THE PRESERVATION OF BACTERIAL CULTURES. I

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Received for publication July 16, 1937

The maintenance of a large collection of stock cultures of bacteria for ready availability is a major task. The method of frequent transfer on a suitable culture medium, as commonly employed, requires not only a great expenditure of time, materials and effort, but also involves the possible loss of certain biological, immunological and cultural characteristics; not to mention the occasional loss of the strain through such agencies as contamination, drying of the medium and contact with accumulating metabolites.

It is the purpose of this paper to present (*a*) a review of the literature on the preservation of bacteria, (*b*) a comparison of three of the methods commonly employed for preserving bacterial cultures and (*c*) an emphasis on an inexpensive and practical method for preserving such cultures as are required to be available at all times for frequent subcultures.

To preserve cultures, and at the same time to maintain them in as nearly as possible their original state, various technics have been advocated. All, however, appear to be based upon one of two principles: First, the prevention of slow-drying of the cultures, or, second, the use of rapid desiccation. A review of the literature has revealed that most of the methods of preservation have been tried only on a limited number of bacterial species; practically none have been subjected to critical study. It is difficult to determine, therefore, whether they would be applicable to a large and varied stock culture collection.

It is our object to evaluate three representative methods by testing them against most of the species commonly maintained

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by teaching laboratories. One of these, perhaps the first attempt to preserve cultures, consisted of sealing the end of the tube containing the actively-growing organisms. This probably originated with Soyka (1887), Soyka and Kral (1888) and Kral (1889), who grew cultures in specially designed glass cylinders and closed the open ends with glass stoppers. Eisenberg (1888) and Czaplewski (1889) found that ordinary tubes sealed with paraffin worked just as well. Löwi (1918) suggested the use of test tubes fitted with ground glass stoppers which were reinforced with gutta percha paper. Others, employing slight variations of Soyka's method and different culture media, have reported successful preservation of many organisms (Ahuja, 1935; Bolley, 1900; Fiorito, 1925; Kiefer, 1923; Lal, 1920, 1925; Lenskaja, 1931; Martini, 1910; Mereshkowsky, 1909; Morax, 1918; Morton, 1935; Sartory and Maheu, 1909; Schultz, 1901; Shennan and Ritchie, 1907; Totire-Ippoliti, 1923 and Truche and Cotoni, 1912).

To determine the efficacy of Soyka's method, cultures were grown in beef-infusion broth, pH 7.2, sealed off in ampoules and stored at room temperature in the dark. Such ampoules were opened periodically and their contents transferred to fresh media. The sub-cultures were examined for viability, colony form and morphology. The results thus obtained follow.

A. Organisms surviving without change. (*a*) After 45 months: *Bacillus anthracis, Bacillus megatherium, Bacillus mesentericus, Bacillus subtilis, Escherichia coli-communior, Shigella dysenteriae* (Shiga and Flexner), *Salmonella enteritidis* (Stanley), *Aerobacter aerogenes, Diplococcus mucosus* (types A and B), *Eberthella typhosa* (4 strains), *Proteus mirabilis, Proteus vulgaris, Staphylococcus albus* (1 strain) and *Staphylococcus aureus* (1 strain). (*b*) After 33 months: *Eberthella typhosa* "R", *Brucella melitensis* "R" and *Micrococcus aurantiacus.*

B. Organisms surviving 30 to 45 months with changes as indicated. Escherichia coli "S" ("R" forms), *Escherichia coli* "R" ("S" and small colony forms), *Shigella dysenteriae* (Strong) "S" ("R" forms), *Salmonella enteritidis* "S" ("R" forms), *Salmonella paratyphi* A "S" (extreme "R" and small colony forms),

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Salmonella pullorum "R" (mucoid borders), *Eberthella typhosa* "S", 1 strain ("R" forms), *Serratia indica* and *Serratia marcesens*, 4 strains (loss of pigment), *Corynebacterium diphtheriae* "R" (small colonies), *Gaffkya tetragena*, 2 strains (small colonies), *Neisseria catarrhalis* (small colonies), *Proteus vulgaris* (small colonies), *Sarcina lutea* (small colonies and loss of pigment), *Staphylococcus aureus* (aureus and albus type of colonies) and *Vibrio proteus* (small colonies).

C. Organisms not surviving on subculture. (*a*) After 30 to 45 months: *Bacillus novus* (Huss), *Alcaligenes fecalis*, *Salmonella paratyphi* A, *Brucella abortus* (bovine and porcine), *Chromobacterium violaceum*, *Corynebacterium hofmannii*, *Corynebacterium xerose*, *Micrococcus flavus*, *roseus* and *tetragenus*, *Neisseria catarrhalis*, *Pasteurella pestis* (*caviae*), *Pseudomonas aeruginosa*, *Sarcina lutea*, *Staphylococcus albus*, *Staphylococcus aureus* (1 strain), *Vibrio metchnikovi* and *Vibrio schuylkilliensis*. (*b*) After 15 to 23 months: *Pseudomonas phosphorescens*, *Alcaligenes bronchisepticus* and *Saccharomyces cerevisiae*.

Summary. Storage of broth cultures in ampoules is not wholly satisfactory. Some species (the species of *Brucella*, *Pseudomonas phosphorescens*, the species of *Corynebacterium*, *Pseudomonas aeruginosa* and *Saccharomyces cerevisiae*) did not survive longer than a few months, and many underwent changes in colony form, which persisted. Similar observations on dissociation have been made by earlier workers and more recently by Hadley (1927, 1937). This method, moreover, is not suitable for many species. Of the organisms studied the method was least adaptable to *Corynebacterium diphtheriae*. The "S" form survived only 3 months while the "R" survived 7 months.

With one exception, all the cultures were grown in infusion broth. In the case of one of the diphtheria "R" strains, the organisms were grown in blood infusion broth. In this particular case, the strain was alive at the end of 45 months, whereas in plain infusion broth, the strains were always dead within 9 months.

The observation that the presence of materials such as blood, peritoneal fluid, etc. would greatly prolong the survival time is

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not new. Foa (1893) suggested that pneumococci could be preserved by storing septicemic blood. Barnabeo (1896) confirmed Foa's results with pneumococci and streptococci. Puntoni (1923, 1924) employed practically the same technic for successfully preserving the gram-negative intestinal rods. Pergher (1927) and Petragnani (1926) applied the method to an even wider range of bacteria. Fragments of infected tissue (Yourevitch, 1930), diluted bile and infusions of various organs (Totire-Ippoliti, 1924) have, likewise, been suggested.

The second method of preservation of cultures to be described is noteworthy in that it attempts not only to preserve the cultures, but also to keep them readily available at all times for multiple transplantation. The genesis of this method is to be found in the studies of Lumière and Chevrotier (1914). They concluded that gonococci could be maintained viable for several months if the cultures were kept either *in vacuo* or sealed with paraffin oil or vaseline. Ungermann (1918) grew other organisms in dilute, inactivated serum overlayed with sterile paraffin oil. Michael (1921) modified the method by adapting it to ordinary solid media, obtaining good results with a wide range of organisms. Nissle (1925) and Dikomeit (1927) observed that even suspensions of organisms remained viable under a layer of sterile paraffin oil or paraffin-oil lanolin mixtures.

The method has been tested only on a few organisms and, in most instances, for a relatively short period (Birkhaug, 1932; Bruni, 1930; Buschke and Langer, 1921; Daranyi, 1928; Kurobawa, 1927; Olsen, 1920; Parish, 1932; Trozky, 1930 and Truche, 1924). Suitable controls usually have been lacking, and the optimum conditions of storage have not been determined. On the other hand, the method attracts because of its simplicity.

EXPERIMENTAL

To evaluate this method the following experiment was carried out. Organisms were grown on appropriate solid media (infusion agar, "blood" agar, etc.), slanted so as not to give too long a slant. Several cultures were prepared from each strain. After good growth had taken place, one slant was covered with sterile

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heavy paraffin oil, or mineral oil, to a height of one centimeter above the top of the slanted surface. For a control, another slant was protected with a rubber cap, a method which had been previously employed with stock cultures. These are illustrated in figure 1. Such pairs of cultures of the same strain were held at the temperature of the incubator, room and refrigerator. To

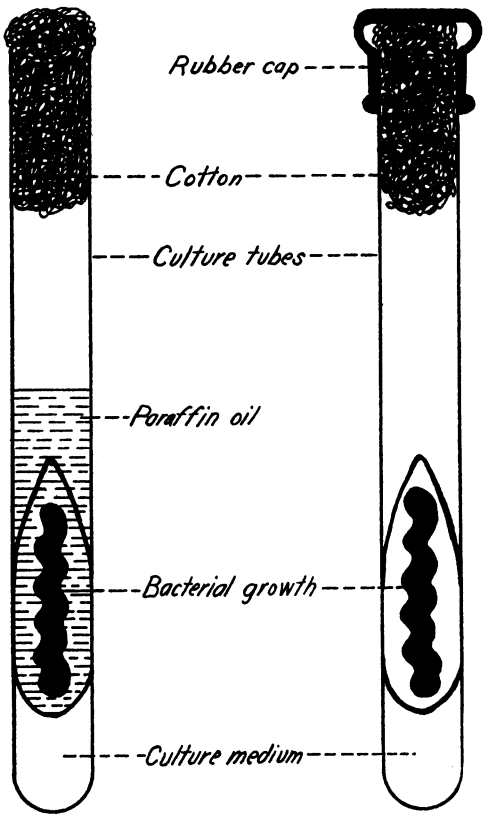

FIG. 1

determine the viability of the cultures under the various conditions, transplants were made from time to time. This was accomplished by fishing off a loopful of the growth, allowing the excess oil to drain off by touching the loop to the inner wall of the tube, then streaking over the surface of fresh medium in the usual manner.

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TABLE 1

Preservation of agar slant cultures under paraffin oil

ORGANISM AND STRAIN	REFRIGERATOR TEMPERATURE (8°–11°C.)				ROOM TEMPERATURE				INCUBATOR TEMPERATURE (37°C.)			
	Test		Control		Test		Control		Test		Control	
	Condition	Interval	Condition	Interval	Condition	Interval	Condition	Interval	Condition	Interval	Condition	Interval
		mos.		*mos.*		*mos.*		*mos.*		*mos.*		*mos.*
Aerobacter aerogenes, P 41	+	12*	+	12*	+	21*	Dead	18				
Alcaligenes bronchisepticus					+	30	+	30				
Alcaligenes fecalis, P 61	+	12*	+	12*	+	21	+	21†	+	21	+	18*
Brucella abortus (bovine) P 63	+	12*	Dead	12	+	21*	Dead	18				
Chromobacterium violaceum, P 104	Dead	12	Dead	12	+	18	Dead	12				
					Dead	21						
Corynebacterium diphtheriae, Park 8[1]	+	3	Dead	3	+	36		†	+	12	+	12
	Dead	4							Dead	23		†
Corynebacterium hofmanii, 811[1]					+	30	+	30	+	12*	+	12*
Corynebacterium xerosis, P 83[1]	+	12*	Dead	12	+	33	Dead	33	+	23*	Dead	10
Diplococcus mucosus, P 10	Dead	12	Dead	12	+	21*	Dead	12	Dead	7	Dead	7
Eberthella typhosa, P 12	+	12*	+	12*	+	21	Dead	18				
Escherichia coli-commune "R," P 94	+	9	+	12	+	18	+	18	Dead	9	Dead	9*
	Dead	12	Dead	18	Dead	21	+	21				
Escherichia coli-commune "S," P 3	+	12*	+	12*	+	20*		†	+	9*	+	9*
Hemophilus influenzae[2]									+	1	Dead	1
									Dead	2		
Hemophilus pertussis[2]	+	12	Dead	12	+	12	Dead	12				
					Dead	21						
Monilia albicans[3]									+	7*	Dead	7
Neisseria catarrhalis, P 66	+	21	+	21	+	21	+	21	+	9*	+	9*
Neisseria gonorrheae[1]									+	13	+	3
											Dead	4
Neisseria intracelluris[1]					Dead	3	Dead	3	+	15	Dead	3
Proteus vulgaris, P 44	+	12*	+	18*	+	21	+	21	+	21	+	18
											Dead	21
Pseudomonas aeruginosa	Dead	12	Dead	12	+	21	+	21	+	30*	Dead	9
Pseudomonas phosphorescens					Dead	30	+	30				

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Saccharomyces cerevisiae					+	10*	Dead	3	+	4	Dead	3
									Dead	7		
Salmonella enteritidis, P 51	Dead	12	+	12*	+	18	+	18	+	18*	+	12*
Salmonella pullorum "R," P 39	Dead	12	+	12	+	21	+	21	Dead	9	Dead	9
			Dead	18								
Sarcina aurantiaca, P 138	+	12*	+	21	+	18	Dead	18	Dead	9	Dead	9
					Dead	21						
Serratia marcescens, P 135	+	12*	+	12	+	21*	Dead	18				
	[illegible]		Dead	18								
Shigella dysenteriae (Flexner), P 109	+	12*	Dead	12	+	21	Dead	18	+	18*	Dead	18
Shigella dysenteriae (Shiga), P 52	+	12*	+	12	Dead	18	+	18	Dead	21	Dead	21
	[illegible]		Dead	18								
Spirillum rubrum, P 104[1]	Dead	12	Dead	12	+	21	Dead	12				
Staphylococcus albus, P 77	+	21	+	21	+	21	+	21	+	21	+	21
Staphylococcus aureus, P 78	+	12*	+	12*	+	21*	Dead	18	+	21	+	21
Staphylococcus citreus, P 150	+	18	+	18								
	Dead	21	+	21	+	33	+	18*	+	21	Dead	18
Streptococcus cardioarthritidis,[1] P 146	+	9*	Dead	9								
Streptococcus fecalis,[1] P 122	+	20		†	+	19*	Dead	18	+	18	+	18
									+	21*	Dead	21
Streptococcus hemolyticus,[1] P 24	+	18	Dead	18	+	19*	Dead	18	+	12*	Dead	4
Streptococcus indifferens,[1] P 26	+	12*	+	12*	+	12*	+	12*				
Streptococcus pneumoniae "M," type I[1]	+	12*	+	12*	+	12*		†				
Streptococcus pneumoniae "S," from type I[1]	+	9*	Dead	7								
Streptococcus pneumoniae "M," type II[1]	+	20*	Dead	9	+	12*	Dead	12				
Streptococcus pneumoniae "S" from type II[1]	+	12*	Dead	12	+	12*	Dead	12				
Streptococcus pneumoniae "M" type III[1]	+	20*	Dead	9	+	12*		†				
Streptococcus viridans,[1] P 25	+	12*	+	12*	+	12*	Dead	12				
	+	20*		†								
Vibrio metchnikovi, P 45	+	18	+	18	+	18	+	18	+	7*	Dead	7
	Dead	21	+	21*	Dead	21	Dead	21				
Vibrio schuylkilliensis, P 8	+	18	+	18	+	18	+	18	+	9*	Dead	9
	Dead	21	+	21	Dead	21	+	21				

+ indicates culture alive.
* Slant washed for making test.
† Culture overgrown with a mold.
[1] Culture maintained on blood agar.
[2] Culture maintained on chocolate agar.
[3] Culture maintained on glucose agar.

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Attention is directed to the following points: (*a*) Unless the oil is well above the uppermost level of the medium, the medium tends to dry out, separate from the wall of the tube and float to the surface of the oil, in which event the organisms are usually found dead. (*b*) The quality of the oil[1] is very important, as any rancidity or toxic substance is harmful to the organisms. (*c*) It is preferable to sterilize the oil in the hot air oven at 150° to 170°C. for one hour; for during autoclaving moisture becomes mixed with the oil, giving it a milky appearance. (*d*) Some precaution is necessary in the flaming of the loop after it has been withdrawn from the oil, since plunging directly into the flame results in spattering. This may be prevented by warming the loop very gently before heating to redness, or by plunging into a beaker of boiling water, then flaming to redness in the usual manner. The technics customarily employed with acid-fast organisms are satisfactory.

The significant results thus far obtained are presented in table 1.

Summary. Forty-four strains, representing a wide variety of bacterial species, have been tested:

Test culture alive, control culture dead	29
Test culture alive, control culture alive	10
Test culture dead, control culture alive	5
Totals: Test cultures alive	39
Control cultures alive	15

The five instances where the control culture survived longer than the culture under oil were *Escherichia coli* "R", *Shigella dysenteriae* (Shiga), *Pseudomonas phosphorescens*, *Vibrio metchnikovi* and *Vibrio schuylkilliensis*. The viable cultures are being held for further observations. The rubber cap on the control cultures probably accounts for the long survival period of the controls, for Birkhaug and Parish report all their controls dead within four weeks. Also we were unable to confirm the favor-

[1] Mineral oil (Heavy), Parke, Davis and Company has proved very satisfactory.

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able results of these authors in regards to *Hemophilus influenzae*. No single temperature is optimum for storage of all bacteria. Room temperature appears to be optimum in the majority of cases, the exceptions being certain of the *Neisseria*, *Hemophilus* and *Streptococcus* groups.

The first two methods described had as their basis the prevention of slow evaporation of the cultures. In contrast, the third method employs the principle of rapid desiccation.

Slow desiccation in air appears to have a decidedly lethal effect. For example, organisms left to dry on glass slides survive no longer than a few days (*Brucella abortus* (Cameron, 1932); *Bacillus anthracis*, *Escherichia coli*, *Corynebacterium diphtheriae*, *Eberthella typhosa*, *Saccharomyces cerevisiae* and *Staphylococcus aureus* (Thurn, 1914); *Vibrio cholerae* (Thurn, 1914; Kitasato, 1889); *Neisseria intracellularis* (Bettencourt and Franca, 1904)). The presence of extraneous material such as silk (Kitasato, 1889); sputum (Bordoni-Uffreduzzi, 1891) and various nutritive substances (Foa and Bordoni-Uffreduzzi, 1887; Abel, 1893, 1897; Latapie, 1918; Winslow and Brooke, 1927; Cameron, 1932) greatly alters the survival period.

Kitasato in 1889, working with *Vibrio cholerae*, observed that the organisms survived longer when desiccator-dried than when air-dried. Germano (1897 a and b) demonstrated quantitatively that diphtheria bacilli, streptococci and pneumococci survived longer when dried in a desiccator over H_2SO_4 than when room-dried. Ficker (1898) was the first to make a critical study of other factors necessary for the preservation of bacteria by desiccation. He concluded that most important were (*a*) the mass and nature of the dried cultures, and (*b*) the menstruum in which the organisms were suspended. In 1908 he reported on the nature of the suspending medium, finding that milk, serum, bouillon, saliva, distilled water, physiological salt solution and urine protected the organisms in the order listed; milk and inactivated serum being best. He also pointed out that another factor in the survival of bacteria is the change in osmotic conditions brought about in drying. Kirstein (1900) found alternate humidity and dryness more destructive than constant dryness.

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He also observed that the dried bacteria lived longer at refrigerator temperature than at room temperature.

Heim (1905, 1907) was the first to report the successful use of drying in a desiccator over a dehydrating agent as a method for preserving a variety of cultures, some surviving two years. He later (1922) used small sterile test tubes instead of glass slides. Brown (1925) reported preserving pneumococci and streptococci by drying on cover-slips in a desiccator over $CaCl_2$. The technic was soon modified, strips of sterile filter paper being substituted for the glass cover-slips and pint milk bottles being used instead of a desiccator. In 1926 he further refined the technic by employing, in some cases, small sterile test tubes instead of the pint milk bottles. In a later report (1932) some strains were reported as remaining viable for as long as 12 years. Harris and Lange (1933), using the method of Brown, found that acid-fast organisms (31 different strains) could be preserved for at least 11 months. Leifson (1936), using a slight modification of Brown's technic, did not obtain commendable results.

Patella (1888) observed that pneumococci dried at 38°C. did not maintain their virulence or viability as long as when dried at a lower temperature (17°C.), but it was not until 1909 that freezing was recommended as a preliminary step to desiccation (Shackell). Hammer (1911) modified Shackell's method in that he dipped strips of paper in bouillon cultures before the freezing and drying process.

Rogers (1914) applied the principle of freezing and drying of bacterial cultures on a large scale. The cultures were frozen by a salt-ice mixture or by means of carbon-dioxide snow. Good results were obtained with the lactic-acid group and the colon group of organisms, questionable results being obtained with yeasts. Rogers found that the loss of viability of the dried cultures was very slow at low temperatures (0°C. or lower), but became more rapid as the temperature of storage increased. This is a confirmation of Ficker's (1898) observations. More cells remained viable in cultures stored *in vacuo* than in hydrogen, carbon dioxide, nitrogen, oxygen or air. The various gaseous environments were least detrimental in the order named. He

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suggested that the method could be applied for the preservation of stock cultures.

Swift (1921) applied the method of drying cultures from the frozen state to streptococci, pneumococci, meningococci and influenza, typhoid, paratyphoid and dysentery bacilli with good results. Otten (1927, 1930) showed that freezing could be eliminated from Swift's technic and he was able thereby to preserve a wide variety of pathogenic bacteria. Pauli (1932) also successfully preserved many cultures without freezing them prior to desiccation, recommending suspension of the organisms in sterile normal horse serum. In 1935 two methods (Elser, Thomas and Steffen, and Flosdorf and Mudd, 1935, 1936) were reported for the preservation of biological products, including microorganisms, by drying from the frozen state. That of Flosdorf and Mudd employed dry-ice for the initial freezing. Elser, Thomas and Steffen reported meningococci and gonococci alive after storage *in vacuo* for 18 years. Rake in 1935 reported the successful preservation of meningococci for periods ranging from 3 to 5 months by freezing the organisms in a dry-ice freezing mixture and drying in a vacuum. In 1937, Swift modified his method so as to employ dry-ice for the freezing agent. Roe (1936) preserved 16 varieties of anaerobes by drying them on strips of filter paper from the frozen state and maintaining in sealed test tubes with a freshly heated piece of $CaCl_2$, thus combining the technics of Brown and Swift.

Experimental

This work was begun in 1932 and of the methods reported in the literature, which employ rapid desiccation, we chose that of Swift (1921). The technic employed was as follows: Heavy suspensions of young bacterial cells were dispensed in amounts of 0.25 cc. into sterile agglutination tubes. These were placed in a salt-ice mixture until frozen (those cultures designated in table 2 by an asterisk were frozen in a dry-ice acetone mixture), then transferred to a desiccator containing a layer of glycerol kept cold by being surrounded with a salt-ice mixture. A dish containing P_2O_5 was placed above the tubes in the desiccator,

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TABLE 2

Preservation of bacteria by the rapid drying technic of Swift

ORGANISM AND STRAIN	INTERVAL AFTER DRYING	CONDITION
	months	
Actinomyces casei, P 133*	3	+
Aerobacter aerogenes, P 41	58	+
Alcaligenes bronchiseptica	40	+
Alcaligenes fecalis, P 61	37	+
Bacillus anthracis, "R," P 60	28	+
Bacillus megatherium, P 97*	24	+
Bacillus mesentericus, P 112*	24	+
Bacillus novus (Huss), P 102*	45	+
Bacillus subtilis, P 7*	17	+
Brucella abortus (bovine) P 63	20	+
Brucella abortus (caprine) P 80	24	+
Brucella abortus (porcine) P 65	22	+
Corynebacterium diphtheriae, Park 8, O 4	24	+
Corynebacterium xerosis, P 83	36	+
Diplococcus mucosus, types A and B	37	+
	58	Dead
Eberthella typhosa, P 16*	45	+
Eberthella typhosa, P 12, 17, 20, 32	58	+
Escherichia coli-commune, "S," P 3	58	+
Escherichia coli-communior, P 101*	4	+
Hemophilus influenzae, P 67	27	+
Lactobacillus acidophilus "S," P 117*	15	+
Micrococcus aurantiacus, P 103*	4	+
Micrococcus roseus, P 100*	16	+
Micrococcus tetragenus, P 40	36	+
Neisseria catarrhalis, P 66*	4	+
Pasteurella pestis (caviae), P 71	58	+
Proteus mirabilis, P 98*	16	+
Proteus vulgaris, P 44*	25	+
Pseudomonas aeruginosa, P 2	58	+
Pseudomonas phosphorescens	12	+
Salmonella enteritidis, P 51	56	+
Salmonella paratyphi A, P 54, 22, 23, 55, 56	36	+
Salmonella paratyphi A, P 56	54	Dead
Salmonella paratyphi A, P 57	57	+
Salmonella paratyphi A, P 58	16	+
	37	Dead
Salmonella paratyphi B, P 33, 34, 35, 36	36	+
Salmonella paratyphi B, P 59	57	+
Salmonella paratyphi B, P 113*, 114*	45	+

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TABLE 2—*Concluded*

ORGANISM AND STRAIN	INTERVAL AFTER DRYING	CONDITION
	months	
Salmonella pullorum, "R," P 39	58	+
Salmonella pullorum, "R," P 47	4	+
	37	Dead
Serratia marcescens, P 4*	45	+
Serratia marcescens, P 4	44	+
Shigella dysenteriae (Flexner) P 21	54	+
Shigella dysenteriae (Flexner) P 109*	57	Dead
Shigella dysenteriae (Shiga) P 52	36	+
	57	Dead
Shigella dysenteriae (Strong) P 53	57	+
Shigella dysenteriae (Y) P 107*	4	+
Staphylococcus albus, P 77	36	+
Staphylococcus aureus, P 5, 37	36	+
Staphylococcus aureus, P 37	58	Dead
Staphylococcus aureus, P 38	58	+
Staphylococcus aureus, P 78	36	+
	58	Dead
Streptococcus fecalis, P 122*	25	+
Streptococcus hemolyticus, P 24	43	+
	57	Dead
Streptococcus hemolyticus, P 73, 74	58	+
Streptococcus indifferens, P 26	36	+
Streptococcus pneumoniae, type I, P 21	58	+
Streptococcus pneumoniae, S from type I	37	+
Streptococcus pneumoniae, type II, P 28	54	+
Streptococcus pneumoniae, S from type II	37	+
	57	Dead
Streptococcus pneumoniae, type III, P 29	36	+
	57	Dead
Streptococcus pneumoniae, S from type III	17	+
	57	Dead
Streptococcus pneumoniae, type V, P 30	60	Dead
Streptococcus pneumoniae, type VI, P 31	60	+
Streptococcus viridans, P 25	57	+
Vibrio metchnikovi, P 45	4	+
	37	Dead
Vibrio schuylkilliensis, P 8	4	Dead

* Cultures frozen in dry ice-acetone mixture (May, 1933).
+ indicates cultures alive and typical.

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the lid placed in position and connected to a vacuum pump (Cenco Hyvac). After the desiccator had been evacuated for 1 to 2 hours, the pump was disconnected and the desiccator placed in the refrigerator. The next day the dried cultures were removed and the open ends of the tubes sealed with paraffin. The preserved cultures were kept at room temperature and in the dark. Those tubes showing a gummy residue were discarded. The results thus far obtained are given in table 2.

Summary. The method has been found to be reasonably trustworthy. However, a number of the strains, which were viable when tested after 3 years, were found dead when duplicate tubes were tested after a period of 57 months (*Shigilla dysenteriae*, *Diplococcus mucosus*, *Salmonella paratyphi* A, *Staphylococcus aureus*, *Streptococcus hemolyticus*, *Streptococcus pneumoniae*, *Vibrio metchnikovi*). Due to the many factors involved in this method, it is impossible to state the reason for the failure of survival of some of the strains. At first it was believed that the breakage of the paraffin seal due to changes in room temperature, especially during the summer months, was responsible for the death of certain of the cultures. However, in other experiments where one portion of the tubes was hermetically sealed, and the other sealed with paraffin in the usual manner, no perceptible difference was noticed in the survival period.

DISCUSSION

From the preliminary studies herein reported, many interesting points have arisen. The preservation of bacterial cultures in ampoules is of little value, if the original characteristics of the strain are to be preserved. Some organisms live only a short time and frequently the cultures undergo dissociation. The method is costly, as culture tubes are lost when they are sealed. It is somewhat dangerous because of the possible shattering of the ampoule when opened. Once an ampoule is opened, a fresh culture has to be sealed off or the contents of the opened ampoule transferred to another tube and resealed.

Preservation of cultures under paraffin oil has many distinct advantages. The cultures are available at all times; numerous

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subcultures can be made without the necessity of discarding the original culture. The method eliminates the use of rubber caps on the culture tubes, thereby bringing about an economy. It, likewise, eliminates the use of wax, cements, etc. which are difficult to remove. It reduces the frequency of contamination to practically nil, especially with molds. No preliminary treatment, such as growth in large amounts, centrifugation, dispensation into special tubes or onto filter paper, is necessary. No special apparatus, such as desiccators and vacuum pumps, is needed. Single colonies or single colony variants are easily preserved without disturbing the stage of development. Practically all the organisms tested live longer under paraffin oil than in the unprotected control cultures. Although good results were not obtained with the one strain of *Shigella dysenteriae* (Shiga) cited in table 1, other strains of the dysentery bacillus and other strains of the Shiga bacillus are being maintained under oil without difficulty. Even in the case of the influenza bacillus where the period of survival under oil was only two months, this was twice as long as in the control culture. With the exception of the influenza bacillus, it is only necessary to make transfers every six months or even once a year. Bucher (1937) states that he maintained a large collection of freshly isolated strains of meningococci and gonococci by making transfers every six months and preserving under paraffin oil.

Preservation of cultures in the dried state by Swift's method has the advantage that many organisms apparently survive longer in the dried state than by other methods of preservation. The cultures can be stored in small containers, which is an aid if storage space is at a minimum or if it is necessary to transport the cultures, and the dry state of the cultures makes them more adaptable to transportation than if they were in the fluid state. The disadvantages of the method are many. Once a tube of the dried culture is opened for subculturing, the culture is lost as far as preservation is concerned, unless one has many duplicate cultures or goes to the trouble of freezing and drying additional cultures from time to time. The method requires growth of the organisms in large quantities. In some cases, such as

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growth of unstable variants, this is not always practical. The method requires a great deal of manipulation, such as centrifugation, dispensation into tubes, freezing and sealing of the tubes. Special apparatus is also required. When removed from the desiccator, it is necessary to wipe the glycerol from the exteriors of the tubes. This is messy. Sealing of the tubes is not without its difficulties. Paraffin and other of the various preparations for that purpose contract and crack due to the cool temperature of the refrigerator and this results in the loss of the cultures. Likewise, the high temperatures encountered in the summer time cause a damage to the seals with subsequent loss of the cultures. It is not practical by the present method to preserve large numbers of cultures individually in the dried state under a vacuum. In view of the fact that numerous workers have reported cultures alive in ampoules for many years, Swift's method has not been under investigation long enough to warrant a definite statement on the longevity of bacteria thus preserved. One is appalled at the lack of critical data on the preservation of cultures by this method. Methods which have been in use for just a few years enjoy such claims as "the cultures would probably keep indefinitely if the seals remained intact." Only by quantitative studies, which have been lacking, will it be possible to venture a definite statement.

SUMMARY

In laboratories where it is necessary to make frequent transplants from stock cultures, a very practical method is to maintain them under sterile paraffin oil. The points in favor of the method are many. (*a*) It greatly reduces the frequency of contamination, especially with molds, thus permitting cultures to be maintained with greater success in surroundings which are not conducive to precise bacteriological work. (*b*) No preliminary treatment of the cultures is necessary. (*c*) Practically all the organisms tested live longer under oil than in the control tubes. (*d*) Changes in cultural and biochemical characteristics —other than the sometimes prolonged lag phase of growth on subculturing—have not been observed. (*e*) The cultures are

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available at all times for transplantation without interfering with the preservation of the stock culture. (*f*) The method is applicable to single colonies or mass cultures. (*g*) It is especially advantageous in working with unstable variants, where occasional transferring to fresh media or growth in mass culture results in a change in the developmental stage of the strain. (*h*) No seals, such as rubber caps, waxes, cements, etc. are needed for the culture tubes. (*i*) No special apparatus is required, such as a centrifuge, desiccator or vacuum pump.

The method of preserving cultures by sealing the tubes in a flame is of questionable practical value, if the original characteristics of a culture are to be preserved. Cultures which remain viable in ampoules frequently show only a few viable organisms when transferred to fresh media and often the viable organisms have dissociated. Other disadvantages are that the cultures are not easily available for subculturing and there is destruction of culture tubes.

Preservation of cultures in the dried state has the advantages that (*a*) less space is required for storage, (*b*) the cultures are more easily transported and (*c*) certain immunological properties apparently are maintained. The disadvantages are that the cost of the special equipment (centrifuge, vacuum pump, etc.) is prohibitive in some laboratories; likewise, the time and effort which must be expended if a large collection of cultures is to be so preserved. Many of the technics for preserving cultures in the dried state are impractical and some technics are more destructive to certain micro-organisms than if the organisms were kept on the original culture medium.

Lack of critical systematic studies upon, and the possibilities of, the method of preservation in the dried state are the basis of additional studies which are now in progress.

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