RESISTANCE OF BACTERIAL SPORES TO THE TRIPHENYLMETHANE DYES.

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PLATE 18.

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The resistance of bacterial spores to dyes is of considerable clinical importance. Dyes are being used extensively as disinfectants in the treatment of wounds; and wounds, particularly in war time, may contain spores of pathogenic bacteria. Such spores may lie dormant for days or months.¹ The bactericidal action of dyes in the body tissues may be lost in a comparatively short time. Gentian violet, for example, ceases to be effective for more than a few hours when injected into the blood stream. How long it remains effective in open wounds or closed cavities is unknown. In this connection the fact may be significant that it does not remain bactericidal long in an alkaline solution although alkali increases its action at first.² Needless to say, if spores fail to be killed by a dye they may lie dormant until its bactericidal action is lost and may then develop and account for secondary infections in wounds previously thought to be sterile. It has been stated that spores are killed by certain dyes, especially by gentian violet.³ The experiments described in the present paper indicate that spores are highly resistant to this dye.

The resistance of spores to dyes is also of theoretical importance. If spores are dye-resistant, the possibility of the development of a dye-fast strain comparable to the arsenic-fast strains of trypanosomes must be considered. It is conceivable that spores developing after most of the bacteriostatic power of a dye has been lost may acquire

¹ Burke, G. S., *J. Infect. Dis.*, 1923, xxxiii, 274.

² Burke, V., and Grieve, I., *Am. J. Med. Sc.*, 1924, clxviii, 98.

³ Churchman, J. W., *J. Exp. Med.*, 1912, xvi, 221; 1921, xxxiii, 569; 1923, xxxvii, 1, 543; *J. Am. Med. Assn.*, 1922, lxxix, 1657; *J. Urol.*, 1924, xi, 1.

dye-fastness, or resistance. Experiments in the present paper indicate that vegetative cells of *Bacillus anthracis* can gain in their ability to grow in the presence of gentian violet.

EXPERIMENTAL.

Experiment 1. To Determine the Resistance of Bacterial Spores to Triphenylmethane Dyes.—The technique adopted was designed to eliminate the possibility of apparent death of spores being due to inhibition by dye carried over in transplants or to death of vegetative cells arising from developing spores.

Technique.—1 cc. of an old culture of *B. anthracis* was placed in a test-tube. 15 cc. of a saturated aqueous solution of gentian violet was added. (The saturated solution was made by adding 6 gm. of gentian violet to 30 cc. of water, warming to 80°C. for 20 minutes, and then filtering.) The dye-spore suspension was placed in the incubator at 37°C. for 24 hours and then plated out in agar in three successive dilutions by the loop method, the first tube of agar receiving a loopful of the spore-dye suspension, the second tube a loopful from the first tube, and so on. A control tube was run, with 0.85 per cent saline in place of dye. It received the same treatment except that just before plating it was heated to 80°C. for 10 minutes to eliminate vegetative cells. The plates were incubated for several days and counts made of those from the third dilutions. Twenty-five anthrax colonies appeared on the third plate of the dye-spore suspension and 375 on the third control plate.

By this procedure the spores are washed in agar to remove the dye. The agar readily absorbs the dye and since there is organic matter present tends to reduce its bactericidal action. This method gives roughly quantitative results. When the exposure to the dye lasts for 24 hours the factor of developing spores in both the dye and saline suspensions must be considered and controlled before accurate quantitative results can be obtained. The technique used is satisfactory for demonstrating the resistance of some of the spores but not for determining accurately the percentage surviving.

The experiment was repeated to confirm the initial finding that some anthrax spores at least are very resistant to gentian violet. Methyl violet gave results similar to gentian violet. This is to be expected since gentian violet is either a particular type of methyl violet or a mixture of methyl and crystal violet. Exposure to saturated gentian violet for 10 minutes at 80°C. gave comparable results

although apparently a slightly smaller percentage of the spores developed. Using *Bacillus subtilis* in place of *Bacillus anthracis* produced similar results. The spores of *Bacillus subtilis* appeared to be somewhat more resistant to gentian violet than the spores of *Bacillus anthracis* as a slightly larger percentage developed after exposure to the dye.

With some disinfectants weaker solutions are more effective than saturated solutions. It was thought that this might account for any discrepancies between the results of our work and those of others. Some spores of *Bacillus anthracis* were found to resist dilutions of gentian violet up to 1:10,000 for 24 hours at 37°C. A saturated solution has greater bactericidal action than weaker solutions.

The results obtained in this experiment indicate that not a few spores of Gram-positive bacteria are highly resistant to the triphenyl-methane dyes. That they are more resistant than the vegetative cells of Gram-negative organisms such as *Bacterium coli* can readily be shown. One need only place a mixture of *Bacillus subtilis* and *Bacterium coli* in a saturated aqueous solution of gentian violet, follow the technique used in this experiment, and a pure culture of *Bacillus subtilis* will result. A relatively shorter exposure will suffice.

Experiment 2. To Determine whether a Dye-Fast Strain of Bacillus anthracis Might Develop upon Exposure to a Non-Bacteriostatic Concentration of Gentian Violet.—

Technique.—The anthrax culture used in this experiment was obtained from a third dilution plate made in an experiment like Experiment 1. This colony apparently had developed from a spore or spores resistant to gentian violet. Strokes from this culture were made on agar plates containing concentrations of gentian violet from 1:100,000 to 1:1,000,000. Extract agar containing 1 per cent dextrose and with a reaction of pH 7.5 was used. From a colony arising in the plate containing the greatest concentration of dye, strokes were made on other plates containing the same and greater concentrations of dye. This process was repeated through a number of generations.

By this method it proved possible to obtain a culture of anthrax that would grow in a concentration of dye at least twice that which inhibited the original culture. Fig. 1 shows two dye-tolerant colonies growing in a dilution of 1:400,000 gentian violet. This represents

the limits of tolerance obtained by this method from one culture. The agar in half the plate contains dye in this concentration. The agar in the other half of the plate is dye-free. The plate shows three strokes, the two lower from a dye-resistant culture and the upper stroke from the stock culture. The stock culture did not contain any organisms capable of growing in this concentration of dye in agar. The tolerance of bacteria for the dye has been found to vary with the pH.²

Agar containing a 1:400,000 dilution of gentian violet is distinctly colored and the colonies are also colored. A microscopic examination of the individual cells of such colonies shows the vegetative cells faintly stained. Since the colonies steadily increase in size the cells must be multiplying despite the presence in them of the dye.

The cells of a transplant of *Bacillus anthracis* as well as the strains may vary in their tolerance for gentian violet. The growth of only two colonies from the many cells stroked on the dye agar in Fig. 1 indicates cell variation in ability to tolerate dye. We believe it indicates cell variation in resistance rather than favorable spots in the agar since the colonies contain dye and strokes from these colonies on similar plates gave identical results. Their growth indicated the ability of only a few cells to start growth and produce colonies in this concentration of dye. In a somewhat weaker solution of the dye a stroke from our dye-resistant strain produced a uniform growth across the dye agar while a stroke from the stock culture failed to produce growth on the dye agar, indicating increased tolerance for many if not all the cells. The presence of dye-resistant and non-resistant strains within a culture of *Bacillus anthracis*, as demonstrated in cultures of *Bacterium coli* by Churchman, should be considered. However, the technique employed by us eliminated in the beginning any non-resistant strains that may have been present.

The colonies appearing on the dye agar grew more slowly than the controls. This delayed growth is not due to a loss of bacteriostatic power of the dye as a result of exposure to the agar. The agar was remelted and restroked with the same result as at first, the colonies that grew on the dye agar starting later and developing more slowly than the control.

Colonies growing in the greater concentrations of dye do not always

appear as typical anthrax colonies (Fig. 2). However, strokes from these atypical colonies on dye-free agar result in the development of typical colonies, and broth cultures injected into guinea pigs cause death with typical symptoms.

DISCUSSION.

The results obtained in Experiment 2 demonstrate that the individual vegetative cells of *Bacillus anthracis* vary in their tolerance for gentian violet and that tolerant strains can be obtained by exposure to non-bacteriostatic concentrations of the dye. These facts may be of clinical importance. The variation in resistance and ability to increase resistance may make possible the development of more or less dye-fast strains of *Bacillus anthracis*, and of other organisms as well, during an infection in which dye therapy is used. The organisms may increase in resistance to the dye just as in resistance to immune bodies.

Dye therapy, as at present applied, may favor the development of dye-fast strains of bacteria. Young and Hill have shown that concentrations of gentian violet greater than 1:140,000 are not well tolerated in the blood stream.⁴ We have shown that *Bacillus anthracis* can develop a resistance to and grow in a dilution of 1:400,000 gentian violet in agar. We do not know the concentration of dye anthrax will resist in the blood stream. The short period during which the dye circulates in full strength, followed by weaker concentrations for some time, favors the development of dye-fast organisms. In open wounds where 1 per cent or even saturated solutions of dyes can be used there should be little opportunity for the development of a dye-fast strain of non-spore-bearing bacteria unless the opportunity is offered in the deeper tissues reached only by the diluted dye. However, spores may resist the dye and then develop after its action is greatly reduced. The resulting cells may develop dye-fastness, gain entrance to the blood stream, and resist intravenous injections. This suggests the advisability of using the maximum strength of dye in all applications with the object of killing the spores if possible and destroying all vegetative cells. Dye

⁴ Young, H. H., and Hill, J. H., *J. Am. Med. Assn.*, 1924, lxxxii, 669.

applications should be frequent enough to destroy late developing spores.

The possibility of enhancing the value of dye therapy by rotating the dye treatment deserves consideration. When two dyes are effective against an organism, or organisms in general, it may be advisable, if it can be shown that dye-fast strains develop in the body, to use the first dye in full strength and if the wound or blood stream is not sterilized to use the other dye in the second application. This may prove more effective than continued application of the same dye since increased resistance to one dye may not be accompanied by increased resistance to all dyes. Rotating the dyes may be more effective than combining two dyes as suggested by Churchman.

Churchman considers it advisable when treating mixed infections to combine two dyes, one effective against Gram-positive organisms and the other effective against Gram-negative organisms. But, as the present work shows, there is ground for the belief that any dye destroying the Gram-negative organisms will also destroy the Gram-positive organisms, exclusive of spores.

The ability of bacteria to increase in tolerance for dyes has a bearing on the testing of antiseptics. Dakin and Dunham in testing the bactericidal action of various dyes in weak concentration for *Staphylococcus aureus* found that after some hours the organisms grew unchecked.⁵ They concluded that, "the dyes in question are readily inactivated and that the presence of apparently unchanged coloring matter in the mixture is no evidence of the continuation of any antiseptic action." In view of our experiments it is possible that the growth of the organisms after the initial inhibition is due to an increase in tolerance for the dye rather than to a lessening in the action of the latter.

CONCLUSIONS.

1. Bacterial spores are highly resistant to the bactericidal action of the triphenylmethane dyes. Many *Bacillus anthracis* and *Bacillus*

⁵ Dakin, H. D., and Dunham, F. K., A handbook of antiseptics, New York, 2nd edition, 1918, 92.

subtilis spores resist a saturated aqueous solution of gentian violet for 24 hours at 37°C. They also resist exposure to the same dye solution for 10 minutes at 80°C.

2. The selective bactericidal action of these dyes applies only to the vegetative cells. Spores of the Gram-positive bacteria are more resistant to these dyes than the vegetative cells of Gram-negative bacteria.

3. The vegetative cells of *Bacillus anthracis* show variation in resistance to the bacteriostatic action of gentian violet. The spores of *Bacillus anthracis* show variation in resistance to the bactericidal action of gentian violet.

4. *Bacillus anthracis* can increase in resistance to the bacteriostatic action of gentian violet and grow in dye dilutions inhibiting the original culture. There is the possibility of pathogenic organisms becoming dye-resistant in the body when exposed to non-bactericidal concentrations. For this reason it is advisable to use the greatest concentration of dye compatible with tissue tolerance.

5. Since the spores of pathogenic bacteria may lie dormant for longer periods than the dyes retain their bacteriostatic action in the body frequent applications of the dye should be made in preventing infections by spore-bearing bacteria.

EXPLANATION OF PLATE 18.

FIG. 1. Strokes of *B. anthracis* on a plate containing gentian violet agar and dye-free agar. The upper stroke is from the stock culture and the two lower strokes from a dye-tolerant culture.

FIG. 2. A slant of gentian violet agar with deeply stained atypical anthrax colonies on the surface.