STUDIES WITH H. PERTUSSIS

II. MAINTENANCE OF CULTURES IN PHASE I

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Shackell’s (1909) method of desiccation from the frozen state was applied first by Hammer (1911) to the preservation of viable bacteria. Rogers (1914), with an improved technique, extended this work on a larger scale to lactic-acid-forming bacilli. Swift (1921) applied the same principle of desiccation in the maintenance of streptococci and pneumococci in the original state of virulence, and similarly Elser, Thomas and Steffen (1935) were able to maintain a wide range of organisms including the meningococcus and gonococcus, in viable state, over a period of many years.

More recently, by use of the Lyophile apparatus (Flosdorf and Mudd, 1935), Siler and his associates (1936) at the Army Medical School have maintained their cultures of S-58 virulent Eberthella typhosa without dissociation over a period of years. Freshly-opened containers of Lyophile organisms are used routinely in preparation of mass cultures for vaccine preparation. Similarly, Welch, Borman, and Mickle (1939) have used the Lyophile apparatus to maintain cultures of Klebsiella pneumoniae in unaltered form.

In the case of Hemophilus pertussis, workers generally rely on fresh isolations to insure that their cultures are in Phase I (Leslie and Gardner’s terminology, 1931). This is the practice recommended for preparation of vaccines by the Referee on Methods and Reagents for the Diagnosis and Control of Whooping Cough

1 This work has been aided by a grant from the United States Public Health Service.
for the Standard Methods Committee on Diagnostic Procedures and Reagents (American Public Health Association Year Book, 1935–1936). In the present report we are able to demonstrate that cultures of *H. pertussis* may be preserved in Phase I for extended periods by desiccation from the frozen state. By this means several advantages may be gained. In systematic studies the same strains may be used over a period of years. Although *H. pertussis* has been generally considered to be of a single type, the recent reports of a Para form (Eldering and Kendrick, 1937, 1938), or an atypical type (Bradford and Slavin, 1937), indicate that such differences must be guarded against. Similarly, differences in hemolytic activity are found. Furthermore, continual isolation is laborious and at times a sufficient number of cases of whooping cough in the proper stage for isolation of the organisms are difficult to find.

**METHOD**

We have used the Cryochem apparatus for carrying out desiccation from the frozen state (Flosdorf and Mudd, 1938). Properly identified cultures isolated from a cough plate are transferred to a test tube slant of Bordet-Gengou medium containing fresh defibrinated blood in appropriate amount. These are harvested by scraping into saline or milk. If in saline, the suspension is added to an equal volume of sterile skimmed milk. This mixture is then distributed in 0.05 ml. amounts into small vials not carrying rubber stoppers and dried according to a procedure previously described (Flosdorf and Mudd, 1935, 1938). With these small volumes, it is advisable to freeze the suspension of bacteria in milk by placing a freezing mixture around the containers after attachment to the manifold of the Cryochem apparatus. By using larger containers, with 0.5 ml. or more of material in each, the cryochemic degassing and self-freezing procedure may be used. In such case, it is not necessary to place a greater number of organisms in the containers, but only to use more milk in order to provide more liquid for evaporation during self-freezing. The Lyophile apparatus likewise may be used; the advantages of the Cryochem procedure lie mostly in economy
and convenience of operation. Similarly, large glass vacuum desiccators may be used for the Cryochem procedure with the containers attached by glass tubes passing through a rubber stopper in the lid (Flosdorf, Boerner, Lukens and Ambler, 1940). However, the limited capacity and the inconvenience and danger of such apparatus makes questionable the advisability of its use routinely, particularly on a large scale.

The desiccation is allowed to continue overnight and then the containers are hermetically sealed by fusion of the glass with a flame. Although we have always evacuated the containers prior to sealing, this may be unnecessary. In the case of guinea-pig complement, hermetically sealing the product in a container filled with air has proven to be quite satisfactory (Flosdorf, Boerner, Lukens and Ambler, 1940). Furthermore, the work of Swift (1921) would indicate that air in contact with at least certain organisms yields satisfactory results. With certain types of equipment, however, sealing in vacuo makes it simpler to maintain sterility.

The sealed containers have been stored at 5° to 8°C. When a fresh culture is desired, a container of the desiccated organisms is opened. After scratching the glass with a file, the container, for breaking, is wrapped under a cloth impregnated with antiseptic to prevent the spreading of dry organisms as air rushes in. Excess antiseptic should be squeezed out of the cloth, however, to avoid sucking the liquid into the container when the vacuum is broken. Sterile water is then added. With a small loop, Bordet-Gengou medium in a petri dish is plated in the usual fashion. In this first culture generation 72 to 96 hours may be required to obtain satisfactory colonies. Colonies may then be picked from the plate and transfers made in the usual fashion.

**TESTING FOR PHASE I CHARACTERISTICS**

The cultures have been found to have all the original characteristics of Phase I with respect to colony form, hemolytic activity, morphology, toxicity, and agglutination. We have tested these properties of cultures grown from organisms maintained in the Cryochem-dried form for over two years. The serological
properties have been tested by using the organisms cultured from Cryochem-preparations to absorb antibodies from serum of rabbits immunized with freshly isolated organisms and then testing the absorbed serum in agglutination with freshly isolated strains as the test antigen. For absorption of agglutinins, 7 ml. of a 1:10 dilution of the Phase I immune serum were incubated 30 minutes at 37°C. and overnight at 6–8° with 1.0 ml. of packed organisms suspended in 2 ml. saline. In Table 1 are recorded the results of a typical experiment. As a control experiment, the serum was treated identically as in absorption to determine the

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<th>ABSORBING ANTIGEN</th>
<th>TEST ANTIGEN</th>
<th>AGGLUTINATION TEST—FINAL SERUM DILUTIONS</th>
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<td>1:100</td>
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<td>&quot;Cryochem Phase I&quot;</td>
<td>Freshly isolated Phase I</td>
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<td>&quot;Cryochem Phase I&quot;</td>
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<td>Phase IV</td>
<td>Freshly isolated Phase I</td>
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<td>Phase IV</td>
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<tr>
<td>None</td>
<td>Freshly isolated Phase I</td>
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<td>None</td>
<td>Phase IV</td>
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effect of the incubation, if any, on the titer of the serum. The effect was negligible.

If the Cryochem-dried organisms had become degraded and had lost their Phase I antigenic structure as a result of long storage in dry form, it should have been made evident by lack of absorption of the agglutinins from the known Phase I antiserum. Included in Table I are recorded the results of a control test in which a known degraded-Phase strain which grows luxuriantly on plain or glycerol-agar was used in an attempt to absorb the Phase I antibody. The agglutination titer against Phase I organisms was not thereby reduced, indicating complete specifi-
city of the Phase I agglutinin absorption. Even two additional absorptions with fresh portions of degraded-Phase organisms, incubating with each additional portion exactly as previously, did not reduce the agglutination titer against Phase I organisms. The cross agglutination titer of the Phase I serum against the degraded-Phase organisms, however, was virtually eliminated in a single absorption with degraded-Phase organisms, and was completely removed in the three absorptions. Absorption of the cross agglutination against degraded-Phase organisms was not quite complete in a single absorption with the Phase I organisms but was complete after two absorptions.

DISCUSSION

The fundamental importance in antibacterial immunity of the combination of agglutinins with surface antigens of bacteria is well established. With non-flagellated organisms the use of such surface reactions as agglutination or phagocytosis therefore provides distinguishing methods of assay for effective surface reactants in either serum or antigen. Complement fixation, and precipitin testing with soluble antigens, are of diagnostic value but do not distinguish surface antigen-antibody combination from phenomena involving other antigens. Consequently we believe the demonstration by agglutinin-absorption of the presence of the Phase I antigenic cellular surface in Cryochem-dried organisms to be the crucial test for the maintenance of pertinent Phase I serological characteristics.

Since Cryochem-dried *H. pertussis* may be maintained without essential alteration of serological and other properties, use of such a drying procedure, as in the case of other organisms, would appear to be justified. We are using this method in our current studies on the antigenic surface-structure of *H. pertussis* (Flosdorf, Kimball, Chambers, 1939) and feel confident that over a period of years we shall be dealing with an organism of essentially constant properties.

*Since going to press, Miller, J. J., and Silverberg, R. J., in an excellent paper, "Agglutinative Reaction in Relation to Pertussis and to Prophylactic Vaccination against Pertussis with Description of a New Technic," J.*
The authors wish to express their appreciation to Dr. Stuart Mudd for his helpful suggestions.

SUMMARY

It has been demonstrated that desiccation from the frozen state may be used to preserve the characteristics of *Hemophilus pertussis* in Phase I. Antigenic properties have been tested by agglutinin absorption and found to be characteristic of Phase I.

REFERENCES


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Immunol., 1939, 37, 207-222, have mentioned that they have used the Lyophile apparatus for keeping their strains. Their data in support of maintenance of Phase I characteristics, however, were not included.
Rogers, L. A. 1914 The preparation of dried cultures. J. Infectious Diseases, 14, 100-123.


