THE EFFECT OF VARIOUS LOW PRESSURES ON THE PER CENT LIVABILITY OF SERRATIA MARCESCENS AND MICROCOCCUS PYOGENES VAR. AUREUS DESICCATED FROM THE FROZEN STATE

Thesis for the Degree of M. S.

MICHIGAN STATE COLLEGE
Robert T. Christian

1952

This is to certify that the

thesis entitled

The effect of various low pressures on the percent liveability of Serratia Marcescens and Micrococcus Pyogenes var. aureous desiccated from the frozen state.

presented by

Robert T. Christian

has been accepted towards fulfillment
of the requirements for

M. S. degree in Bacteriology

W. J. Stafseth
Major professor

Date July 31, 1952

THE EFFECT OF VARIOUS LOW PRESSURES ON THE PER CENT LIVABILITY OF *SERRATIA MARCESCENS* AND *MICROCOCCUS PYOGENES* VAR. *AUREUS* DESICCATED FROM THE FROZEN STATE

By

ROBERT T. CHRISTIAN

A THESIS

Submitted to the School of Graduate Studies of Michigan State College of Agriculture and Applied Science in partial fulfillment of the requirements for the degree of

MASTER OF SCIENCE

Department of Bacteriology

1952

TABLE OF CONTENTS

To Joan

ACKNOWLEDGMENTS

The author wishes to thank Dr. J. J. Stockton, Assistant Professor of Bacteriology, for suggesting this problem and guiding it to its conclusion.

INTRODUCTION

The preservation of bacteria in their normal physiological and antigenic state over long periods of time has for years been a problem to the bacteriologist. A number of methods of preservation have been developed, many of which have shown some degree of promise. Most were discarded either because they were not satisfactory for the preservation of the more fastidious organisms or because of the low per cent viability of the preserved cultures. The most generally satisfactory process developed to date is the freeze-drying, or lyophilizing, process.

Freeze-drying, with certain variations, has been used for the preservation of bacteria since the beginning of the present century. In the past decade it has achieved prominence as a means of preserving various biological products, antibiotics, and blood fractions. However, there still remain many questions in the mind of the bacteriologist, a few of which are: How many of the organisms survive? Are there any changes in the biological characteristics of the organisms? What are the physical factors to be considered in lyophilization? Some of these

questions have been at least partially answered. The physical factors to be considered have been reviewed by Rogers (1914), and the per cent viability by Naylor and Smith (1946).

Various investigators have agreed that the best way to store lyophilized organisms is in a vacuum, but there have not been reported quantitative studies of the effect of low pressures on bacteria during storage. Most assuredly, the lower the pressure at the time of sealing, the more likely are the desiccated cultures to survive. Laboratory equipment available in most biological laboratories is not of the caliber to allow drying and sealing at pressures appreciably lower than about 50 microns of mercury. Other factors remaining constant, how does the per cent viability of a culture sealed at 50 microns of mercury pressure compare with that of a culture sealed at 100, 200, 500, or 1,000 microns of mercury pressure? What is the upper limit of sealing pressure at and above which no appreciable viability can be expected to remain during a reasonable storage period?

Two organisms were chosen in attempts to answer these two questions. They were Serratia marcescens and Micrococcus pyogenes var. aureus. The former was chosen since it is

relatively susceptible to the drying process (Naylor and Smith, 1949), and the latter because of its high resistance to unfavorable conditions, including drying.

HISTORICAL REVIEW

That clothes dry and ice slowly "wastes away" on a very cold day is an age-old observation. The knowledge of this phenomenon is old in science, too. It was old in 1813, when William Hyde Wollaston demonstrated freezing by evaporation and sublimation before the Royal Society of London. It is because of the simplicity and clarity with which he demonstrated the relationship between vapor pressure, temperature, and cooling by evaporation that his experiment is still cited today.

> That a fluid from which a portion is evaporated, becomes cooler in consequence of the heat absorbed by that part which assumes the gaseous state; that fluids rise in the state of vapors at lower temperatures when the pressure of the atmosphere is removed and consequently may be cooled to a lower degree by evaporation "in vacuo" than in open air are facts too well known to need confirmation before members of this society.

However, feeling that a new mode of demonstration of an established principle deserved attention, he described a little instrument for demonstrating this phenomenon.

> Let a glass tube be taken, having its internal diameter about 1/8 of an inch, with a ball at each extremity of about one inch in diameter and let the tube be bent to a

> right angle at a distance one-half inch from each ball. One of these balls should contain a little water (if the ball be more than half full, it will be liable to burst by the expansion of water in freezing) and the remaining cavity should be as perfect a vacuum as can be readily obtained . . . .
>
> When an instrument of this description has been successfully exhausted, if the ball that is empty be immersed in a freezing mixture of salt and snow, the other ball, though at the distance of two or three feet, will be frozen solid in the course of a very few minutes. The vapor contained in the empty ball is condensed by the common operation of cold and vacuum produced by this condensation gives opportunity for fresh quantity to arise from the opposite ball, with proportional reduction of its temperature.

Professor John Leslie used sulfuric acid to absorb the vapor under vacuum in an experiment that obtained to the same end.

These two early investigators demonstrated the principles of freezing by vacuum and sublimation, but to them it was merely the demonstration of a physical principle for which they suggested no practical application.

The modern process of freeze-drying is to a great extent the work of the biologists who needed a way to preserve labile biological products. With the progress of bacteriology and serology the development of a dependable method for the preservation of serums and bacterial cultures became imperative. A successful method for preserving serum was described by Martin, in 1896. He filtered the serum, which was kept at 40 degrees centigrade, through a Chamberland candle and

collected the water vapor in a cooled condenser connected to a vacuum pump. The stability of this dried serum was recognized by Erlich.

D'Arsonval and Bordas (1906) carried out low-temperature distillations (15° C.) in a similar manner. Their apparatus approached that now used for freeze-drying. With a better vacuum pump they could have frozen the serum and dried from the frozen state. Flosdorf (1949) stated that other workers, including Noguchi, Friedberg, Massol and Grysezi, Massol and Nowaczynski, Predtetschensky and Grigorowitsch, described similar methods for drying serum in vacuo. However, none of these workers dried from the frozen state.

During this time, other investigators showed that bacteria could be subjected to very low temperatures and still remain viable. Macfadyen (1900) froze bacteria in liquid air (-190° C.) and stored them in that medium for 24 hours without destroying their viability. He later reported that exposure to liquid hydrogen at a temperature of -252 degrees centigrade (21° absolute) did not kill all the cells.

Rogers (1914) stated that Paul and Pratt dried staphylococci on garnets and kept them in liquid air for one month. They found

no change in the reaction of the cells to disinfectants. Smith and Swingle (1905) observed that most cells of a bacterial culture survived freezing, and that the critical temperature was about 0 degrees centigrade. They felt that those organisms that could survive 0 degrees centigrade could survive absolute zero. Further experiments showed that repeated freezing and thawing slowly reduced the number of viable cells in a culture. It has since been shown that the death of the organism is due to the disruption of the cell by ice crystals. Quick freezing greatly reduces the size of the crystals formed, and therefore reduces the number of organisms killed.

The above evidence indicates that bacteria (and probably all biological materials) are not greatly harmed by freezing, that dried products are more stable than hydrated products, and that drying from the frozen state is possible.

It comes as no surprise, therefore, that Shackell (1909), while attempting to preserve glycogen in the liver, minced the tissue and dried it from the frozen state. After freezing the material in an ice-salt mixture, he placed it in a desiccator containing sulfuric acid as the desiccant, and dried the material in vacuo. Harris and Shackell (1911), using Shackell's method,

dried the brains of rabbits infected with rabies virus and found that the tissue retained its infectivity for 4 months. They believed that organisms were killed by slow drying because of the high concentration of salts produced.

Hammer (1911) was the first to use the freeze-dry method to preserve bacterial cultures. He placed strips of filter paper, impregnated with bacteria, in test tubes, froze them in an ice-salt mixture, and put the tubes in a desiccator. The desiccator, containing sulfuric acid as the desiccant, was then sealed and evacuated. After 48 hours, the desiccator, which had been kept in ice, was allowed to come to atmospheric pressure. The organisms thus preserved were viable for 57 days.

Shattock and Dudgeon (1912) dried films of bacteria on glass cover slips and stored them in the presence of charcoal at the temperature of liquid air (-190° C.). Although the results as to the viability of the cells at this temperature were inconclusive, they did determine the resistance of the dried cells to heat and to ultraviolet light. They found dried cultures to be more resistant to heat, although their resistance to ultraviolet light was unchanged.

Rogers (1914) found that the dried "starter cultures" used at that time in the dairy industry were very poor products. While investigating the methods used to produce these cultures, he found that most of the organisms were killed during the drying process. His first attempt to find a better method of preservation consisted in drying the organisms rapidly at a lowered temperature under reduced pressure. The drying was hastened by bubbling warmed air through the culture. This method, while very successful, was deemed too expensive for the average laboratory. He next investigated drying from the frozen state. After testing various desiccants, including calcium chloride, sulfuric acid, phosphorus pentoxide, and a cooled desiccating chamber, he found that calcium chloride was too slow and that the temperature he was able to obtain in the cooled chamber was insufficient to lower the vapor pressure to a practical level. He was most successful using a chemical desiccant. In reviewing the conditions necessary for the survival of cells in the dried state, Rogers stated: "The nearer the cell approaches an absolute dormant condition the longer its actual death will be postponed." The lower the metabolic activity of the organism the longer will be its survival. The conditions

which control this activity are the moisture content, the temperature at which the culture is held, and the nature of the gas with which it is in contact. If the moisture content is below 5 per cent, the activity of the cell will be very low, but lowering the moisture content below 5 per cent does not substantially increase the survival rate. Bacteria succumb to unfavorable conditions most rapidly at the temperature at which they grow most rapidly. Rogers observed that the loss of bacteria at 0 degrees centigrade was within the limit of experimental error, while at 17 degrees centigrade there was a 30 per cent loss, and at 30 degrees centigrade there was a 60 per cent loss. The results with various gases showed the greatest loss of activity to be in oxygen or in air, and the least loss of activity to be in a vacuum.

Swift (1921) introduced a method for freeze drying that remained the standard process for many years. The organisms were first concentrated, and most of the supernatant fluid decanted. The tubes were than placed in glycerol in a desiccator, the desiccator surrounded with an ice-salt solution, and the organisms frozen in the desiccator at -4 to -6 degrees centigrade. After the cultures were frozen, the desiccator was sealed and the apparatus placed in an ice box for 12 hours.

At the end of this period, the tubes were brought to atmospheric pressure, sealed with paraffin, and stored in the dark at room temperature.

Brown (1925) published two papers detailing three methods for the preservation of bacterial cultures. The first method consisted in simply drying the organisms on glass cover slips and storing them under vacuum. The second involved impregnating strips of filter paper with bacterial cultures and drying them in vacuo, after which the papers were sealed in tubes containing a small amount of calcium chloride. The third method was a modification to permit storage of larger numbers of the filter paper strips. Pint milk bottles fitted with ground-glass covers were substituted for the tubes. They were sealed by evacuating them in a desiccator, then allowing the atmospheric pressure to enter suddenly. This sealed the bottles by forcing the covers down on the bottles. Cultures preserved in this way were viable for two years.

Meanwhile, this process was being used for the preservation of other biologics. Sawyer, Lloyd, and Kileken (1929) preserved yellow-fever virus by freezing the blood of monkeys that had the disease. Blood was drawn on the first day of the attack

and frozen by Swift's (1921) method. The dried product was then sealed in tubes containing a small amount of calcium chloride.

Hartley, Eagleton, and O'Kell (1923) successfully dried serum and hemolysin under reduced pressure by passing a stream of warm air through the tubes. Complement, however, lost 70 per cent of its activity when treated this way. Craig's (1931) method of drying complement yielded a more satisfactory product. Fresh guinea pig serum was placed in a desiccator containing calcium chloride, and the chamber evacuated by means of a vacuum pump, until the serum froze. The desiccator was allowed to stand at room temperature for 6 hours, after which the dried complement was stored at low temperature in vacuo.

Elser, Thomas, and Steffen (1935) reported the investigations in freeze-drying begun by Elser in 1909. Using inactivated serum as a suspending medium, he had lyophilized cultures of such sensitive organisms as pneumococci and meningococci which, he reported, were viable after 15 years of storage in vacuo. These investigators developed the first practical cold condenser which was the basis of the commercial application of the freeze-drying process.

Flosdorf and Mudd (1935) reported work similar to that begun by Elser (1935). They described lyophilizing units of capacities suitable for all needs from those of a small laboratory to a semicommercial plant. Their publication contained definite procedures and drawings of the equipment. The apparatus consisted of a manifold which was continuous with the condensing chamber. The condensing chamber was kept at -78 degrees centigrade by immersing it in a dry ice-organic solvent bath. A suitable vacuum pump was connected to the condensing chamber. The maximum pressure allowable was 0.70 millimeter of mercury, but lyophilization should be carried out much below this, preferably at 0.010 to 0.050 millimeter of mercury. The material to be lyophilized was shell frozen at the temperature of dry ice. (The layer of serum should not be more than 3.0 millimeters thick.) The material was kept frozen by the latent heat of sublimation. This was the first paper on lyophilization by the man who was destined to become the authority in this field. Flosdorf, in his many papers and excellent book, has covered this field completely. Although he has contributed relatively little to the theoretical knowledge of lyophilization of bacteria, the tools he has given the bacteriologist are invaluable.

As so often happens when an adequate tool is placed in the hands of scientists, there was a great deal of work done in the next few years. Eagle, Strauss, and Stimer (1935) showed that complement could be dried by the method of Flosdorf and Mudd. They demonstrated that complement processed in this way and stored for 18 months in the ice box was indistinguishable from complement prepared from freshly drawn guinea pig blood. Rivers and Ward (1935) showed that vaccinia virus mixed with pure gum acacia and dried from the frozen state was suitable for intradermal injection in humans after storage for 1 month at 38 degrees centigrade. Flosdorf and Kimball (1940) reported that lyophilization preserved cultures of Hemophilus pertussis in Phase I for long periods.

Flosdorf and Mudd (1938) described the last major change in the freeze-drying process. Because dry ice is the most expensive ingredient in the procedure, they attempted to find a cheaper substitute for it. Anhydrous calcium sulfate was found to be most satisfactory, because of its low original cost and the fact that it could be regenerated. The process using this chemical desiccant is called "Cryochem." The cost of drying one liter of serum by the "Cryochem" method was between forty

and seventy-five cents. Drying a like amount of serum using the "lyophil" method cost between three and twenty dollars.

Morton and Pulaski (1938) reviewed the whole field of pure-culture preservation. They compared freeze-drying (Swift, 1921) to several other methods. They found that the freeze-drying process gave good results but was much more expensive in time and equipment than the other methods tested.

Leifson (1936) dried bacteria on perforated glass beads and on other substances. The dried cultures were stored in vacuo after the method of Brown (1925).

Swift (1937) reported several modifications of his original method. He now froze the organisms in a glycerol-dry ice mixture and dried them in a desiccator kept in an insulated box. He repeated his earlier procedure of sealing the organisms under atmospheric pressure with paraffin.

Rahn (1945) reviewed physical methods of sterilization and discussed many points that apply to this work. While discussing drying as a means of sterilization, he pointed out that although bacteria and viruses survive well in the dried state, there is a steady decrease in viability. At 37 degrees centigrade, death was logarithmic, and its cause was found to be

oxidation. Cultures of Vibrio comma slowly lost their viability when kept at warm temperatures in a high vacuum over phosphorus pentoxide.

While discussing death by freezing, Rahn (1945) pointed out that most organisms could not grow at lowered temperatures; in fact, they died slowly. Ninety-five per cent of the organisms of a young culture of Escherichia coli were killed by cooling them rapidly from 45 to 10 degrees centigrade; however, gradual cooling did not kill the organisms. Slow freezing formed ice crystals which punctured the plasma membrane of the bacterial cell, killing the organism. Very rapid freezing, on the other hand, produced a different picture. The water was changed to a glass-like amorphous mass. Rapid freezing and thawing two hundred times did not kill the tubercle bacillus.

Lord Stamp (1947) dried bacteria, suspended in gelatin, ascorbic acid, and other agents, on paraffin discs. He also lyophilized cultures by the method of Flosdorf (1935). He found that gelatin was a better suspending medium than blood, serum, mucin, or polyvinyl alcohol, under the conditions of his test. The addition of ascorbic acid increased the survival rate, but the addition of glutathione had no effect. He also tested the

relative merits of slow drying versus rapid drying. Slow drying on paraffin discs in the presence of phosphorus pentoxide under vacuum gave approximately three times the survival rate obtained with rapid drying by lyophilization. Viability under these conditions was reduced to one-half after 2 years of storage, and to one-quarter after 3 years. No change was found in chromogenicity or virulence during this period.

Naylor and Smith (1946) dried S. marcescens in various ways. They first dried the organisms, as did Stamp (1947), on paraffin discs in the presence of phosphorus pentoxide in vacuo at 4 degrees centigrade. After 24 hours, the vacuum was released and the discs stored at room temperature and atmospheric pressure in the presence of phosphorus pentoxide. Storing bacteria in this way, they reported, gave less than 0.1 per cent survival after 10 days of storage. If the cells were suspended in a 1.5 per cent ascorbic acid and 1.0 per cent mannite solution and dried as above 45 per cent survival was recorded after the same period. There was no further decrease in viability during a 5-month storage period.

They next investigated lyophilization as a means of preserving cultures. The cells were first concentrated, then

resuspended in a solution containing ascorbic acid, thiourea, ammonium chloride, and dextrin. The optimum concentration of these substances was found to be:

ascorbic acid	0.5 per cent
thiourea	0.5 per cent
ammonium chloride	0.5 per cent
dextrin	2.0 per cent

One milliliter of the culture was shell frozen in a 10-milliliter ampoule by spinning it rapidly in a dry ice-alcohol mixture.

Investigation of the effect of moisture on viability showed a decrease in viability as the moisture content of the cells was reduced from 2.1 per cent to 0.42 per cent.

The following data show their findings regarding the viability of cells stored in air, nitrogen, and vacuum:

air	9 per cent
nitrogen	28 per cent
vacuum	99 per cent

They stated that 100 per cent counts were obtained under optimum conditions of drying and storage.

Proom and Hemmons (1949) reported their experience in preserving between 1,500 and 2,000 strains of bacteria over a number of years. They used the method of Swift (1937), with a few modifications. The cultures were grown on agar slopes,

then emulsified in nutrient broth. The emulsion was put into small test tubes and frozen in a dry ice-acetone mixture in a desiccator containing phosphorus pentoxide. The desiccator was then evacuated. The following morning the tap of the desiccator was closed, the vacuum pump turned off, and the unit allowed to come to room temperature. Dry nitrogen from a cylinder was run slowly into the desiccator. The tubes were then removed and labeled. This completed the primary drying. For the secondary drying, the tubes were again placed in the desiccator and redried in a high vacuum at room temperature over phosphorus pentoxide for 7 days. Dry nitrogen was then run into the desiccator, and the tubes were removed and sealed.

The organisms tested for resistance to drying fell into three groups. Typical of the first group is M. pyogenes var. aureus, which is very resistant and remains viable for many years. E. coli is representative of the second group. Many of these cultures die after a few years of storage. An example of the third group is Neisseria intracellularis. These cultures begin to die after two years' storage.

In an attempt to simulate long-term storage conditions, the desiccates were heated to 60, 80, and 100 degrees centigrade

for 1 hour. There was evidence that the loss in viability ran parallel with the storage loss at lower temperatures. Although they realized that "it is always possible that heating to high temperatures introduces factors which would not operate at room temperatures . . . in practice it has proved a useful method of estimating probable storage loss."

Atkins, Moses, and Gray (1949) found that when yeasts were lyophilized, only very small numbers survived, and that variants were found in more than 50 per cent of the revitalized cultures.

Hutton, Hilmoe, and Roberts (1951) developed an elaborate apparatus for the control of many factors during the freeze-drying process. They were able to control the temperature of the ice film before and during drying, to vary the rate of drying, and to stop the process of drying at any stage. Using Brucella abortus as the test organism, they achieved a much higher percentage of viability if the temperature of the frozen culture was never allowed to rise above 30 degrees centigrade. They also showed that there is an optimum rate of sublimation for the greatest recovery of cells. Their results showed a

direct relationship between viability and the per cent of moisture remaining in the dried cultures.

The present status of freeze-drying is summed up by Harris (1951) in his paper reviewing the laboratory application of the process. He says, "There seem as yet to be no general rules for the successful preservation of all bacterial cultures, and if the culture alone cannot be dried there is an almost unlimited choice of protein-containing compounds and mixtures which may be tested for preservative action."

MATERIALS AND METHODS

Lyophilization of S. marcescens was carried out following the procedure outlined by Naylor and Smith (1946). The cells were grown in a liquid medium composed of 1.0 per cent Difco tryptone, 0.5 per cent Difco yeast extract, 0.1 per cent glucose, 0.2 per cent disodium phosphate, and 0.1 per cent sodium dihydrogen phosphate at a pH of 6.8 to 7.0. This medium, hereafter referred to as Medium I, was dispensed in 250-milliliter amounts in 500-milliliter Erlenmeyer flasks. Stock cultures were maintained on slants of the following composition: 1.0 per cent Difco tryptone, 0.5 per cent Difco yeast extract, 0.5 per cent glucose, and 1.5 per cent agar. Hereafter, this will be referred to as Medium II. To obtain good pigment production, stock cultures were incubated at room temperature.

Approximately two standard loopfuls of the stock culture were transferred to Medium I. According to Naylor and Smith (1946), aeration proved to be a very important factor in the production of resistant cells in this medium. For this reason,

the liquid culture was agitated continuously during incubation at 30 to 38 degrees centigrade. Agitation was accomplished by the use of a Burrell Shaker employing amplitudes of 16 millimeters and a frequency of 170 cycles per minute. After 18 to 24 hours of continuous shaking, the cells were concentrated by centrifugation, subjecting the culture to a relative centrifugal force of approximately 1,400 times gravity for 1 hour. The supernatant was then decanted, and approximately 3 milliliters of diluent, composed of 0.5 per cent ascorbic acid, 0.5 per cent thiourea, 0.5 per cent ammonium chloride, and 2.0 per cent dextrin, was added to the sediment.

The cells were resuspended, using a sterile wire loop, the material pooled, and about 20 milliliters of diluent added. These cultures were then shaken on the shaking machine for 60 minutes (amplitude 13.5 mm, frequency 150 cycles per minute), and then plated to determine the initial count.

Serum vials (Kimble, A3-179, 2 ml) were washed with distilled water, calibrated at 1 milliliter, plugged lightly with nonabsorbent cotton, and sterilized by dry heat for 2 hours at 160 degrees centigrade. One milliliter of the cell suspension was introduced into the vials with a 1-milliliter tuberculin

syringe. The material was shell frozen in a dry ice-alcohol bath at -70 to -78 degrees centigrade, revolving the vials so that two-thirds of the wall was covered with an even layer of the frozen cell suspension. The vials were stored in the freezing bath until they were placed on the manifold of the drying apparatus.

The lyophilizing apparatus consisted of a Cenco Megovac pump, to which was attached a sixteen-outlet manifold which held the vials by means of pressure tubing, and a condenser filled with Drierite (anhydrous calcium sulphate). A Stokes-McLeod pressure gauge was connected to the desiccating chamber. The system was evacuated to a pressure of 300 microns of mercury or less before the vials were attached to the system. The vials were exposed to existing room conditions during the process of sublimation, and no attempt was made to maintain controlled conditions of temperature and relative humidity. Lyophilization was allowed to proceed for 24 hours, then the vials were sealed with a cross-fire hand torch. Vials were sealed at pressures varying from 50 to 1,000 microns of mercury, as determined by the Stokes-McLeod gauge.

The lyophilized cultures were stored in the dark at room temperature for periods of from 1 week to 4 months. They were reconstituted by adding sterile saline (0.9 per cent) to the 1-milliliter mark. Dilutions were made and plated on Medium II. The plates were incubated at 30 degrees centigrade, and counts made after 24 to 48 hours.

The same general procedure was followed in lyophilizing cultures of M. pyogenes var. aureus. Stock cultures were maintained on Difco tryptose agar. Difco tryptose broth was inoculated with the organism and incubated without shaking for 48 hours at 35 degrees centigrade. The culture was concentrated by centrifugation, resuspended in sterile skim milk, and then tubed, frozen, dried, and sealed exactly as were cultures of S. marcescens. After approximately 3 months, the cultures were reconstituted, dilutions were made, and they were plated on Difco tryptose agar.

RESULTS

TABLE I

DATA PERTAINING TO EXPERIMENTS WITH S. MARCESCENS LYOPHILIZED FOR 24 HOURS AT APPROXIMATELY 100 MICRONS OF MERCURY

Date Sealed	Date Opened	No.	Original Count	Final Count	% Viability	Pressure in Microns of Mercury
2-26	3-5	1	127×10^8	265×10^6	2.0200	60
2-26	5-13	2	127×10^8	53×10^7	4.3300	60
2-26	5-13	5	127×10^8	102×10^6	0.7850	135
2-26	3-5	6	127×10^8	210×10^6	1.5800	135
2-26	3-5	8	127×10^8	55×10^7	4.3600	190
2-26	5-13	9	127×10^8	122×10^6	0.9610	400
2-26	3-5	10	127×10^8	100×10^5	0.0788	400
2-26	3-5	12	127×10^8	113×10^5	0.0890	550
2-26	3-5	13	127×10^8	470×10^6	0.3700	675

TABLE II

DATA PERTAINING TO EXPERIMENTS WITH PYOG. VAR. AUREUS LYOPHILIZED FOR 24 HOURS AT APPROXIMATELY 100 MICRONS OF MERCURY

Date Sealed	Date Opened	No.	Original Count	Final Count	% Viability	Pressure in Microns of Mercury
4-11	6-3	1	47×10^7	34×10^6	7.230	100
4-11	6-3	3	47×10^7	248×10^5	5.280	175
4-11	6-3	5	47×10^7	197×10^5	4.190	275
4-11	6-3	7	47×10^7	210×10^5	5.010	475
4-11	6-3	9	47×10^7	204×10^5	4.340	400
4-11	6-3	11	47×10^7	45×10^5	0.957	800
4-11	6-3	15	47×10^7	31×10^6	6.000	1100
4-11	6-5	2	47×10^7	284×10^5	6.040	100
4-11	6-5	4	47×10^7	58×10^6	13.000	175
4-11	6-5	6	47×10^7	97×10^5	2.060	275
4-11	6-5	8	47×10^7	125×10^5	2.660	475
4-11	6-5	13	47×10^7	42×10^5	0.892	700
4-11	6-5	16	47×10^7	266×10^4	0.566	1100
4-18	6-10	1	78×10^7	46×10^7	59.000	70

TABLE II (Continued)

Date Sealed	Date Opened	No.	Original Count	Final Count	% Viability	Pressure in Microns of Mercury
4-18	6-10	3	78×10^7	45×10^7	57.700	150
4-18	6-10	5	78×10^7	255×10^6	32.700	275
4-18	6-10	7	78×10^7	195×10^6	25.000	350
4-18	6-10	9	78×10^7	172×10^6	22.000	410
4-18	6-10	11	78×10^7	140×10^6	17.800	500
4-18	6-12	2	78×10^7	34×10^7	43.600	70
4-18	6-12	6	78×10^7	82×10^7	105.000	275
4-18	6-12	8	78×10^7	37×10^7	47.400	350
4-18	6-12	12	78×10^7	34×10^7	43.600	500
4-18	6-12	13	78×10^7	62×10^5	1.260	700
4-18	6-12	14	78×10^7	261×10^6	25.800	700
4-22	6-13	1	32×10^6	30×10^5	9.380	65
4-22	6-13	2	32×10^6	66×10^5	20.600	65
4-22	6-13	4	32×10^6	224×10^4	7.010	100
4-22	6-13	5	32×10^6	200×10^4	6.250	275
4-22	6-13	6	32×10^6	219×10^4	6.840	275

TABLE II (Continued)

Date Sealed	Date Opened	No.	Original Count	Final Count	% Viability	Pressure in Microns of Mercury
4-22	6-13	7	32×10^6	33×10^5	10.300	275
4-22	6-13	8	32×10^6	130×10^5	40.600	275
4-22	6-13	9	32×10^6	69×10^4	2.500	425
4-22	6-13	10	32×10^6	49×10^4	1.530	425
4-22	6-13	11	32×10^6	87×10^4	2.750	450
4-22	6-14	12	32×10^6	89×10^4	2.780	450
4-22	6-14	13	32×10^6	45×10^4	1.410	750
4-22	6-14	14	32×10^6	151×10^4	4.720	750
4-22	6-14	15	32×10^6	114×10^4	3.560	950

Figure 1. Plot of S. marcescens showing per cent viable cells as per cent determined by plate counts when sealed and stored at the indicated pressure.

FIGURE 1

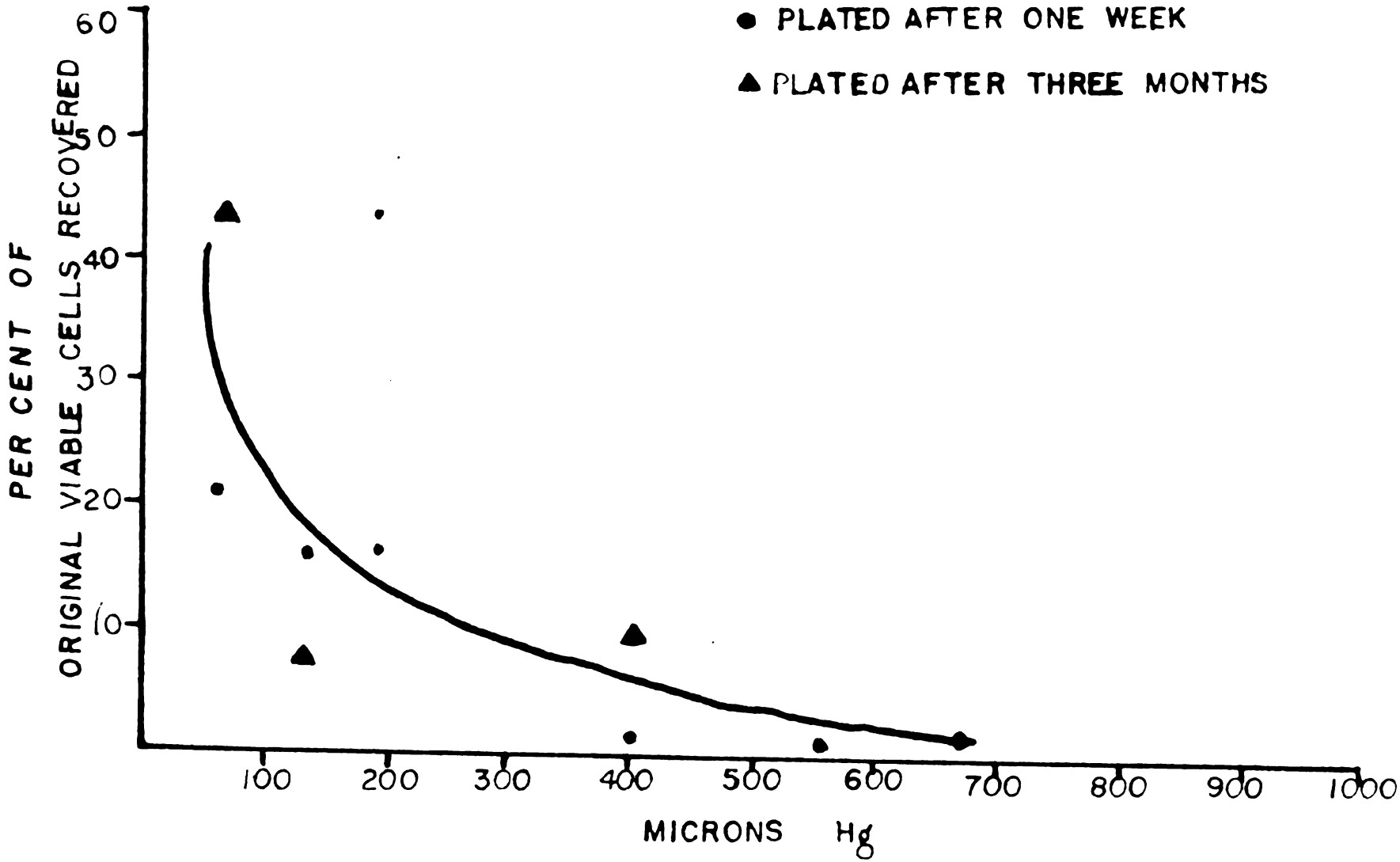

● PLATED AFTER ONE WEEK
▲ PLATED AFTER THREE MONTHS
PER CENT OF ORIGINAL VIABLE CELLS RECOVERED
60
50
40
30
20
10
100
200
300
400
500
600
700
800
900
1000
MICRONS Hg

Figure 2. Plot of Micro. pyo. var. aureus showing per cent viable cells as per cent determined by plate counts when sealed and stored at the indicated pressure.

FIGURE 2

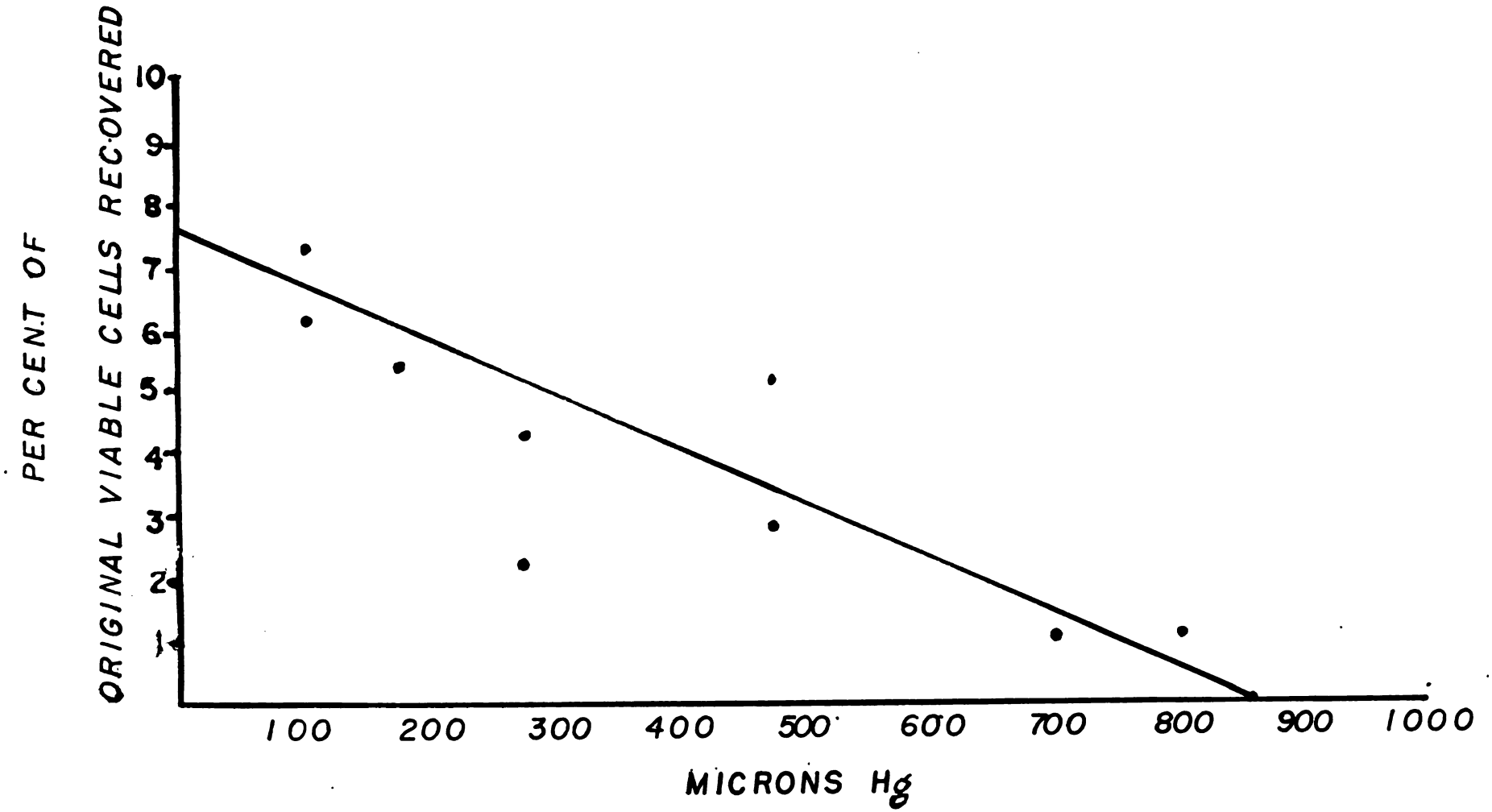

PER CENT OF
ORIGINAL VIABLE CELLS RECOVERED
10
9
8
7
6
5
4
3
2
1
100
200
300
400
500
600
700
800
900
1000
MICRONS Hg

Figure 3. Plot of Micro. pyo. var. aureus showing per cent viable cells as per cent determined by plate counts when sealed and stored at the indicated pressure.

FIGURE 3

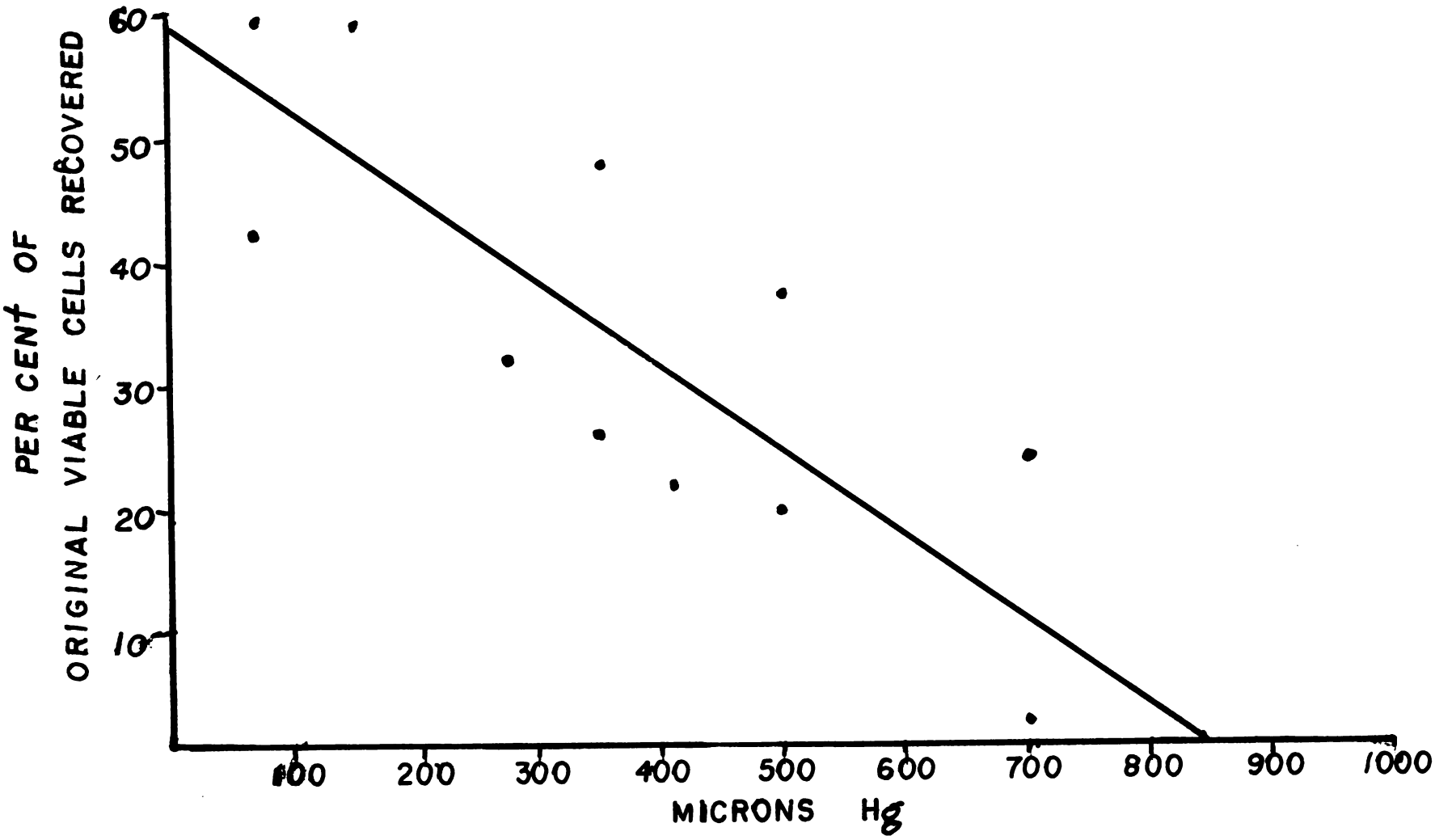
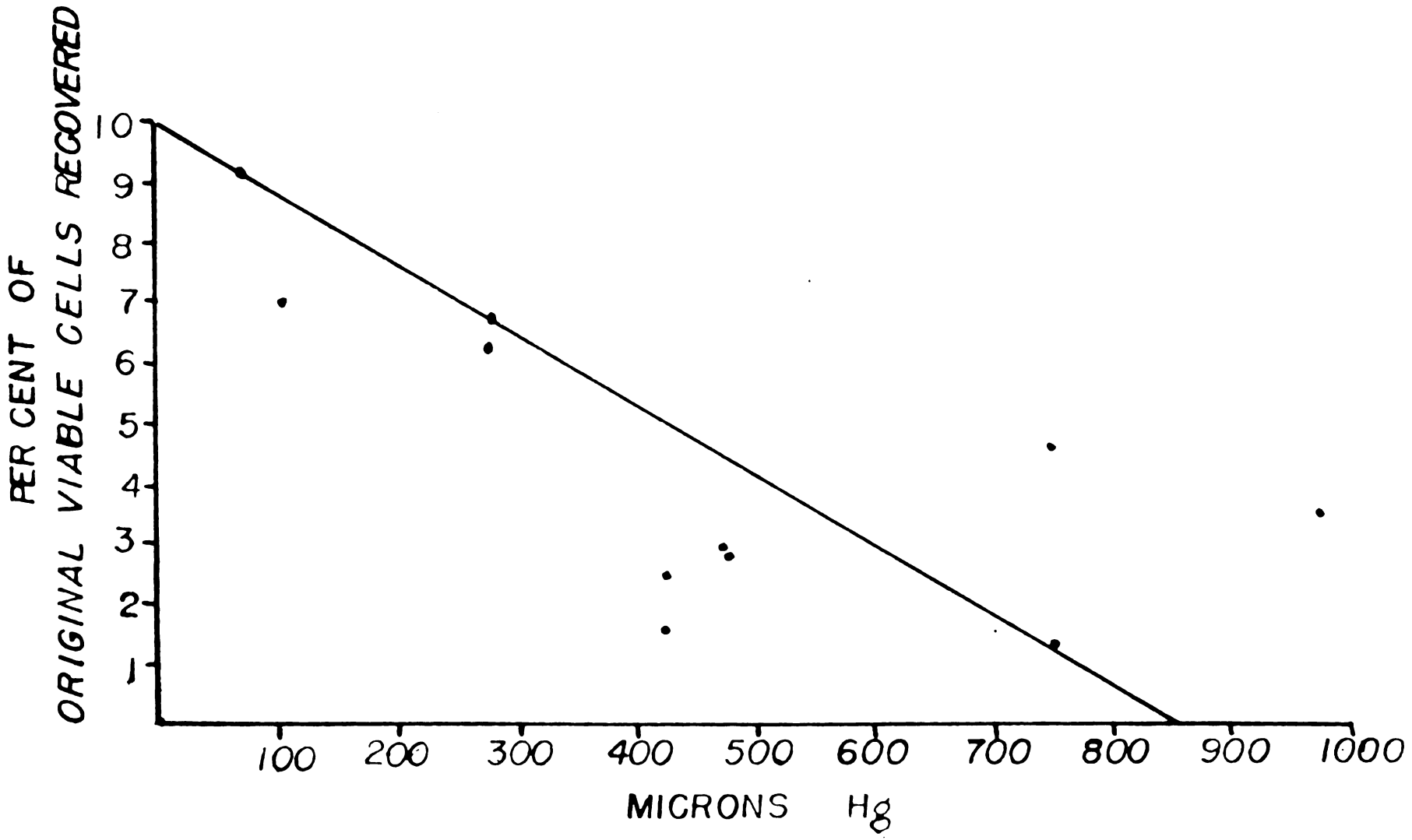

PER CENT OF ORIGINAL VIABLE CELLS RECOVERED
60
50
40
30
20
10
100
200
300
400
500
600
700
800
900
1000
MICRONS Hg

Figure 4. Plot of Micro. pyo. var. aureus showing per cent viable cells as per cent determined by plate counts when sealed and stored at the indicated pressure.

FIGURE 4


PER CENT OF
ORIGINAL VIABLE CELLS RECOVERED
10
9
8
7
6
5
4
3
2
1
100
200
300
400
500
600
700
800
900
1000
MICRONS Hg

DISCUSSION

The above data show a definite correlation between the pressure under which a dried culture is stored and the per cent survival of the organisms. This relationship is more evident with M. pyogenes var. aureus than with S. marcescens, probably because a larger number of cultures of M. pyogenes were plated at the same time, and the methods had been better developed. Even considering this, S. marcescens showed the same pressure-survival relationship.

The theoretical pressure above which M. pyogenes could not be stored is approximately 835 microns of mercury, a figure obtained by extrapolating to the abcissa (Figures 2, 3, 4). The experimental values are very close to this value. It is evident that some organisms survived the theoretically maximum pressure, but the per cent is very low. This is not surprising, since Swift (1921) and Hammer (1911) preserved organisms by drying and storing them at atmospheric pressure and room temperature. The fact that the data from three experiments fall in a relatively narrow range gives support to these conclusions.

During the early experiments using S. marcescens, more than 100 per cent of the organisms were recovered on plating. Proom and Hemmons (1949) reported a similar condition, and concluded that it was due to a more explicit disaggregation of the clumps of bacteria. In these experiments, the concentrated culture was thoroughly shaken to break up the clumps before plating. Thus, there were no clumps to be broken up during freezing, and the viability was consistently below 100 per cent.

The data in Figure 1 suggest that a higher per cent recovery could have been obtained if the pressure under which the tubes were sealed had been lower. It seems that if a pressure of 1 or 2 microns of mercury could have been obtained, as reported by Naylor and Smith (1946), viability would have been 100 per cent. It is unfortunate that pressures below 50 microns of mercury could not be obtained on the apparatus used.

Figure 1 shows an attempt to determine the effect of storage time on the viability of S. marcescens. Cultures were plated after 1 week and after 3 months of storage, and the results were plotted on the same graph. There seems to be little, if any, difference in the number of organisms surviving.

SUMMARY

A correlation was found to exist between the pressure under which a culture was stored and the per cent viability of lyophilized cultures of S. marcescens and M. pyo. var. aureus. Eight hundred thirty-five microns of mercury was found to be the highest pressure under which M. pyo. var. aureus could be stored, under the conditions of drying as described herein.

The fact that there was little batch-to-batch reproducibility is not of great importance. It must be remembered that only the pressure was controlled, and that such factors as rate of sublimation and per cent of residual moisture, reported as being of great importance by Hutton et al. (1951), were variable.

REFERENCES

ATKIN, L., MOSES, W., and GRAY, P. P. 1949. The preservation of yeast cultures by lyophilization. J. of Bact., 57(6), 575-578.

BROWN, J. H. 1925. The preservation of bacteria in vacuo. Abstracts of Bacteriology, IX, 8.

BROWN, J. H. 1926. Vacuum tubes for the storage and shipment of bacteria. Science, 46, 429-430.

D'ARSONVAL and BORDAS, F. "Les basses temperatures et l'analyse chimique," Compt. rend. acad. sci., 142, 1058-1059 (1906).

EAGLE, H., STRAUSS, H., and STEINER, R. 1935. The use in the Wasserman fraction of a uniform and stable dehydrated complement. Amer. J. Clin. Path., 5, 173-179.

ELSER, E., THOMAS, R. A., and STEFFEN, G. L. 1935. The desiccation of sera and other biological products (including microorganisms) in the frozen state with preservation of the original qualities of products so treated. J. Immunol., 28, 433-473.

FLOSDORF, E. W. 1949. Freeze-drying. Reinhold Publishing Co., New York.

FLOSDORF, E. W., HULL, L. W., and MUDD, S. 1945. Drying by sublimation. J. of Immunol., 50, 21-45.

FLOSDORF, E. W., and KIMBALL, A. C. 1940. Studies with H. pertussis. II. Maintainance of cultures in Phase I. J. Bacti., 39, 255-261.

FLOSDORF, E. W., and MUDD, S. 1935. Procedure and apparatus for preservation in "Lyophile" from serum and other biological substances. J. Immunol., 29, 389-425.

FLOSDORF, E. W., and MUDD, S. 1938. An improved procedure and apparatus for preservation of sera, microorganisms and other substances: the Cryochem process. J. Immunol., 34, 469-490.

HAMMER, B. W. 1911. A note on the vacuum desiccation of bacteria. J. of Med. Res., 24, 527-530.

HARRIS, R. J. C. 1951. A review of laboratory freeze-drying with particular reference to viruses. Vacuum, 1, 11-22.

HARRIS, D. L., and SHACKELL. 1911. The effect of vacuum desiccation upon the virus of rabies with remarks upon a new method. J. of the Am. Pub. Health Assn.

HARTLEY, P., EAGLETON, A. J., and O'KELL, C. C. 1923. The desiccation of serum and other protein solutions with special reference to the reagents used in the Wasserman reaction. J. Path. and Bact., 26, 53-65.

HUTTON, R. S., HELMOE, R. J., and ROBERTS, J. L. 1951. Some physical factors that influence the survival of Brucella abortus during freeze-drying. J. of Bact., 61, 309-319.

LEIFSON, E. 1936. The preservation of bacteria by drying in vacuo. Amer. J. Hyg., 32(2), 231-236.

MACFADYEN, A. 1899. On the influence of the temperature of liquid air on bacteria. A. Proc. Roy Soc. London, LXVI, 180-182.

MACFADYEN, A., and ROWLAND, S. 1900. Influence of the temperature of liquid hydrogen on bacteria. A. Proc. Roy. Soc. London, LXVI, 488-489.

MORTON, H. E., and PULASKI. 1938. The preservation of bacterial cultures. J. of Bact., 35(2), 163-183.

NAYLOR, H. B., and SMITH, P. A. 1946. Factors affecting the viability of Serratia marcescens during dehydration and storage. J. Bact., 52, 565-573.

PROOM, H., and HEMMONS. 1949. The drying and preservation of bacterial cultures. J. Microbiol., 3, 7-18.

RAHN, O. 1945. Physical methods of sterilization of microorganisms. Bact. Rev., 9(1), 1-47.

RIVERS, T. M., and WARD, S. M. 1935. Further observations on the cultivation of vaccine virus for Jennerian prophylaxis in man. J. Exp. Med., 58(5), 635-648.

ROGERS, L. A. 1914. The preservation of dried cultures. J. Infectious Dis., 14, 100-123.

SAWYER, W. A., LLOYD, W. D. M., and KITCHEN, S. F. 1929. The preservation of yellow fever virus. J. Exptl. Med., 50, 1-13.

SHACKELL, L. F. 1909. An improved method of desiccation with some applications to biological problems. Am. J. Physiol., 24, 325-340.

SHATTOCK, S. G., and DUDGEON, L. S. 1912. Certain results of drying non-sporing bacteria in charcoal liquid air vacuum. Proc. Roy. Soc. London, Series B., LXXXV, 127-138.

SMITH, F., and SWINGLE, B. 1905. The effect of freezing on bacteria. Science, ns 21, 481-482.

STAMP, T. C. (LORD). 1947. The preservation of bacteria by drying. J. Gen. Microbiol., 1, 251-265.

SWIFT, H. F. 1921. Preservation of stock cultures of bacteria by freezing and drying. J. Exper. Med., 33, 69-75.

SWIFT, H. F. 1937. A simple method for preserving bacterial cultures by freezing and drying. J. Bact., 33(4), 411-421.