PHYSICAL ACTION OF SURFACE-ACTIVE CATIONS UPON BACTERIA

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A considerable amount of work has been reported on the surface-active cations, and several workers have shown that, although these compounds are highly germicidal, they are also highly bacteriostatic in relatively high dilutions. Although these compounds are highly bacteriostatic, there has been little work done to distinguish between actual germicidal activity and bacteriostatic action. Baker, Harrison, and Miller (1941) made the first attempt to separate bacteriostatic from germicidal action by the use of phospholipids. They found that the effect of the cationic compounds upon the test bacteria was seriously impaired when the bacteria were added simultaneously with the phospholipids. They were, however, unable to revive the bacteria once they had been in contact with the cationic compound. Valko and DuBois (1944) were the first workers to show the so-called reversibility of the surface-active cations. They were able by the use of "duponol PC" to neutralize in part the effect of a 1:3,000 dilution of "zephiran" upon *Staphylococcus aureus* after 10 minutes' exposure. Using *Escherichia coli* and *Eberthella typhosa*, they were able to obtain reversal up to 5 minutes. The measurement that they used for kill or survival was the absence or presence of growth in the broth subculture tubes. As mentioned by Hotchkiss (1946) this is a qualitative rather than a quantitative procedure and assumes either complete killing or no killing at all rather than the percentage surviving. Unfortunately, although such data show that neutralization has occurred, there is no information upon the degree of neutralization. The work presented in this paper further substantiates the fact that the action of the surface-active cations is reversible. The techniques used vary from those of previous workers in the method of approach and in that they are quantitative.

EXPERIMENTAL METHODS AND RESULTS

These studies were initiated originally by the fact that when spores of *Bacillus subtilis* were exposed to surface-active cations, complete killing was obtained after short periods of exposure. The writers were firmly convinced that the bacterial spores were not susceptible to this degree to the action of these compounds and that the apparent killing was actually a bacteriostatic action. When the treated spores were subsequently treated with anionic neutralizing agents, a few spores recovered from their inhibition, but the apparent percentage of killing was still exceedingly high. It was finally decided to attempt the removal of the excess surface-active agent by centrifugation of the treated spores followed by repeated washing with sterile distilled water or physiological saline. This procedure gave

a high percentage of recovery of the spores, far greater than that obtained by neutralization by an anionic compound.

In the preparation of the spore suspensions, *Bacillus subtilis* was grown on the surface of plain agar for 7 days, after which time the growth was harvested in distilled water and the resulting suspension heated to 80 C for 10 minutes for the purpose of destroying the vegetative cells and the heat-susceptible spores. This suspension was immediately cooled and preserved by storage at 10 C for subsequent use.

In the conduct of the experiments a constant amount of the spore suspension was treated in duplicate with the cationic compound giving a final concentration of the compound of 1:2,000 based upon the anhydrous material. A control containing the same amount of spores suspended in distilled water was carried along to determine as closely as possible the number of spores being treated in the various steps. These samples were centrifuged until the spores were thrown down, the supernatant was removed, sterile distilled water was added to the original volume, and an aliquot was removed for determining the bacterial count.

TABLE 1

The reversal of bacteriostatic effect of 1:2,000 "B.T.C." on spores of Bacillus subtilis by centrifugation

TREATMENT	NO RINSE	1ST RINSE	2ND RINSE	3RD RINSE
None		151,000	100,000	150,000
1	0	0	590	1,900
2	0	0	160	2,700

The suspension was then centrifuged a second and third time, the supernatant being removed and a bacterial count being made each time. Another untreated control was allowed to stand during the course of the experiment and was plated at the termination of the experiment. The data are presented in table 1. From these data it can readily be seen that there is a definite revival of the theoretically killed spores as shown by the comparison of the bacterial count of the first rinse and the treated control. Furthermore, this revival increases with the greater number of rinses.

In the foregoing experiments it was noted in every instance that the bacterial counts of the various dilutions plated failed to follow the normal pattern, e.g., the low dilution gave a lower count per plate than did the higher dilution, although the number of colonies was within the normal number of colonies (25 to 250) permissible for counting. This could only mean that the simple process of dilution and shaking was further reversing the action of the surface-active cations.

Following the foregoing observation, an experiment was conducted to determine whether or not this reversal could be demonstrated by a simple dilution technique. The tubes were prepared in the same manner as in the previous experiment. From these tubes 1-ml samples were drawn at 2-minute intervals for 20 minutes, placed in 99 ml of sterile distilled water, and shaken vigorously 50

times. This diluted suspension was plated in amounts of 1 ml and 0.1 ml, and 1 ml of a 1:100 dilution. A control suspension of the spores in distilled water was plated before the experiment was run, held during the course of the experiment, and then plated again at its termination to determine whether or not there had been any large change in the population during the course of the experiment due to external factors. Table 2 gives a typical set of results. The data show that no germicidal action occurs after the first 2 minutes of exposure. The bacterial population remains practically constant for the entire 20 minutes of exposure. It can be seen that in the 1:2,000 dilution of the cationic agent practically all of the spores were recovered, in the 1:1,000 dilution a kill of 50 per cent was apparent, and in the 1:500 dilution the kill was increased to approximately 90 per cent.

TABLE 2

The reversal of bacteriostatic effect of "B.T.C." on spores of Bacillus subtilis by dilution and shaking

EXPOSURE TIME	NUMBER OF BACTERIA PER ML		
	Trial 1 1:2,000	Trial 2 1:1,000	Trial 3 1:500
Initial count	310,000	770,000	2,320,000
2 minutes	149,000	310,000	206,000
4 minutes	136,000	384,000	206,000
6 minutes	256,000	204,000	196,000
8 minutes	218,000	428,000	160,000
10 minutes	272,000	276,000	118,000
12 minutes	140,000	224,000	141,000
14 minutes	248,000	168,000	126,000
16 minutes	264,000	254,000	124,000
18 minutes	240,000	134,000	114,000
20 minutes	278,000	168,000	158,000

Data are presented in table 3 showing the effect of dilution by nutrient agar upon the number of colonies appearing on the plates. It is interesting to note that although the 1-ml and 0.1-ml portions came from the same flask, which was the first dilution of the treated suspension, the plate count from the 0.1-ml portion in practically all cases yielded a greater number of colonies than did the 1-ml portion. This would indicate that the dilution resulting from mixing the organisms in the nutrient agar tended to eliminate the bacteriostatic action. This would be in addition to the reversing action of the agar itself as reported by Quisno *et al.* (1946).

Much work has been reported on the electrophoretic mobilities of bacterial cells: some in regard to their pathogenicity (Frampton and Hildebrand, 1944), some regarding the stage of growth of an individual organism (Moyer, 1936*a*), some regarding the effect of cations in general upon organisms (Moyer, 1936*b*), and most recently some on the effect of surface-active agents upon various bacterial cells (Dyar and Ordal, 1946). Electrophoretic mobility, using a technique

similar to that used by the foregoing workers, was used to show the removal of surface-active agents from the surface of bacteria by washing procedures.

A simple electrophoretic cell was used with a depth of 600 microns. The distance between the electrodes was 3.4 cm. To keep from encountering various currents within the electrophoretic chamber of this type, all determinations were made with the microscope focused at a distance of 400 microns below the bottom of the cover glass. This depth was chosen because it has been shown by other workers that, in a 600-micron cell, the most stationary levels are at 200 and 400 microns from the bottom of the cell. The lower level (200 microns from the bottom of the cell) was chosen for observation because the spores are relatively heavy and tend to settle toward the bottom of the cell. A constant potential of 20.0 volts was used for all tests, and a current of 1 milliampere was never exceeded.

TABLE 3

The reversal of bacteriostatic effect on spores of Bacillus subtilis by agar plating after treatment with 1:500 dilution of "B.T.C." as demonstrated by comparative counts obtained with 1-ml and 0.1-ml dilution agar plates

SAMPLE NUMBER	NUMBER OF COLONIES APPEARING ON THE PLATES	
	1.0 ml	0.1 ml
1	14	206
2	33	206
3	40	196
4	45	160
5	11	118
6	63	141
7	18	126
8	63	124
9	122	114
10	66	158

In making the electrophoretic tests, the time for a single spore in the electrical field to traverse 200 microns in one direction was observed and recorded. The poles of the cell were then reversed and the time for the same spore to travel back to its original position was determined. This was done for the purpose of nullifying any currents, convection or otherwise, which may have been present at that level in the preparation. Five determinations on each preparation were made in this fashion, the 10 time intervals were averaged, and the mean was used for the calculation of the rate of speed of the spores in the electrical field.

In the tests, the samples were centrifuged and washed in the same manner used to obtain the results in table 1. Aliquots were removed after each washing and the electrophoretic mobilities determined. The concentrations of the surface-active cations were 1:1,000, 1:2,000, and 1:4,000. The mobility rate of the untreated spore suspension was determined as a control.

The data for the foregoing experiment using "roccal" as the surface-active

cation are given in table 4. Similar experiments were conducted using "B.T.C." and "tetrosan" as the surface-active cations, and also using as test organisms *Escherichia coli* and *Staphylococcus aureus*. The data for the last mentioned are not given because they present identically the same picture as that presented in table 4.

In the foregoing experiments no attempt was made to regulate the pH by the use of a buffering system since preliminary experiments showed only negligible differences between mobilities in the buffered and unbuffered solutions. The data show that when the organisms were treated with a 1:1,000 solution of the cationic agent, the spores changed from a negative mobility of 19 microns per second to a positive mobility of 18 microns per second. After one washing the positive charge was lost by the removal of the cationic substance from the surface of the spore and from the solution. The spore now showed a negative mobility of 16.9 microns per second. A second washing increased the negative mobility to a point greater than the negative mobility of the untreated spores.

TABLE 4

The electrophoretic mobilities of spores of Bacillus subtilis when treated with "roccal" followed by centrifugation and washing with distilled water

TREATMENT	MOBILITIES—μ/SEC IN 20 VOLT FIELDS		
	Dilution of cationic agent		
	1:1,000	1:2,000	1:4,000
No wash	+18.6	+16.7	+7.0
First wash	−16.9	−16.9	−20.3
Second wash	−25.5	−25.9	−19.8
None	−19.0	−19.4	−19.4

The symbols + and − represent electrical charges on spores.

These data confirm the bacteriological finding that dilution will remove the cationic compounds from the surface of the spores.

In the light of the results above, further studies were conducted on *Escherichia coli*, *Eberthella typhosa*, and *Staphylococcus aureus* using the dilution technique as a method of reversing the action of the cationic compounds. Dilutions of 1:5,000, 1:6,000, and 1:10,000 of the surface-active cations were used with an inoculum of over 10,000,000 organisms per ml. In no instance, either by shaking or centrifuging, could the organisms be recovered after an exposure of 3 minutes or more. In most cases a count of 1 or 2 colonies was found on the plates containing the 1:100 dilution of the cationic agent and organism suspension after an exposure of 1 minute. In one case a count of 1,700 per ml was obtained after the exposure of *Staphylococcus aureus* for 1 minute. This dropped to 300 per ml in 2 minutes, and then to zero in 3 minutes. This, then, would seem to indicate that vegetative cells are killed or sufficiently hampered by the action of the cation so that their recovery by either of the methods, washing or centrifuging, is not possible. Dilutions of the cationic compound greater than 1:10,000 were

not used. This policy was adopted since these compounds are used in the field in dilutions of less than 1:10,000.

In making the electrophoretic studies, the question arose as to whether or not clumping of the organisms would occur if the concentration of the cationic agent was such that it just neutralized the surface charge of the organisms, or if any clumping occurred spontaneously during the transition of the organism from a negative to a positive charge owing to the adsorption of the compound. To determine whether or not such was the case, tests were run on *Escherichia coli*, *Eberthella typhosa*, *Pseudomonas aeruginosa*, and *Staphylococcus aureus*. Twenty-four-hour broth cultures of these organisms were centrifuged to remove the broth, and were then resuspended in sterile water by vigorous shaking to assure the breaking up of all clumps that might have been formed by centrifugation. These suspensions were then added to varying dilutions of three different quaternaries, namely, "roccal," "hyamine 1622," and "B.T.C." Glass cover slips were placed in the dilutions of cationic substances before the organism suspensions were added to facilitate the clumping of the organisms upon a glass surface, as has been reported previously. After the addition of the organism suspension, the cover slips were removed, placed upon a clean slide, and examined by using dark-field illumination. The following results represent the three quaternaries collectively since no differences in their reactions were noted.

Pseudomonas aeruginosa—clumping occurred up to a dilution of 1:4,000. No clumping in dilution of 1:5,000 or greater.

Escherichia coli—clumping occurred up to a dilution of 1:2,000. No clumping in dilutions of 1:4,000 or greater, with the exception of 1:60,000, which showed no clumping immediately but did after a 24-hour period.

Eberthella typhosa and *Staphylococcus aureus*—no clumping in any dilutions from 1:1,000 to 1:100,000.

It will be recalled that in a previous portion of the paper dealing with reversal, it was noted that when working with dilutions commonly used for disinfection purposes, namely, 1:1,000 to 1:7,000, no reversal of the cationic action could be obtained with vegetative cells even though it was shown electrophoretically that the compound had been washed from the surface of the organism by shaking, which process was sufficient effectively to disperse any clumps present. This, then, would seem to show that the organisms had either been killed before the clumping occurred, or, more logically, that the compound adsorbed on the surface of the organisms continues its action even though the organisms are clumped.

It is of interest to note that in conducting the foregoing experiments the dark-field illumination showed those organisms that had been killed or immobilized by the cationic agent to have a much higher refractive index than normal organisms or those organisms present in the solution which had not as yet been immobilized.

An application of the possible reversal by dilution and shaking was attempted by the authors while collecting data for another paper (Mallmann, Kivela, and Turney, 1946). While taking swabs of beverage glasses treated with 150 ppm of a surface-active cation, the swab bottles were shaken 30 to 50 times to thor-

oughly fluff the cotton of the swab immediately after the swab had been taken. In this manner it was hoped that the compound could be washed from the surface of the organism. The fact that possible bacteriostasis may occur from the compound carried over on the swab was disregarded since, from previous experiments carried on by the authors, it has been quite conclusively shown that the extremely high dilutions of the compound resulting in the swab bottles would have no effect upon the bacterial population. The results showed no difference in count between the shaken and unshaken swabs, which again demonstrates that the organisms present on the glasses were evidently killed before the action of the cationic agent was reversed.

In the light of the studies cited on electrical charges and the apparent adsorption of the cationic compounds on the surface of the organism, an attempt was made to correlate these with a possible mode of action of the cationic surface-active agents. Hotchkiss (1946) reported that the cell contents of organisms had been found in the supernatant fluid after the organisms had been acted upon by surface-active cations, and he believed that this was due to the denaturation of the cell wall, which thereby destroyed its selective permeability and allowed the cell fluids to escape into the suspending fluid. These authors thought that, in addition to the cell wall breakdown, the osmotic pressure exerted by these compounds might increase the amount of these cell fluids in the surrounding medium. Thus a study of the osmotic pressure of a surface-active cation was made, using "roccal" as a typical compound.

The results obtained are what would normally be expected judging from the molecular weight of this compound. The osmotic pressure exerted by the compound, as determined by a purely physical test, would not seem to exert an appreciable effect. However, this does not reveal the entire condition. It should be remembered that in very dilute solutions of a cationic compound enough is adsorbed on the surface of the organism to change its charge from a negative one to a positive one. This, then, would indicate that the concentration of the compound upon the surface of the organism is much greater than the concentration of the compound in the solution. The authors believe that this adsorbed material exerts an osmotic pressure comparable to its concentration on the bacterial cell. This means that an organism in a 1:1,000 dilution of a cationic compound would not be exposed to an osmotic pressure of only a 1:1,000 dilution but would be subjected to an osmotic pressure greatly in excess of that, owing to the high concentration of the compound upon the organism. This osmotic pressure would tend to draw the cell fluids through the disrupted cell wall into the surrounding fluids. This belief was further strengthened by Dyar (1947), who observed, while employing a 1:300 dilution of cetyl pyridinium chloride for a cell wall stain, that the examination of the bacterial cells in water under a cover slip showed the cytoplasm to be shrunk away from the cell wall.

It is quite probable that the surface-active agents are adsorbed on the surface of the bacterial cells in sufficient concentration to interfere with the osmotic balance of the organism and its surrounding menstruum, and in this manner may prevent the intake of nutrients. This would help to explain why these

compounds are so highly bacteriostatic in high dilutions, and why the bacteriostatic effect can be removed by dilution and vigorous shaking or by the use of neutralizing agents. Further, it would explain partially why reversal can be obtained using spores, since their cell wall is much thicker than that of vegetative cells and consequently more resistant to chemical changes as well as osmotic effects.

SUMMARY

The bacteriostatic effect of surface-active cations on bacterial spores can be reversed by dilution and shaking in distilled water or physiological saline solution.

The reversal of the bacteriostatic effect of surface-active cations by the removal of the cations adsorbed on the bacterial spores by dilution was proved by demonstrating that negative mobilities of the spores were restored by washing the cells with distilled water.

The high osmotic pressure exerted on the bacterial cell by the adsorbed surface-active cation may explain, in part, the destruction of vegetative cells by the discharge of cell fluids into the suspending solution.

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