THE ANTIBACTERIAL ACTION OF SURFACE ACTIVE CATIONS[1]

E. I. VALKO AND A. S. DuBOIS

Onyx Oil and Chemical Company, Jersey City, N. J.

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The antibacterial action of surface-active cations was reported by Hartmann and Kägi (1928) on the basis of observations made by Doerr. Domagk (1935) gave a detailed description of the bacteriological properties of a very potent member of this group. Notwithstanding the considerable attention which these discoveries aroused, as shown by the number of investigations reported in the literature, apparently no attempt has been made to relate the antibacterial action of surface-active cations to that of other types of toxic cations.

The observations on the reversibility of the antibacterial effect of the surface-active cations, and on the protective action on bacteria exerted by relatively harmless cations against the action of toxic cations, which are described in this paper, indicate that there is a close relationship between the antibacterial behavior of metallic and dye cations and that of surface-active cations. It appears that the basis for the antibacterial action of all these cations can be satisfactorily described in terms of a cationic exchange by the bacteria. It is not claimed that this concept explains the ultimate mechanism of the antibacterial action, but nevertheless it probably is a necessary step in this direction and makes possible the elimination of other proposals which have been put forward.

The most striking example of the reversibility of the antibacterial action has been presented by Engelhardt (1922), although the reversibility of the action of mercuric chloride on bacteria was already known (Geppert, 1889; Süpfle and Müller, 1920). Engelhardt demonstrated that *Staphylococcus aureus* remained viable after a two-hour exposure to 1% mercuric chloride, when thereafter the poison was removed by washing. When hydrogen sulphide was used for detoxication, even the effects of a 72-hour exposure to 1% mercuric chloride proved reversible. Similar results were obtained with anthrax spores.

Simon and Wood (1914), adopted Ehrlich's conception of a combination between the antibacterial agent and some of the nutriceptors of the bacteria as the basis for the inhibiting action of basic dyes and assumed the existence of acidic groups in the structure of the bacterial organism with which the ammonium groups of the inhibiting dyes would unite. The cell dies, according to them, because a sufficient number of its nutriceptors have been thrown out of action, bringing about its starvation or inability to multiply.

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A brief account of the observations reported in this paper was given at the joint meeting of the New Jersey and New York branches of the Society of American Bacteriologists, Princeton, N. J., May 16, 1942. (J. Bact., **44**, 394 (1942).)

Stearn and Stearn (1926, 1928) proposed a general theory of reversible adsorption by the acidic groups of the bacteria as the basis for the antibacterial action of cations, including those of the heavy metal salts and basic dyes as well as the cations of the substituted aniline type. McCalla (1940), recently drew attention to the experiments of Engelhardt and carried out similar ones on *Escherichia coli*. He described these as an example of cationic exchange by bacteria. Our own results, presented below, supply a confirmation for this theory and permit its extension to surface active cations.

EXPERIMENTS AND RESULTS

The *Eberthella typhosa* and *Staphylococcus aureus* used in these experiments had the phenol resistance required by the F. D. A. method (Ruehle and Brewer, 1931). The F. D. A. culture medium was used except in the experiments on acriflavine.

Zephiran (technical) which is a 10% aqueous solution of mixed alkyl dimethyl benzyl ammonium chlorides, was purchased from the General Dyestuffs Corp., New York, N. Y.; cetyl pyridinium bromide, was prepared by the condensation of cetyl bromide with pyridine and recrystallized twice from acetone. The phenol coefficient of the Zephiran, (100% active) was 187, and that of the cetyl pyridinium bromide 400, tested against *S. aureus* at 37°C.

The N-stearoyl diethylene triamine lactate was prepared by condensation of commercial stearic acid with diethylene triamine and subsequent neutralization with lactic acid; the N-dodecyl dimethyl benzyl ammonium chloride by reaction of benzyl chloride with distilled dodecyl dimethyl amine; the benzotriazolium derivatives by the method of Kuhn and Westphal (1940).

Acriflavine (Neutral, U.S.P.) was purchased from the National Aniline and Chemical Co., New York, N. Y. Duponol PC, a pharmaceutical grade of sodium dodecyl sulfate, was purchased from E. I. duPont de Nemours, Inc., Wilmington, Del.

Our first series of experiments have been carried out in order to find out whether the antibacterial action of the surface-active cations could be reversed by detoxication. After several unsuccessful experiments using picric acid, soap, etc. we finally selected sodium dodecyl sulfate (Duponol PC) as detoxifying agent.

The procedure for this series consisted in adding 0.5 ml. of the required dilution of the antibacterial agent to 15 ml. distilled water at 37°C. This was then inoculated with 0.5 ml. of a 22–26 hr. old culture of the organism. After certain periods, indicated in the tables as "time of exposure," either 0.5 or 0.75 ml. of an equivalent dilution of the reviving agent was added. Subcultures were made after varying periods, denoted in the tables as "time of detoxication," by transferring five 4-mm. loopfuls into nutrient broth. The tubes were incubated at 37°C. for 48 hours and examined for growth. In the tables, + indicates growth, i.e., revival; 0 absence of growth, i.e., no revival.

From each medication tube, a subculture was made before adding the reviving agents. In no case was growth observed.

In each set of tests, a control was carried out using both the antibacterial and reviving agents in the same tube, but without bacteria and a loopful was transferred into broth. The absence of growth, observed in all cases, demonstrated the sterility of the solutions of the antibacterial and reviving agents.

A number of revived cultures were subcultured. Growth was observed in all these cases, giving further evidence of the viability of the revived bacteria.

TABLE 1

Reversibility after treatment with various concentrations of Zephiran

Organism: *S. aureus*
Time of exposure: 5 minutes
Time of detoxication: 5 minutes

CONCENTRATION OF ZEPHIRAN*	DUPONOL PC	
	100 per cent equivalent	150 per cent equivalent
1:1000	0	0
1:3000	+	+
1:15,000	+	+

* In all the tables, the dilution of Zephiran is expressed relative to the active ingredients and not to the commercial product.

TABLE 2

Reversibility after various times of exposure and of detoxication

Organism: *S. aureus*
Concentration of Zephiran: 1:3000

TIME OF DETOXICATION	TIME OF EXPOSURE (MINUTES)			
	3½	5	10	30
	100 PER CENT EQUIVALENT DUPONOL PC			
minutes				
1	+	+		0
5	+	+	+	0
10	+	+	+	0
30	+		+	0
	150 PER CENT EQUIVALENT DUPONOL PC			
1	+	+	0	
5	+	+	0	0
10	+	+	0	
30	0	0	0	

Table 1 shows that under the conditions of the experiments, *S. aureus* exposed to the action of 1:1000 Zephiran, representing 15 times the so-called "killing" concentration, as determined and defined by the F.D.A. method, could not be revived. However, after exposure to a concentration which is still 5 times the "killing" concentration, revival could be accomplished by detoxication with one as well as one-and-one-half equivalent of the surface active anion.

Table 2 shows that revival is possible only when the time of exposure to the antibacterial agent does not reach a certain limit. Furthermore, it demonstrates the fact that when the antibacterial effect is reversible, the detoxicating treatment does not require more than one minute. In a few cases, which are not reported in the table, when only half a minute was allowed for detoxication, revival was likewise accomplished. Through prolonged contact with excess detoxicating agents, the bacteria may again lose their viability. This is somewhat surprising because mere exposure of the bacteria to the concentration of Duponol present during detoxication, does not inhibit their growth in subculture. Obviously,

TABLE 3

Revival experiments using cetyl pyridinium bromide against S. aureus

Concentration of cetyl pyridinium bromide: 1:3000
Time of exposure: 5 minutes

TIME OF DETOXICATION	100 PER CENT EQUIVALENT DUPONOL PC
minutes	
$2\frac{1}{2}$	+
5	+
10	+
30	+

TABLE 4

Revival experiments with Escherichia coli

Concentration of Zephiran: 1:3000
Time of exposure: 5 minutes

TIME OF DETOXICATION	DUPONOL PC	
	100 per cent equivalent	150 per cent equivalent
minutes		
$2\frac{1}{2}$	+	0
5	+	0
10	+	0
30	+	0

there was a difference between the behaviour of the revived culture and that of the fresh ones.

Table 3 demonstrates that the effect of cetyl pyridinium bromide on *S. aureus*, can also be reversed.

Table 4 shows that the effect of Zephiran on the gram-negative bacterium, *Escherichia coli*, is reversible under the same conditions as with the gram-positive organism, *S. aureus*.

Table 5 shows that this is also true with *Eberthella typhosa*.

Our second series of experiments represent an attempt to obtain information as to whether or not the adsorbability and specific toxicity of cations can be distinguished as factors of their antibacterial action.

It is evident that the antibacterial action of cations (or of any other substance) is more closely dependent on the actual amount of the cations on or in the bacteria than on the concentration of the cations in the solution with which the bacteria are treated. It is to be assumed that a shift of the normal cationic (or ionic) balance in the bacterial organism alone would affect the bacteria. The extent of the damage may be determined alone by the extent of the shift regardless of the specific nature of the cation causing it or it may depend upon the latter. In the first case the adsorbability alone is the determining factor, whereas in the second the specific toxicity is a contributing factor. The investigation of the effect of the simultaneous application of a different cation was expected to give some indication of the relative contribution of these two factors. This was carried out by determining the "killing" dilution of mixtures consisting of a highly toxic cation, e.g. N-dodecyl dimethyl benzyl ammonium chloride and small amount of a relatively harmless cation, e.g. N-hexadecyl dimethyl benzyl ammonium chloride. The dilution of the toxic cation in these mixtures was

TABLE 5

Revival experiments with Eberthella typhosa

Concentration of Zephiran: 1:3000

Time of exposure: 5 minutes

TIME OF DETOXICATION	DUPONOL PC	
	100 per cent equivalent	150 per cent equivalent
minutes		
5	+	0
10	+	0
30	+	0

compared with its "killing" dilution when applied alone. If the "killing" dilution in the mixture is higher, then the addition of the second component has an antagonistic effect. This was observed in all cases. A further indication of this antagonistic effect was found in the comparison of the antibacterial effect of pure 1-dodecyl-3-ethyl benzotriazolium ethosulfate with that of a preparation of the same compound containing 25% of higher homologs, which have a lower antibacterial efficiency.

In these experiments the F. D. A. method of determining phenol coefficient was followed, using *S. aureus* at 37°C.

The results obtained are summarized in Table 6. Similar results have been obtained using *E. typhosa* at 20°C., although in this case the effect was not so pronounced.

The data indicate that the effect of relatively harmless surface active cations on the antibacterial action of highly toxic surface action cations, is not only not additive, but definitely subtractive. This phenomenon can easily be explained by assuming that the adsorption equilibrium of the toxic cations by bacteria is shifted through the competition of the harmless cations for the same spaces

(presumably the carboxylic groups of the protein material) on the bacteria. Further systematic investigations of the protective effect of harmless cations would be required in order to provide some approximate information on the relative contribution of the affinity and specific toxicity of the cations to their antibacterial action.

TABLE 6

Protective action of relatively harmless cations

	DILUTION KILLING IN 10 BUT NOT 5 MINUTES
Compounds tested separately:	
N-Dodecyl dimethyl benzyl ammonium chloride (A)	1:40,000
N-Stearoyl diethylene triamine lactate (B)	1:1250
N-Hexadecyl dimethyl benzyl ammonium chloride (C)	1:15,000
N-Octadecyl dimethyl benzyl ammonium chloride (D)	1:8000
Above compounds tested in mixtures:	
Mixture of A with 20% B*	1:27,500
Mixture of A with 25% C*	1:27,500
Mixture of A with 25% D*	1:28,000
Pure and mixed benzotriazolium compounds:	
1-Dodecyl-3-ethyl benzotriazolium ethosulfate† (E)	1:7500
1-Alkyl (C_{12} to C_{16}) benzotriazolium ethosulfate (consisting of about 75% E)	1:5000

* The killing dilution is that of A alone.

† We could not confirm the high germicidal value claimed for the compound by Kuhn and Westphal (1940). This is in agreement with the observation of Rawlins, Sweet and Joslyn (1943).

TABLE 7

Antagonistic effect of sodium dodecyl sulfate on acriflavine

ACRIFLAVINE (1:2000)	E. COLI	DUPONOL PC (1:2000)	GROWTH	
			In broth	On Endo agar
ml.	*ml.*	*ml.*		
0.2	0.1	0.2	0	0
0.2	0.1	0.3	0	0
0.2	0.1	0.4	0	0
0.2	0.1	0.6	+	+
0.2	0.1	0.8	+	+

A third series of experiments were suggested by McIlwain's (1941) observation that the inhibiting and killing action of acriflavine can be prevented and reversed by tryptic casein, yeast extract and similar materials.

Our experiments were carried out in order to determine the antagonistic effect of sodium dodecyl sulfate to acriflavine. They were conducted by inoculating tubes containing a protein-free mineral medium described by McIlwain and

various dilutions of acriflavine or of acriflavine plus Duponol PC, with *E. coli* and incubating at 37°C. Prevention of inhibition, i.e. the antagonistic effect, was confirmed by plating on Endo agar. The results are summarized in Table 7. The results obtained indicate that a high molecular anion can at certain concentrations, inhibit the antibacterial action of acriflavine.

DISCUSSION

The experiments presented above are, to our knowledge, the first ones to indicate the reversibility of the antibacterial ("killing") effect of surface active cations. Baker, Harrison and Miller (1941) recently attempted reversal with phospholipids which, as they demonstrated inhibit the antibacterial effect of surface active cations when simultaneously applied to the bacteria. Their results were negative, possibly because the time of exposure of the bacteria to the toxic cation, 30 minutes, was too long or because the phospholipids are not powerful enough detoxicating agents. Our results are, however, by no means surprising in view of the successful revival experiments with mercuric chloride quoted in the introduction.

The initial process in the action of surface-active cations can be satisfactorily described as a reversible adsorption by the bacteria, which function as cationic exchangers. There is ample evidence for such a function of the bacteria (McCalla, 1941). It is remarkable that the electrochemical equivalent weight of bacteria as measured by cationic exchange is about 800–1300, which agrees fairly well with that of such animal proteins as albumins. Stearn and Stearn (1926) had previously assumed that the electrochemical equivalent weight of bacteria is essentially equal to that of its proteins. Since McCalla has confirmed this assumption and observed the rapid and complete exchange of cations by bacteria it seems that cations of all types can quickly penetrate the bacteria and that their adsorption depends on their specific attraction for the acidic groups of the bacteria. It does not seem warranted to consider the surface activity of the toxic cations as an important factor of their antibacterial action. Surface-active cations probably penetrate bacteria just as basic dyes do and it is not correct to regard their adsorption as a surface phenomenon. The surface activity and the adsorbability both depend on certain features of the structure of the cations. These may be equally pronounced in certain toxic cations, e.g. in those considered here. However, other types of toxic cations e.g. mercuric or dye ions, are strongly adsorbable and yet have only little surface activity.

Obviously, the demonstration of reversibility presented by Engelhardt (1922), McCalla (1940), as well as ourselves, cast considerable doubt on the correctness of such expressions, as bactericidal versus bacteriostatic action as these are sometimes currently used. The effect is now sometimes (although incorrectly) called bactericidal if the inhibition of the bacterial growth persists in the subculture. In view of the possibilities of a reversible toxication, this means only that the detoxication is not accomplished by mere dilution with the culture medium. On the other hand, in the case of complete reversibility a sufficient dilution should accomplish detoxication and revival. With surface active

cations, as with mercuric chloride, the cultures after exposure to a "killing" concentration could be diluted with the nutrient medium far below the so-called "killing" concentration without showing any revival. We believe that the basis for this phenomenon is the fact that the establishment of the desorption equilibrium requires, under certain circumstances, a comparatively long time. It may be, that during this desorption process, secondary, irreversible changes occur in the bacteria preventing their recovery.

The protective action of relatively harmless cations against the action of toxic cations of similar type belongs to the general category of antagonistic phenomena. Cases of so-called chemotherapeutic interference (Findlay and Wenyon, 1939), showing the protective action of relatively harmless dye cations against toxic cations on micro-organisms obviously belong to the same group. Hoffman, Schweitzer and Dalby (1941) reported recently the subtractive effect exerted by a weak fungistatic fatty acid on a strongly fungistatic one. They suggested two possible explanations: (1) the presence of a molecular aggregate comprising both kind of molecules, (2) a competitive absorption. In our case, because of the extremely low concentration of the antibacterial ions, we believe that the second explanation is more likely.

The dependence of the antibacterial action of cations (and anions) on the pH, as first observed by Browning, Gulbransen and Kennaway (1919) on acridine, can be described in terms of a competitive ionic exchange by the bacteria. This was done for dye ions by Stearn and Stearn (1924) as well as by McCalla (1941). Gershenfeld *et al.* (1941) and Scales and Kemp (1941) reported the completely analogous dependence of the antibacterial action of surface-active cations and anions on the pH. The inhibitory action of surface-active cations increases with increasing alkalinity and that of the anions, with increasing acidity and this holds for both gram-negative and gram-positive organisms. The statement that surface-active anions do not prevent the growth of gram-negative bacteria (cf. Dubos, 1942) holds only for neutral or alkaline media. If the pH is low enough surface-active anions do show an antibacterial effect against gram-positive organisms. The correlation of the analogous behaviour of dyes with the electrochemical properties and possibly with the lipoid content of the bacteria was discussed by Stearn and Stearn (1928).

Since Woods' (1940) discovery of the effect of p-aminobenzoic acid on the inhibitory action of sulfanilamide, increasing attention has been directed to the phenomenon of antagonistic effects. The experiments reported in this paper deal with two types of antagonism: (*a*) antagonism by direct chemical inactivation of the toxic substance, e.g. inactivation of mercury by hydrogen sulfide or of a surface-active cation by a surface-active anion, (*b*) antagonism by competitive adsorption, as assumed for the effect of relatively harmless adsorption cations.

Fildes (1940), showed that the mercapto compounds are able to prevent the antibacterial action of mercuric chloride. McIlwain (1941) demonstrated that the antibacterial action of acriflavine can be prevented by nucleic acid, tryptic casein, etc. Both authors recognize the tendency of these antagonistic agents to combine with the respective toxic cations. Nevertheless, both of them, but

more explicitly McIlwain, incline toward the assumption that the reversal of the toxic action is due to the fact that the antagonistic agents function as essential metabolites or as substitutes for essential metabolites, which the bacteria cannot produce as a consequence of toxication.

Our own experiments have shown that sodium dodecyl sulfate does inhibit the antibacterial action of acriflavine. It is certainly unnecessary to assume that sodium dodecyl sulfate can act as an essential metabolite. In all cases where the antagonistic effect can be related to direct chemical inactivation of the toxic substance, another explanation of the effect is unnecessary and has little likelihood. As mercapto compounds combine with mercury compounds and as nucleic acid and tryptic casein adsorb acriflavine, the assumption of a direct inactivation is a satisfactory explanation unless and until it is shown that the magnitude of the effect is greater than can be expected on the basis of the chemical equivalency of the antagonistic substance. This has been shown in the case of the p-amino benzoic acid-sulfanilamide system. However, consideration of the state of ionization of sulfanilamide and p-amino-benzoic acid (Schmelkes *et al.*, 1942; Bell and Roblin, 1942) indicates that the possibility of a mode of action by competitive adsorption is not completely eliminated, even in this case.

The reversible adsorption by ionic exchange has to be considered only as a first process in the mechanism of the antibacterial action of surface active cations. Little is known about the course of events which follow this primary process and finally lead to the death of the bacteria; however, the observations listed below may give some indication.

Anson (1929) discovered the remarkable ability of surface-active cations and anions to denature proteins. Although it is safe to assume that the adsorption of the surface-active ions is responsible for the denaturation, it cannot be said whether or not the protein denaturation plays a decisive role in the antibacterial action. Quastel and Wheatley (1931) demonstrated that enzymes can be inactivated by dye ions. This action can be reversed, e.g. by phosphate ions. They attribute this phenomenon to ionic adsorption. Kuhn and Bielig (1940) confirmed Anson's observation that surface-active cations denature proteins and they found that chloroplastin and other symplexes can be split by such cations. They observed that a precipitation of proteins by surface-active cations occurs on the alkaline side of their iso-electric points. Among these were enzymes such as pepsin, trypsin, insulin, catalase. Kuhn and Bielig point out the importance of these observations in relation to any further theory of disinfection. Peck (1942) demonstrated the inhibition of the proteolytic action of trypsin by soaps and its reversal by calcium salts.

SUMMARY

1. The "killing" action of surface-active cations on bacteria can be reversed, under certain conditions, by detoxication with a high molecular anion.

2. The antibacterial behaviour of surface-active cations is in agreement with that of toxic metallic ions and dye cations. They can be considered as a phenomenon of ionic exchange by bacteria.

3. The antibacterial action of acriflavine can be reversed by detoxication with a high molecular anion.

4. Observations are presented that demonstrate the protective action on bacteria of relatively harmless cations against toxic cations. This can likewise be considered as a case of ionic exchange.

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