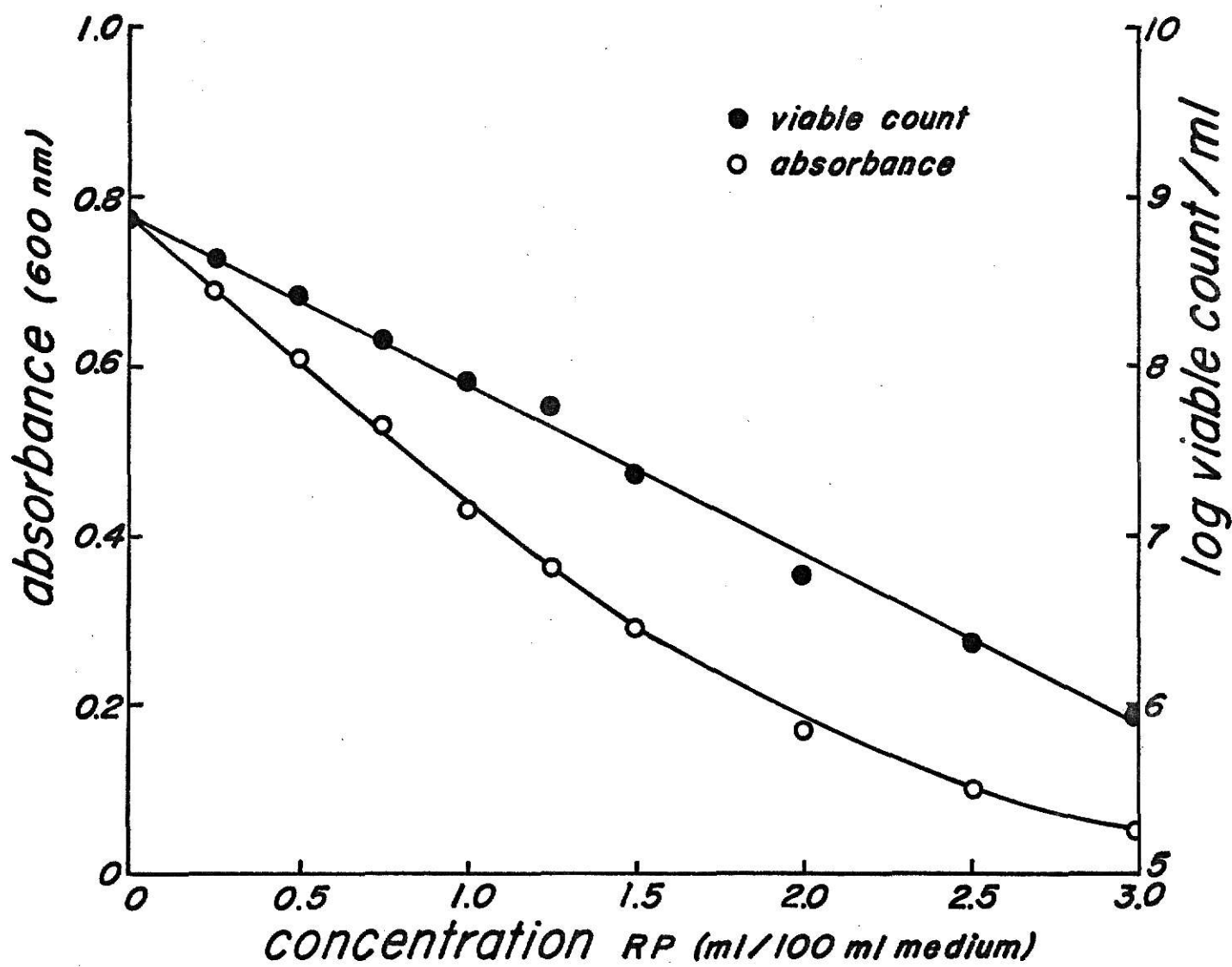


Figure 2.

Effects of RP on 9-10 hr cells of B. popilliae. Cells were grown in JB broth medium containing varying concentrations of RP. Each point represents average from 3 samples read in triplicate.



antimicrobial agents might be useful in controlling contamination during routine culture of the fastidious milky disease bacteria.

Viable counts decreased approximately ten-fold with the addition of 1.0 ml of CNV or RP. The medium with 1.0 ml of CNV per 100 ml contained 7.5 mcg cholistimethate, 12.5 units nystatin and 3.0 mcg vancomycin and the medium with 1.0 ml RP per 100 ml contained 10.0 mcg ristocetin and 25.0 units polymyxin B per ml of medium. Figures 1 and 2 indicate that viable count and optical density of 9-10 hr cells decreased expotentially with increased concentration of antibiotics.

Microscopic examination of these cultures indicated that sublethal concentrations of CNV and RP elicited some morphological change in the bacteria. Marked enlargement of cells was seen with higher concentrations of the agents. Because these antibiotics in low concentration showed gross morphological changes, some effect on the cell wall or cell membrane possibly occurs. Sensitive organisms are affected at these sites. Cholistimethate, which is a polypeptide antibiotic of the polymyxin group and has an antibacterial spectrum that includes a number of gram-negative organisms, affects the cell membrane (Schwartz et al. 1960, Kurihara and Suzuki 1953). Vancomycin disrupts the cell wall and is highly active against gram-positive cocci (Jordon 1961, Russel 1964). Nystatin has no effect on bacteria, but its site of action is the protoplasmic membrane of the fungus (Kinsky 1962, Lampen

et al. 1962, Marini et al. 1961). Ristocetin is believed to have the same mode of action as penicillin, the cell wall (American Medical Association 1960). Polymyxin B is active against gram-negative bacteria and affects the cell membrane (Few and Schulman 1953, Newton 1956, Rhodes et al. 1953).

Inhibitory levels of CNV and RP varied remarkably with the age of the inoculating culture of B. popilliae. The results of concentration of CNV and RP on different age cultures is given in Fig. 3 and 4 respectively. Concentrations inhibiting young cultures usually had no significant effect upon older cultures. Higher concentrations (3.0 ml/100 ml medium) of these agents had very little effect on 24 hr cells, but partially inhibited growth of 4 to 5-hr cells.

Studies were conducted to determine the resistance of B. popilliae to CNV and RP in solid JB medium. Results indicated that 0.5 ml CNV and RP per 100 ml medium was the highest concentration allowing uninterrupted growth of this organism. Colony size was slightly diminished, but viable count remained essentially the same in the presence or absence of this concentration of the antibiotics. The control count on JB medium was 1.1×10^9 cells per ml. The count on JB medium with 0.5 ml CNV per 100 ml medium was 9.0×10^8 and with 0.5 ml RP per 100 ml medium was 1.1×10^9 . In accordance with these results, 0.5 ml of each agent was added to all 100 ml aliquots of plating medium in all subsequent experiments.

Figure 3.

Effects of CNV on different age cells of B. popilliae. Cells were grown in JB broth medium containing varying concentrations of CNV. Each point represents average from 3 samples read in triplicate.

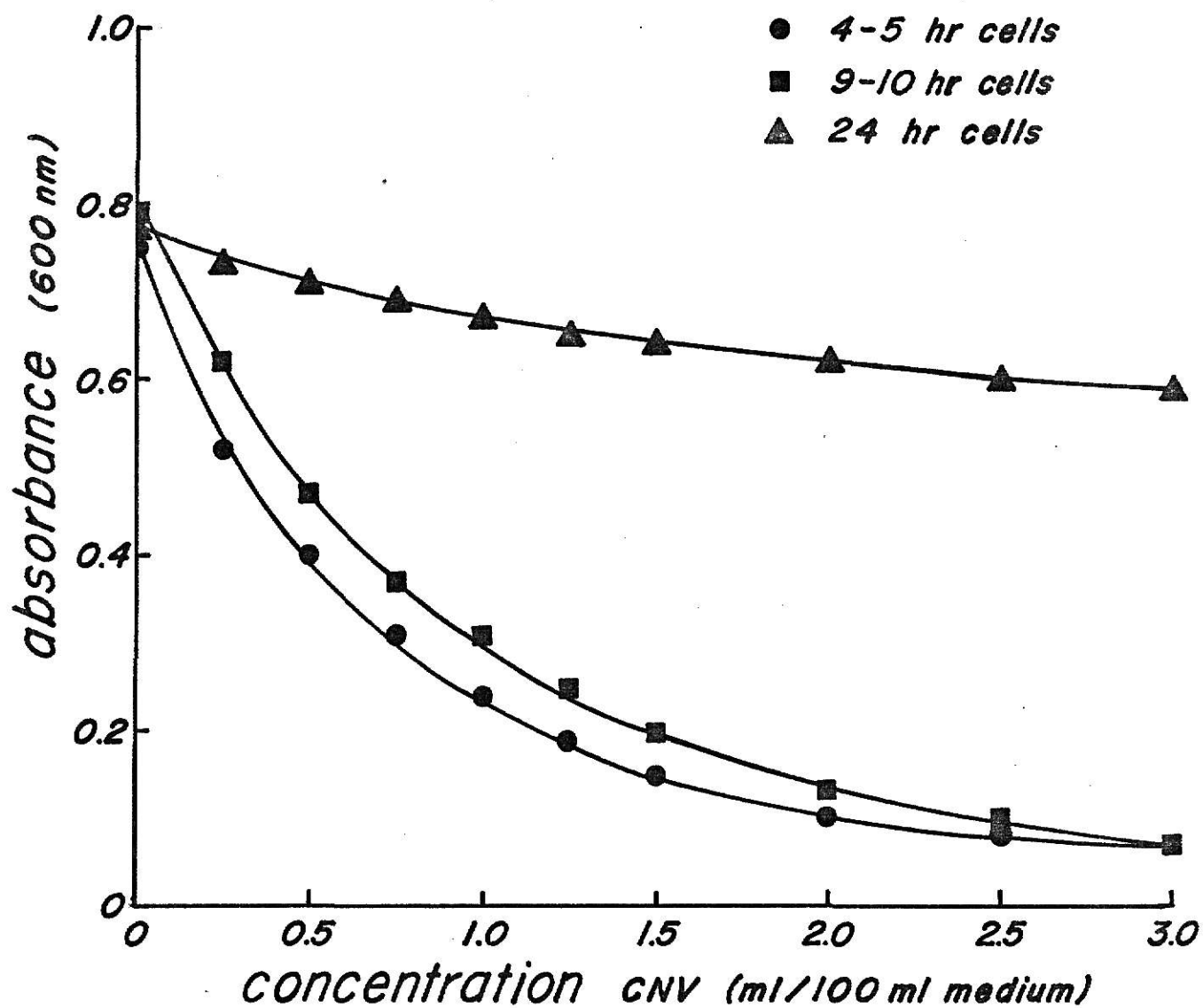
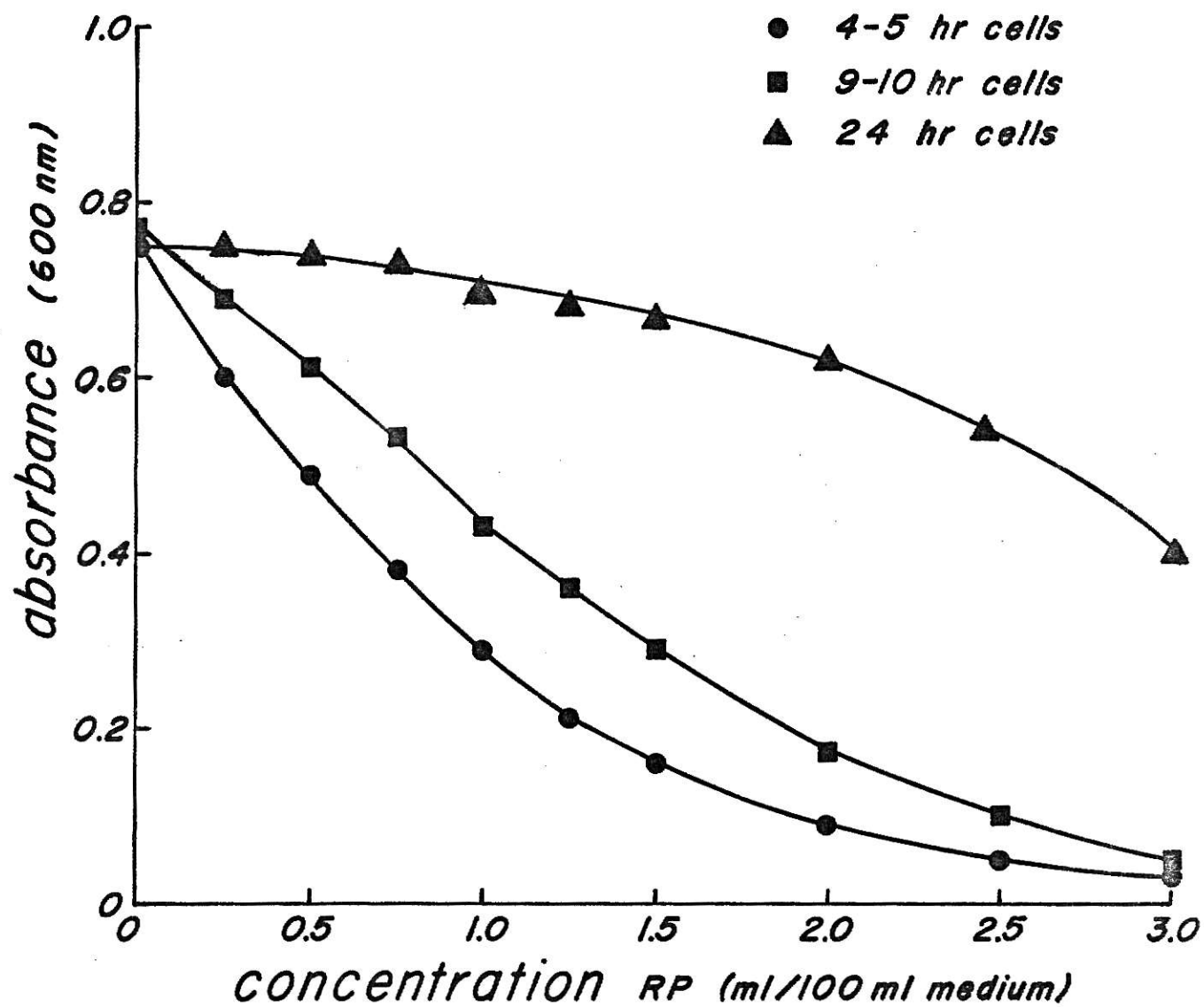


Figure 4.

Effects of RP on different age cells of B. popilliae. Cells were grown in JB broth medium containing varying concentrations of RP. Each point represents average from 3 samples read in triplicate.



Growth of B. subtilis was inhibited in the presence of CNV and RP. Minimal concentrations of both agents completely inhibited growth of this organism in liquid and solid medium. Investigations with other organisms suggest that these antimicrobial mixtures could also be used to inhibit growth of many common contaminants (Pridham et al. 1965).

Effects of CNV and RP on Lyophilized Cells

Effects of CNV and RP on lyophilized B. popilliae were studied for two reasons. First, contamination might be controlled by using antimicrobial agents during enumeration of lyophilized cells. Antibiotics could be added to the medium used for determination of per cent viability. Second, vegetative and lyophilized cells might differ in their response to these agents. If differences are discovered, these variations might be advantageous in determining the reason for instability of the lyophilized cells in moist soil.

Results of growing lyophilized cells in the presence of CNV and RP are shown in Table 4. Counts were obtained by re-suspending the lyophilized pellets and plating on JB medium containing the specific antibiotic. Concentrations of CNV and RP were 0.5 ml/100 ml medium.

Highest viability was obtained using CNV. Since this antimicrobial mixture also reduced contamination, its incorporation into media used for viable counts is suggested. Concentrations of 0.5 ml CNV per 100 ml medium appear to be optimal

TABLE 4. EFFECTS OF ANTIMICROBIAL AGENTS ON
VIABILITY OF LYOPHILIZED B. POPILLIAE

Experiment	Antimicrobial Agent	Cells/ml	% of Control Cells Recovered
1 ^a	Control	6.76×10^8 ^b	-
1	CNV ^c	4.02×10^8	59.5
1	RP ^c	3.17×10^8	46.9
2	Control	1.52×10^9	-
2	CNV	9.71×10^8	63.8
2	RP	5.97×10^8	39.3

^aall vials of an experiment were from the same lyophilization

^baverage of three vials

^cconcentration of CNV and RP was 0.5 ml/100 ml medium

- - -

for inhibiting contaminating organisms and allowing growth of B. popilliae.

Differences in effect of the agents upon vegetative and lyophilized cells were not appreciable. Therefore, it seems that the action of CNV and RP upon vegetative and lyophilized cells is quite similar. This relative similarity of the two physical states agrees with investigations using other methods for studying these states.

Effects of CNV and RP on Viability of
Lyophilized Cells in Soil

Further research, concerned with the use of lyophilized B. popilliae as a control agent will undoubtedly be involved with this organism's viability in soil. Since previous studies in this area have been hindered by contaminating organisms present in the soil, investigations were undertaken to determine if the incorporation of CNV and RP into the medium would inhibit these contaminants. Results indicated that contamination was reduced during routine viable counts of different soil samples.

Viability of the lyophilized cells present in soil and enumerated on a medium containing CNV and RP was also studied. Data from such studies are presented in Table 5. Again, viability was greater using CNV and viability of cells with RP was diminished. Reduced viability of the lyophilized cells inoculated into soils can be observed by comparing Tables 4 and 5. Viability of lyophilized cells on RP was approximately 43% (Table 4), but when plated from soil the viability was reduced to approximately 7% (Table 5). The reason for this agent affecting cells after they have been in soil is not understood.

TABLE 5. EFFECTS OF ANTIMICROBIAL AGENTS ON
VIABILITY OF LYOPHILIZED B. POPILLIAE
IN SOIL

Soil Sample ^a	Antimicrobial Agent	Cells/ml	% of Control Cells Recovered
4	Control	1.05×10^7	-
4	CNV ^b	7.16×10^6	68.2
4	RP ^c	9.44×10^5	9.0
6	Control	5.57×10^6	-
6	CNV	2.90×10^6	52.1
6	RP	2.60×10^5	4.7
8	Control	1.70×10^7	-
8	CNV	5.29×10^6	31.1
8	RP	1.17×10^6	6.9

^aall soil samples were of loam soil

^bconcentration of CNV was 0.75 ml/100 ml medium

^cconcentration of RP was 0.5 ml/100 ml medium

SUMMARY AND CONCLUSIONS

Since B. popilliae is being employed commercially for biological control of the Japanese beetle, the public health significance of data reported was of interest.

It was determined that this organism is lethal to mice if given in very large dosages. An LD_{50} value of 3.10×10^9 was calculated by the method of Reed and Muench. Deaths occurred rapidly, usually within 24 hr of injection. The possibility of such a rapid death being caused by a mechanical obstruction of some vital organ was investigated. Heat-killed cells were injected for this determination and it was discovered that infection was not produced upon injection of 2.0×10^{10} cells, which is almost ten-fold more organisms than the number of viable cells required to cause death. Therefore, it was concluded that death was probably due to a heat labile toxin.

Deaths occurred upon intraperitoneal injection but not upon subcutaneous injection of even ten-fold greater numbers of organisms. This indicates that pathogenicity of B. popilliae is dependent upon the route of injection. Lesions were apparent in all mice at the site of the injection.

Koch's postulates were proven by isolating organisms from mice that had succumbed to infection with B. popilliae and reinjecting these isolated organisms into susceptible mice. The organisms causing infection were isolated and grown

in pure culture by inoculating a medium containing selective antibiotics. Death was reproduced by injecting mice with the organisms in pure culture.

Lyophilized B. popilliae was slightly more infective for mice than were fresh broth cultured cells. The LD₅₀ of lyophilized cells was calculated to be 3.39×10^8 . The difference was of interest, because of the proposal to use lyophilized cells for the control agent.

Since B. subtilis is normally considered to be avirulent to warm-blooded animals, its LD₅₀ was obtained and compared with the LD₅₀ of B. popilliae. B. subtilis had an LD₅₀ of 4.23×10^8 which is almost ten-fold fewer organisms than required by B. popilliae to cause 50% mortality. Because of this relative avirulence and the extremely large numbers of organisms required for infection, there seems to be little danger to the public in the commercial uses of B. popilliae to control insect populations.

It was concluded that antimicrobial agents CNV and RP could be used to reduce contamination during routine growth of B. popilliae. Therefore, incorporation of these antibiotics into solid medium used for enumeration is suggested. In contrast, growth of B. subtilis was completely inhibited in the presence of these agents. It was also determined that inhibitory levels of CNV and RP for B. popilliae varied remarkably with the age of the inoculating culture.

Vegetative and lyophilized cells were very similar in their response to these antibiotics. Highest viability of

lyophilized B. popilliae was obtained using CNV. Viability of lyophilized cells present in soil was greatest when plated on medium containing CNV and was diminished in the presence of RP. Viability of lyophilized cells on RP was approximately 43%, but when plated from soil the viability was reduced to approximately 7%.

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THE PATHOGENICITY OF BACILLUS POPILLIAE FOR MICE. EFFECTS OF
CERTAIN ANTIMICROBIAL AGENTS ON VIABILITY
OF BACILLUS POPILLIAE.

by

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ABSTRACT

Pathogenicity for mice of the insect pathogen Bacillus popilliae was studied. It was determined that this organism is lethal to mice if given in very large dosages. The LD₅₀ of the vegetative cells was 3.10×10^9 . Isolation of B. popilliae from mice that had succumbed to infection and re-injecting these isolated organisms into susceptible mice resulted in death. Deaths did not occur upon subcutaneous injection of ten-fold greater numbers of organisms than the LD₅₀ dose for the intraperitoneal route and no symptoms of infection occurred upon injection of ten-fold more heat-killed cells than viable cells.

Lyophilized B. popilliae was slightly more lethal for mice than were fresh broth cultured cells. The LD₅₀ of lyophilized cells was calculated to be 3.39×10^8 .

The LD₅₀ of B. subtilis was determined to give a comparison of pathogenicity. Approximately ten-fold fewer organisms of B. subtilis than B. popilliae were required to cause 50% mortality.

The effects of antimicrobial mixtures, cholistimethate, nystatin, vancomycin (CNV) and ristocetin, polymyxin B (RP) on B. popilliae were investigated. The results suggest that these antibiotics might be useful in controlling contamination during culture of the fastidious milky disease bacteria.. Inhibitory levels of CNV and RP varied remarkably with the age

of the inoculating culture. In contrast, growth of B. subtilis was completely inhibited in the presence of these antimicrobial agents.