# PRESERVATION OF STOCK CULTURES OF BACTERIA BY FREEZING AND DRYING.*

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The object of this communication is to present the results of the prolonged preservation of stock cultures of bacteria after freezing and drying, and to describe the method used.

The benefit of a method that will assure the preservation of cultures of bacteria without frequent manipulation is self-evident. As type organisms are isolated or identified it is desirable to keep the original state of virulence or to retain their cultural characteristics. In addition to the danger of contamination from frequent manipulation, there is the well known tendency for many bacteria to lose their virulence or other biological characteristics from repeated transfers on artificial media. During the course of many investigations it is often of advantage to keep all the bacteria isolated until there is time for more detailed study. A method that will permit the shipping of cultures from one city or country to another without the bulk of culture media, or without danger of breakage or death of the bacteria, has obvious advantages.

The principle of keeping bacteria in a dormant state by desiccation after freezing has been known for several years. Shackell (1) was one of the first to call attention to its advantages. He showed, first, that after freezing, substances such as tissue could be uniformly dried; second, that serum retained its complement and antibody activity unaltered for weeks; and third, that rabies virus in rabbit brains did not lose its virulence when kept in this manner. Hammer (2) dried bacteria previously frozen on strips of filter paper, and kept them from 54 to 57 days. Controls, dried without freezing, were killed immediately. Shattock and Dudgeon (3) showed that organisms dried on charcoal without freezing were usually killed in from 4 to 40 days. *B. pyocyaneus*, however, lived for at least 7

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months. Macfadyen (4) and Macfadyen and Rowland (5) demonstrated that practically all bacteria as well as yeast could be frozen and reduced to a temperature of liquid hydrogen, *i.e* −252°C., and later recovered in a viable and unchanged condition. Rogers (6) finally applied the principle of freezing and drying on a large scale so that mass cultures of lactic acid-forming bacilli could be preserved for commercial purposes. He suggested the use of this method for the preservation of stock cultures for the laboratory.

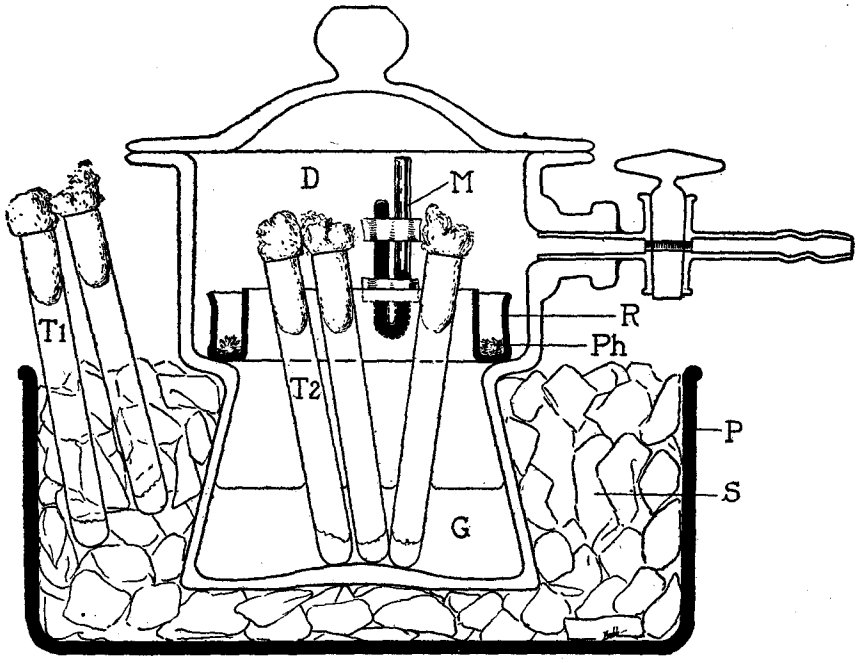
For the past 6 years we have been using this method in the preservation of stock cultures of streptococci and pneumococci. Recently upon attempting to recover these bacteria it was found that if they were originally prepared under proper conditions they were viable after a period of from 2 to 4 years. Comparison of the tubes from which the organisms could not be recovered, with those from which they were easily recovered, showed that the physical state of the dried material had much to do with the viability of the organisms. For example, of twenty-two tubes in which it was possible to recover bacteria, nineteen were in a condition of "dry foam" and three in a condition of "flaky gum." Among eleven with which failure occurred, nine were distinctly gummy, one showed a "flaky gum," and one a "dried foam." This physical state with the accompanying success or failure was present immediately after the organisms were dried or after 4 years. The results showed that it was necessary to maintain the frozen state until drying was complete; this led to the devising of the present method in which the tubes are immersed in glycerol which acts as a medium for the conduction of cold from a salt-ice mixture.

## *Method.*

*Apparatus* (*Text-Fig. 1*).—A desiccator (*D*) is chosen with broad opposing surfaces on cover and stop-cock. It is usually necessary to regrind all opposing glass surfaces. On the inner side of the desiccator is fastened with adhesive plaster a small mercury manometer (*M*) with one open and one closed end. In the upper part of the desiccator is a receptacle (*R*) for the desiccating salt; if phosphorus pentoxide (*Ph*) is used this container can be of copper. In the bottom of the desiccator is placed about 4 cm. of commercial glycerol (*G*). A pan (*P*) is provided, large enough to hold the desiccator and

a salt-ice mixture (*S*) which is packed around the latter. It is necessary to have a mechanical pump that will give a vacuum as low as 2 or 3 mm. of mercury; preliminary exhaustion of the larger part of the air can be conveniently carried out with the ordinary water pump.

*Preparation of Cultures.*—The object to be attained is a maximum number of young actively growing forms of the bacteria in a minimum




TEXT-FIG. 1. Diagrammatic cross-section of the apparatus used for freezing and drying bacterial cultures for preservation. *D*, desiccator; *M*, mercury manometer; *R*, copper receptacle for the phosphorus pentoxide (*Ph*); *G*, glycerol in the bottom of the desiccator; *P*, pan for the salt-ice mixture (*S*); *T 1*, position of a tube of culture during freezing; *T 2*, position of a tube of culture during desiccation.

of fluid. It is necessary to have enough fluid to form a small amount of ice. If fluid media are used it is well to grow the organisms for 15 to 18 hours, centrifugalize the culture, and discard most of the supernatant broth. The concentrated bacteria can then be pipetted into a number of small tubes and frozen. With certain bacteria it

is necessary to use the growth from solid media. In this case the growth should be scraped off into a small amount of condensation water or of broth that has been added, and the suspension subsequently pipetted into tubes. We have found it convenient to use small tubes, about 10 cm. long and from 5 to 10 mm. in diameter.

*Manipulation.*—After the culture has been placed in the small tubes as above described the desiccator containing the glycerol is set in the pan containing the salt and ice mixture; the tubes as well are immersed in this mixture and the cultures frozen (position *T 1*, Text-fig. 1). Simultaneously the temperature of the glycerol is reduced to minus 4–6°C. After careful lubrication of the desiccator cover and stop-cock and after the glycerol has reached a proper temperature and the bacterial suspension is well frozen, the tubes are immersed in the glycerol (position *T 2*), the phosphorus pentoxide is put in the copper pan, and the cover placed securely on the desiccator. The air is then exhausted from the desiccator, first with an ordinary water pump and finally with the high vacuum pump, during all this time the desiccator being kept in the salt-ice mixture. When the proper degree of vacuum has been obtained the whole apparatus is placed in the ice box where it is left until desiccation is complete.[1] This time, in our experience, is usually about 12 hours. When it is certain that desiccation is complete the tubes are removed from the desiccator, the cotton stoppers pushed down into them, and melted paraffin is poured in until the tubes are thoroughly sealed. It is usually necessary to paraffin the tubes two or three times in order to insure complete sealing. If it is desired to keep the organisms for many years the tubes may be sealed by melting and fusing the open ends of the tubes. After the tubes have been well labelled, they can be kept at room temperature, preferably in the dark.

The appearance of the properly dried culture is that of a very light, spongy, flaky material. If it is separated from the sides of the tube it can be shaken about and looks like dried lather made from shaving soap.

*Recovery of Organisms.*—The organisms may be recovered in one of several ways. For such bacteria as the cocci, and those of the

[1] If a frigo ice box is available it is advisable to place the desiccator at a temperature below zero.

colon, typhoid, and dysentery group, as well as other organisms that grow easily in broth, after the removal of the stoppers, broth may be added directly to the dried powder and the tubes incubated. Organisms like meningococci or others that grow better on solid media are best recovered by picking up a small amount of the dried material on a platinum loop and smearing it over the surface of suitable media, after it has been moistened in the water of condensation.

Results obtained by this method with various types of organisms are given below.

*Streptococci and Pneumococci.*—Out of fifteen strains of non-hemolytic streptococci kept from 22 to 40 months, all except two showed growth like that of the original culture, with similar fermentation reactions. These two, however, showed similar types of fermentation reactions with all the subcultures recovered; it is therefore probable that the change in fermentation occurred before the organisms were frozen. In the strains which originally showed a moderate degree of virulence, the virulence was maintained after the recovery of the organism. Four strains of hemolytic streptococci have been recovered after a period of from 42 to 51 months. Three strains of pneumococci were preserved in this manner and upon recovery several months later had retained their original virulence and reaction to type serum. One of the strains that had been accustomed to grow on 75 per cent bile retained this property in the subcultures.

*Meningococci.*—It has been found best to grow meningococci on solid media, scrape off the growth in a small amount of water of condensation, and freeze the resulting suspension. An experiment was performed to determine how small an amount of meningococci might be preserved in this manner; it was found that one loopful suspended in 0.2 cc. of broth or condensation water could be kept for a period of at least 2 months following desiccation. No tests were made after longer intervals. In the recovery of meningococci it has been found much more satisfactory to smear the dried powder upon the surface of freshly prepared blood agar or dextrose serum agar; the addition of blood dextrose broth to the dried powder has yielded more uncertain results. *Bacillus influenzæ* is best treated in the same manner as meningococci.

*Other Organisms.*—Typhoid, paratyphoid, and dysentery bacilli have been frozen following growth either in broth or on solid media. They were easily recovered by adding broth to the dried culture.

Other organisms of the bacteria group have not been tested because it is felt that those mentioned above represent the various types. There are few bacteria more delicate and more difficult to maintain in stock cultures than the meningococci and *Bacillus influenzæ*. Attempts have been made to preserve the spirochete of relapsing fever recovered from the blood of rats inoculated with these organisms, but the freezing seems to be sufficient to kill completely all the organisms. It is probable that spirochetes in general are not susceptible to preservation in this form, as a number of different strains have been shown to succumb to freezing. No work has been carried out by us with filterable viruses, but Harris and Shackell (7) have shown that the virulence of rabies virus is retained in this manner. Rous[2] has been able to preserve the virus of chicken sarcoma by a similar method for 7 years.

## SUMMARY.

Attention is called to the fact that bacteria may be preserved for a long time by desiccation in the frozen state. It has been shown that it is necessary to maintain the frozen condition until desiccation is complete; if the fluid melts before the moisture is completely removed, the organisms are killed, probably because of the concentration of the salts upon the surface of the bacteria. By the simple expedient of immersing the tubes of organisms in glycerol contained in a desiccator and subsequently keeping the whole apparatus in a salt-ice mixture until drying is complete, the organisms are easily maintained in the frozen state, and dry properly. Bacteria preserved in this manner retain their cultural, biochemical, and immunological characters for prolonged periods.

[2] Rous, P., personal communication.

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