THE RESISTANCE OF DEHYDRATED PNEUMOCOCCI TO CHEMICALS AND HEAT

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It is well recognized that desiccation not only prevents bacterial growth, but under certain conditions leads to the death of many microörganisms. Leaving aside the extreme resistance of spore-bearing bacteria, it is known, however, that even relatively delicate bacteria such as the typhoid bacillus and the pneumococcus, although they may be destroyed in a thin layer from aqueous suspension when slowly desiccated, remain alive after drying to a constant weight when assembled in large masses (Reichel). The survival of the pneumococcus in the dried spleen of mice dead from infection of a virulent culture is made use of as current procedure in conserving both cultures and virulence.

The condition of "suspended animation" of dehydrated bacteria is in itself a matter for interesting speculation. Of more tangible import is the fact that the presence of water is necessary to stain bacteria (Churchman (1928)) and that dehydrated bacteria are relatively unaffected by many active disinfectants in anhydrous alcoholic solution (Ford (1927)). It seems less well known that desiccated bacteria are also relatively very resistant to heat.

Our interest in submitting desiccated pneumococci to a new and more complete analysis as regards resistance to chemicals and heat, lay first in affording another basis of comparison between bacteria and certain filterable viruses, since the latter are also known to withstand desiccation. And, again, it was thought to be interesting to test the relative resistance of colonial dissociants ("Rough" and "Smooth" forms) of a given pneumococcus to heat

and chemicals. Such dissociants are of particular interest since they are correlated with differences in virulence.

EXPERIMENTAL

Organism

The organism used in this work is a Type I pneumococcus. It is a highly virulent strain which has been kept in the laboratory for some time and known as No. 94. One culture from this strain was carried in broth for four to six months and became avirulent. A second culture from this same strain was kept virulent by mouse passage. Mice were regularly killed by 1/100,000 of a cubic centimeter in twenty-four hours. The virulent and the non-virulent cultures used in these experiments came, then, from one original strain. Single cell isolation was not resorted to, as the strain had been plated and purified many times. The virulent strain was repeatedly observed on plating as "Smooth" in colonial form, whereas the avirulent strain was "Rough."

Preparation of dried material

An actively growing broth culture was used to inoculate flasks of infusion broth, at pH 7.8, and these flasks were incubated for from eighteen to twenty-four hours. The broth was removed to large centrifuge tubes and centrifugalized and the sediment collected and placed in a vacuum desiccator at room temperature over calcium chloride until dry. When dry, usually in about two days, the sediment was ground to a fine powder in an agate mortar until there were no particles large enough to be visible to the eye. This dried ground powder reached constant weight after about twenty-four hours in the desiccator.

Amount of dried material used

A small standardized curette was found to contain, when level-full, approximately 4 mgm. of the dried material. One curette load was used for each determination.

Many lots of the dried material were prepared in separate small test tubes which were kept in the desiccator so that the material could be used in the experiments within a week of the time of

preparation. Other tubes were sealed and kept in the dark at room temperature for later tests. The number of viable organisms in the dried material was found by plating to vary within fairly narrow limits during the usual period of ten days to two weeks after drying. In the four milligrams used, there were roughly from 200 to 400 million living organisms.

Preparation of alcoholic solutions of bactericidal chemicals

So-called "absolute" alcohol (99.7 per cent) was added to a large bottle half full of quick lime and left in contact for at least a week. Precautions were taken to protect this alcohol from exposure to air so that it would remain water free. On testing with anhydrous copper sulphate the alcohol appeared to be water free. One gram of the disinfectant to be tested was dissolved in 100 grams of this anhydrous alcohol, (i.e., 125 cc.) so as to obtain a one per cent solution by weight.

Technique of tests

To a plugged and sterilized test tube, 8 by 100 mm., previously drawn out in a flame so as to make a constriction near the middle, a curette load of the dried material was added and, on tapping, this material fell to the bottom of the tube. Approximately 0.75 cc. of the alcoholic solution of the disinfectant was added by a capillary pipette and the tube sealed in a flame at the constriction. The sealed bulb thus formed was incubated for twelve hours at 37°C. Following the treatment period of twelve hours, the bulb tip was flamed and broken open. The contents were removed by a sterile capillary pipette to a sterile, rubber-stoppered centrifuge tube containing at least 5 cc. absolute alcohol. This tube was centrifugalized until it was clear and the sediment was packed in the bottom: the supernatant wash alcohol was decanted and 10 cc. of fresh alcohol added. The sediment was again suspended in the alcohol by shaking and the tube again centrifugalized. This operation was repeated with a second washing with 10 cc. of alcohol. Thus the sediment was washed in three lots of alcohol and the disinfectant thereby almost entirely removed. Following the decanting of the last wash alcohol

the centrifuge tube with sediment was placed in the vacuum desiccator until the alcohol had evaporated, which usually took about an hour. About 7 cc. of broth was now added to the centrifuge tube and a sterile cotton stopper substituted for the rubber stopper. The tube was incubated for four days with daily observations. A tube showing growth was examined microscopically so as to rule out possible contamination. At the end of the four day period all tubes not showing growth were reinoculated with a loop of a broth culture of pneumococci and again incubated. This step acted as a check to show that the broth did not contain enough disinfectant to have a bacteriostatic effect.

Control tests of the destructive effect of chemicals employed on "moist" pneumococci

The chemicals: alcohols, salts of heavy metals, dye stuffs and others, that we have tested on dried pneumococci were all known or suspected to be destructive for most bacteria under their normal conditions of growth. A control check of the bactericidal effect of most of these materials or cultures in aqueous suspension seemed desirable. For this purpose the original cultures used to prepare the dehydrated preparations were employed as follows: eighteen to twenty-four-hour cultures in the standardized broth were found on plating to contain 200 to 600 million organisms per cubic centimeter. Two cubic centimeter amounts of this culture were replaced in separate small tubes and centrifugalized until clear. The sediments after removal of the supernatant fluid contained, then, something less than 400 to 1000 million as compared with 200 to 400 million employed in dried culture experiments. In other words there were more moist bacteria used in these tests than dried bacteria.

To each sediment the chemical, when soluble in broth or Ringer's solution, was added in solution in this nutritive fluid in 1 cc. amounts. Chemicals like alcohol, chloroform, xylol and the essential oils were added to the moist sedimented culture intact. These mixtures were allowed to stand at room temperature for eighteen to twenty-four hours in well-stoppered tubes. The

tubes were centrifugalized, the supernatants decanted, and 5 cc. of broth were added to the entire sediment. To avoid continued action of the disinfectant the tubes were shaken and 1 cc. of the suspended organisms was added to a fresh 5 cc. tube of broth. Growth was allowed for four days at incubator temperature and, when positive, was checked morphologically.

SURVIVAL OF DESICCATED PNEUMOCOCCI

It is known that the completely desiccated blood or organs of mice dead of pneumococcus infection not only contain living pneumococci for many months, but that these organisms retain their virulence unaltered, (Neufeld and Schnitzer (1928)). Griffith is stated by these authors to have obtained positive cultures by these methods for as long as three years. Our dried preparations, not of infected organs, but of bacteria, have been found to contain living organisms for eighteen months.

There is a marked decrease of living bacteria in the process of desiccation itself however rapidly it be performed. By plating out the original broth culture, and deducting the number of organisms found, also by plating, in the supernatant fluid after centrifugalization, the number of living bacteria present in the sediment may be determined. After complete desiccation the total weight of dried bacteria per cubic centimeter of original culture may be calculated. From this, by plating, the number of living dried bacteria and their percentile relationship to those present in an aliquot part of original culture was determined.

Two estimates of this sort indicated:

1. In a rapid desiccation, two days from original culture to apparent dryness: Constant weight fourth day; 8.62 per cent of Rough pneumococci survived, and 7.28 per cent of Smooth pneumococci survived.

2. In less rapid desiccation, four days from original culture to apparent dryness and constant weight: 0.546 per cent of Rough pneumococci survived; 0.042 per cent of Smooth pneumococci survived.

It is evident that slight variations in technic suffice to affect the resistance of pneumococci to desiccation. A single attempt to

obtain dried organisms in larger numbers by growth in 1 per cent glucose broth, in which it is known that more acid is produced, resulted in a sterile desiccate.

The greater resistance of the "Rough" organisms to desiccation should be recalled in consideration with results presently to be given.

TABLE 1

*The destructive effect of various anhydrous solvents on moist and on dried smooth pneumococci**

CHEMICAL EMPLOYED	MOIST CULTURE	DRIED CULTURE
Ethyl alcohol	0*	+*
Amyl alcohol	—	+
Butyl alcohol	—	+
Caprylic alcohol	—	+
Acetone	0	+
Aniline	—	+
Amyl acetate	—	+
Benzene	0	+
Benzaldehyde	0	+
Carbon tetrachloride	0	+
Chloroform	0	+
Ether	0	+
Ethyl acetate	0	+
Ethylene chloride	0	+
Petroleum ether	0	+
Toluene	0	+
Xylene	0	+

* The plus sign (+) indicates that the pneumococci survived the exposure, as shown by growth; the "0" sign indicates complete destruction with negative growth; the dash (—) indicates "not tested."

THE EFFECT OF VARIOUS ANHYDROUS SOLVENTS ON MOIST AND DEHYDRATED CULTURES OF PNEUMOCOCCI

In table 1 the effect of various anhydrous solvents on moist and on dehydrated preparations of "Smooth" pneumococci is shown.

It is evident that none of the solvents tried destroyed dehydrated pneumococci whereas all were completely destructive for moist cultures of pneumococci. The series of solvents were also tested after mixture with equal parts of anhydrous ethyl alcohol and were likewise without effect on the dried bacteria.

It is evident that only such other bactericidal chemicals could be tried on dried bacteria as are soluble in one of the anhydrous solvents. As a matter of fact we have tested only certain ones that are soluble in anhydrous ethyl alcohol.

TABLE 2

The destructive effect of various compounds of mercury in solution of ethyl alcohol on moist and dried pneumococci in both "S" (virulent) and "R" (avirulent) forms

COMPOUND AND DILUTION	MOIST		DRIED	
	"S"	"R"	"S"	"R"
Mercuric chloride:				
1 per cent	0	0	+	0
0.1 per cent	0	0	+	0
0.01 per cent	0	0	+	+
0.001 per cent	0	+	+	•+
Mercuric cyanide:				
1 per cent	—	—	0	0
0.1 per cent	—	—	0	0
0.01 per cent	—	—	+	0
0.001 per cent	—	—	+	±*
Mercuric iodide:				
1 per cent	—	—	0	0
0.1 per cent	—	—	0	0
0.01 per cent	—	—	+	+
0.001 per cent	—	—	+	+
Mercurochrome:				
Saturated†	—	—	0	0
10 per cent	—	—	+	0
1 per cent	—	—	+	+
0.1 per cent	—	—	+	+

* These dilutions were always run in duplicate; ± indicates that one tube was sterile and the other showed growth.)

† Mercurochrome is only slightly soluble in alcohol. In this series we have employed a saturated solution and percentages of it.

THE DESTRUCTIVE ACTION OF CERTAIN HEAVY METALS ON DRIED PNEUMOCOCCI

Bichloride of mercury is known to be highly destructive for all bacteria in dilutions of from 1:10,000 to 1:100,000 (Zinsser) (table 2).

It is evident, then, that these compounds of mercury are destructive of "R" dried pneumococci, although less so than for the organisms in watery suspension. The virulent strain of our pneumococcus ("Smooth") was more susceptible when moist than the "rough" but much more resistant than the rough when dried.

Silver nitrate is another well known chemical disinfectant which kills cocci under normal conditions in dilutions of 1:1,000 (Zinsser in table adapted from Fleugge, p. 84). It was likewise destructive of our dried pneumococci for the virulent strain in alcoholic solution in similar concentrations (1:1,000). It killed the dried avirulent strain at times, but not regularly, in a dilution of 1:10,000.

The effect of specific pneumococcidal chemicals on moist and dried pneumococci

Ethyl-hydrocuprein (Optochin)[1] has long been known to be actively, and more or less specifically, destructive for the pneumococcus (Morgenroth and Levy (1912). It has been found to sterilize cultures of this microörganism within twenty-four hours in an aqueous dilution in proportions of from 1:400,000 to less than 1:1,000,000 (Morgenroth (1914). We have found our preparation active in aqueous solution on moist cultures diluted 1:100,000. With an old and less active preparation of optochin, the moist "S" strain was killed in higher dilution than the moist "R" preparation.

Sodium desoxycholate[2] is the most active component responsible for the specific lytic effect of bile on the pneumococcus. Kozlonski (1925) found this salt active in broth cultures in a dilution 1:1,000, whereas bile was effective only in a dilution of 1:10. In our control experiments on moist cultures, a 0.02 per cent solution of sodium desoxycholate in broth completely destroyed the pneumococci.

[1] (Optochin hydrochloride (Zimmer and C. Vereinigte Chinin Fbrik. Frankfort, Germany as employed by Morgenroth) kindly furnished us by Dr. Oswald T. Avery of the Hospital of The Rockefeller Institute was used in our experiments.)

[2] (Desoxycholic acid was also furnished us by Dr. Avery. A sodium salt was obtained by dissolving in NaOH.)

Both optochin and desoxycholic acid are readily soluble in alcohol. As shown in table 3 neither substance dissolved in alcohol has the slightest effect in 1 per cent solution or less on dehydrated pneumococci whether "R" or "S."

TABLE 3

Effect of optochin and desoxycholic acid in alcoholic solution on dehydrated pneumococci

CHEMICAL AND DILUTION	DRIED "S"	DRIED "R"	MOIST "S"
Optochin:			
1 per cent	+	—	—
0.1 per cent	+	+	0
0.01 per cent	+	+	—
0.001 per cent	+	+	0
Desoxycholic acid:			
1 per cent	+	+	—
0.1 per cent	+	+	0
0.01 per cent	+	+	—
0.001 per cent	+	+	—

A few controls indicate the destruction produced by these substances in broth on moist cultures.

TABLE 4

Effect of phenol and of iodine in alcoholic solutions on dehydrated pneumococci

CHEMICAL AND DILUTION	DRIED "S"	DRIED "R"
Phenol:		
5 per cent	+	+
1 per cent	+	+
Iodine:		
1 per cent	+	+
0.1 per cent	+	+
0.01 per cent	—	+

THE ACTION OF IODINE AND PHENOL IN ALCOHOLIC SOLUTION ON DRIED PNEUMOCOCCI

Both iodine and phenol are recognized disinfectants. Carbolic acid is said to destroy pneumococci in dilution of 1:60. It was found to destroy our moist pneumococci when dissolved in broth or Ringer's solution in a dilution of 1:200. Neither sub-

stance was active in the concentrations tested in alcoholic solution on dried preparations as shown in table 4.

THE ACTION OF DYE STUFFS ON DRIED PNEUMOCOCCI

Many dye stuffs, particularly gentian violet, are known to be very destructive for bacteria and particularly for those that retain the Gram stain. Pneumococci "R" and "S" were destroyed when moist in a dilution of gentian violet of 0.1 per cent; they are then apparently more resistant to this dye than streptococci (Gay and Morrison) although no direct comparison has been made. When tested on dehydrated pneumococci, whether virulent or avirulent, alcoholic solutions of gentian violet and of basic fuchsin had not the least destructive action in 1 per cent solution.

THE ACTION OF ESSENTIAL OILS ON DRIED PNEUMOCOCCI

Omeltschenko (1891) tested the germicidal properties of a considerable number of essential oils on the typhoid bacillus, the tubercle bacillus and anthrax spores. He found their effect due not only to a direct action, but particularly to their vapors and respective vapor tension. The oils of cinnamon, fennel and lavender were most effective. The action of the vapors was much less effective on dried bacteria. Destruction of the bacteria tested was accompanied by the appearance of granules and their failure to stain. Our preliminary tests on the direct destructive effect of arachis (peanut), and of cinnamon oil indicated that their addition to moist cultures was definitely germicidal under the conditions that have been specified. We then tested a number of these essential oils, both alone and admixed with equal parts of alcohol on the dehydrated bacteria.

The following oils were tested:

Arachis	Origanum both Crete and pure
Cedar	Sandalwood
Cinnamon	Tansy
Citronella	Thyme
Clove	Wintergreen

All these oils were inactive on dried pneumococci except clove, tansy and, in one test, cinnamon oil. It may be that this excep-

tional action is due to admixture of water with the oil, which possibility is rendered more likely in view of the fact that a mixture of equal parts of these particular oils with water-free alcohol was, in no case, active on the dried preparations.

EFFECT OF HEAT ON MOIST AND DRIED PNEUMOCOCCI BOTH "R" AND "S" VARIETIES

It is generally accepted that the thermal death point for pneumococcus as given by Sternberg is 52°C. for ten minutes. We

TABLE 5

Thermal death points of "moist" "R" and "S" cultures of pneumococcus, Type I

C.	15 MINUTES		30 MINUTES		1 HOUR	
	"R"	"S"	"R"	"S"	"R"	"S"
52°	+	+	+	+	+	0
56°	+	0	+	0	0	0
60°	0	0	0	0	0	0

TABLE 6

Thermal death points of desiccated "R" and "S" cultures of pneumococcus, Type I

C.	15 MINUTES		30 MINUTES		1 HOUR	
	"R"	"S"	"R"	"S"	"R"	"S"
65°	+	+	+	+	+	+
100°	+	+	+	+	+	+
110°	—	—	+	+	—	—
115°	—	—	+	+	—	—
120°	—	—	0	0	—	—
125°	—	—	0	0	—	—
150°	—	—	0	0	—	—

find no reference to the relative resistance of "R" and "S" strains of this or indeed of other organisms nor is there reference to the effect of heat on desiccated bacterial cultures.

In tables 5 and 6 are given our determinations of thermal death points which have been consistently repeated.

Technic. "Moist" bacteria were heated in the form of 1 cc. of an eighteen to twenty-four-hour culture in sealed tubes, at the temperatures and for the periods indicated.

Dry bacteria were heated in 6 mgm. amounts in sealed dry tubes immersed in water (65° to 100°) or in a dry sterilizer (110° to 150°) at the temperatures and for the periods indicated.

CONCLUSIONS

Pneumococcus Type I in the form of both virulent and avirulent ("Smooth" and "Rough") dissociants is susceptible when grown in broth to the usual disinfectants heavy metals, dye stuffs, anhydrous solvents, phenol and iodine; and to certain more or less specific substances such as optochin and bile salts. In two instances it could be shown (mercuric chloride, optochin) that the "Smooth" organism was more readily killed than the "Rough" form.

When the microörganisms are collected by centrifugalization and rapidly dried to constant weight over $CaCl_2$ a large proportion of the cells are killed, the surviving percentage depending on the technique employed. The surviving pneumococci may continue to decrease in number but some, at all events, survive for as yet undetermined periods—for eighteen months at least.

Desiccated but living pneumococci of both forms "R" and "S" are not killed in the absence of water by alcoholic solutions of the substances described except in the case of the heavy metals (mercury salts, silver nitrate). Dried "S" pneumococci, contrary to the findings in moist cultures, are more resistant to mercuric chloride than the "R" forms.

The thermal death point of moist "R" pneumococci (56°) is distinctly higher than that of moist "S" pneumococci. When the two dissociants are dried they are both resist heating to 115° for 30 minutes but are killed by exposure to temperatures of 120° and above.

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