EFFECT OF INORGANIC CATIONS ON BACTERICIDAL ACTIVITY OF ANIONIC SURFACTANTS

J. G. VOSS

Miami Valley Laboratories, The Procter & Gamble Co., Cincinnati, Ohio

Received for publication 11 March 1963

ABSTRACT

Voss, J. G. (Procter & Gamble Co., Cincinnati, Ohio). Effect of inorganic cations on bactericidal activity of anionic surfactants. J. Bacteriol. **86**:207–211. 1963.—The bactericidal effectiveness of two alkyl benzene sulfonates and of three other types of anionic surfactants against *Staphylococcus aureus* is increased in the presence of low concentrations of divalent cations, especially alkaline earths and metals of group IIB of the periodic table. The cations may act by decreasing the negative charge at the cell surface and increasing adsorption of the surfactant anions, leading to damage to the cytoplasmic membrane and death of the cell. Increased adsorption of surfactant is also found with *Escherichia coli*, but does not lead to death of the cell.

Synthetic detergents are now widely used for purposes of cleaning and sanitation. The most commonly used synthetic surfactant is the anionic alkyl benzene sulfonate (C_{12}ABS), in which the alkyl is a branched-chain dodecyl group composed of four propylene units. This compound has negligible bactericidal activity against gram-negative bacteria, but appreciable activity against gram-positive species.

In preliminary studies of the bactericidal activity of C_{12}ABS, 0.1 M phosphate buffer was used to control pH. An unexpected increase in bactericidal activity of C_{12}ABS against *Staphylococcus aureus* was observed. Addition of other salts (NaCl, KCl) instead of the buffer showed this to be a nonspecific effect of electrolytes, rather than solely an effect of pH. The work reported here shows that the activity of C_{12}ABS and other anionic surfactants against *S. aureus* may be greatly increased by the addition of some divalent inorganic cations.

MATERIALS AND METHODS

Five anionic surfactants were studied. The C_{12}ABS was approximately 99% surfactant; C_{15}ABS, with a pentapropylene side chain, was of similar purity. Lauryl glyceryl ether sulfonate was 98.5% surfactant. Lauryl trioxyethylene sulfate was used as a paste containing 55.4% surfactant plus water and a considerable amount of Na_2SO_4. Lauryl sulfate was used as a product containing 90% surfactant and 10% inorganic salts. All three lauryl derivatives were prepared from fatty alcohol produced by reduction of coconut oil fatty acids, and containing predominantly lauryl alcohol. All of the surfactants were used as the sodium salts.

The test organisms, *S. aureus* ATCC 6538 and *Escherichia coli* ATCC 10536, were grown for 24 hr at 37 C in Brain Heart Infusion broth (Difco), centrifuged, washed once in water, and resuspended in water to the original volume. A 2-ml portion of the suspension of washed cells was added to 18 ml of the test solution at 37 C; the final pH was 5.9 to 6.8. The initial population was 100 to 150 million per ml for both organisms, by plate count on Brain Heart Infusion Agar. Plate counts were made in duplicate after 10-min exposure of the cells to the test solution at 37 C.

Electrophoretic measurements on suspensions of *S. aureus* were made by moving-boundary electrophoresis, using a Perkin-Elmer model 38A instrument.

Adsorption of C_{12}ABS by *S. aureus* and *E. coli* was determined with S^{35}-tagged C_{12}ABS, with a specific activity of 10 to 16 mc/g. The adsorption was carried out for 15 min at 37 C in a volume of 10 ml; the cells were then centrifuged, resuspended in water, and sampled for radioassay. Because the adsorption is readily reversible, the cells could not be washed without loss of a large fraction of the adsorbed $C_{12}ABS^{35}$. Calculations of adsorption were corrected for an average 1% carry-over of the supernatant. The samples were assayed for radioactivity by counting in a liquid scintillation counter, using a Packard Tri-Carb spectrometer.

Nitrogen was determined by the micro-Kjeldahl method.

RESULTS

The results of studies on the influence of a number of salts on the bactericidal activity of C_{12}ABS against *S. aureus* are shown in Table 1. Under the conditions of the test, average survival after exposure to 25 ppm of C_{12}ABS alone was 85%. No differences between the effects of sulfates and chlorides were observed; different cations, however, varied markedly in their effect on the bactericidal activity of C_{12}ABS. The most effective were divalent alkaline earth cations, and especially those metals belonging to group IIB of the periodic table. It will be noted that increased bactericidal activity could still be observed at salt concentrations which were lower than the concentration of C_{12}ABS; addition of a quantity of $CaCl_2$ insufficient to convert all of the C_{12}ABS to the calcium salt still permitted only 28% survival of the test organism. With the exception of $HgCl_2$, none of these salts displayed significant bactericidal activity when tested alone at the concentrations shown. Cells stained after death showed no visible alterations and no loss of their gram-positive character.

Other work showed that the effect of $CaCl_2$ in increasing bactericidal activity was not diminished in the presence of a tenfold molar excess of NaCl; no antagonism between the cations was observed.

Since divalent cations had proved effective, the effect of a "divalent organic cation" was tested. 1,4-Dimethyl-1,4-diazoniabicyclo-(2,2,2)-octane diiodide was prepared by reaction of triethylene diamine with excess methyl iodide, and was recrystallized from methanol. This compound, with two positively charged N atoms, was ineffective in increasing the bactericidal activity of 25 ppm of C_{12}ABS, when added at a concentration of 5×10^{-4} M.

Because the activity of C_{12}ABS had been studied most extensively in the presence of $CaCl_2$, the behavior of four other anionic surfactants in the presence of this salt was also determined. The data in Table 2 show that the effect of $CaCl_2$ in increasing bactericidal activity

TABLE 1. *Per cent survival of Staphylococcus aureus after 10-min exposure at 37 C to 25 ppm of C_{12}ABS (0.00007 M)[a] plus indicated salts*

Salt	Molar concn of salt									
	0.15	0.05	0.01	0.0025	0.0005	0.0001	0.00005	0.000025	0.00001	0.000001
NaCl	1.4	1.0 (2)[b]	61							
Na_2SO_4		6.1	61	79						
KCl	1.4									
LiCl					>10					
$MgSO_4$		0.034	0.036	3.8						
$MgCl_2$		0.017	0.025	2.5	8.8					
$CaCl_2$		0.017	0.035 (3)	0.055 (2)	5.0 (10)	14 (3)	31 (4)	28	45	
$ZnCl_2$					0.35					
$BaCl_2$					6.0					
$CuSO_4$					3.5					
$MnCl_2$					8.0					
$CdCl_2$					0.086 (2)		6.6		>20	
$HgCl_2$							0.0089[c]		14[d]	25[e]
$SrCl_2$					19					
$FeCl_3$					>25					
$AlCl_3$					>25					
$SnCl_4$					>25					

[a] Used alone, 25 ppm of C_{12}ABS gave 85% survival.
[b] Parenthetical figures indicate number of replicate determinations.
[c] $HgCl_2$ control (no C_{12}ABS): 0.21% survival.
[d] $HgCl_2$ control: 17% survival.
[e] $HgCl_2$ control: 39% survival.

TABLE 2. *Per cent survival of Staphylococcus aureus after 10-min exposure at 37 C to mixtures of anionic surfactants with $CaCl_2$*

Surfactant	Concn		$CaCl_2$	Survival	
	ppm		M	%	
C_{12}ABS	25	(7×10^{-5} M)	—	85	(16)*
	25		5×10^{-4}	5.0	(10)
	25		10^{-4}	14	(3)
	25		5×10^{-5}	31	(4)
C_{15}ABS	25	(6×10^{-5} M)	—	24	
	25		5×10^{-4}	0.12	
	25		10^{-4}	0.15	
	25		5×10^{-5}	0.26	
Lauryl sulfate	25	(8×10^{-5} M)	—	56	
	25		10^{-3}	25	
	25		5×10^{-5}	24	
	50		—	35	
	50		10^{-3}	0.52	
	50		5×10^{-5}	4.1	
Lauryl glyceryl ether sulfonate	25	(6×10^{-5} M)	—	89	
	25		5×10^{-4}	30	
	50		—	63	
	50		5×10^{-4}	2.6	
	100		—	28	(2)
	100		5×10^{-4}	0.36	(2)
Lauryl trioxyethylene sulfate	50	(12×10^{-5} M)	—	25	
	50		5×10^{-4}	8.6	
	100		—	1.8	
	100		5×10^{-4}	0.55	
None		—	5×10^{-4}	100	

* Parenthetical figures indicate number of replicate determinations.

against *S. aureus* is not limited to C_{12}ABS but is also apparent with other detergent types. The effect was greatest, however, with the two alkyl benzene sulfonates.

It seemed probable that increased kill of *S. aureus* by C_{12}ABS in the presence of added electrolyte was due to increased adsorption of the anionic surfactant by the negatively charged cell. Therefore, the adsorption of reduced concentrations of $C_{12}ABS^{35}$ was determined in the presence and absence of added $CaCl_2$. The influence of $CaCl_2$ on adsorption of $C_{12}ABS^{35}$ by the insusceptible *E. coli* was similarly determined. The results in Table 3 show that the adsorption of $C_{12}ABS^{35}$ by *S. aureus* is indeed increased by $CaCl_2$. Interestingly, although low concentrations of C_{12}ABS have little or no killing action on *E. coli*, the adsorption of $C_{12}ABS^{35}$ by this organism is somewhat greater than that by the susceptible *S. aureus*, and is similarly increased by $CaCl_2$.

Because of its effect on adsorption of C_{12}ABS, a preliminary investigation of the influence of $CaCl_2$ on the electrophoretic behavior of *S. aureus* was carried out. Cells were suspended in 0.01 M $CaCl_2$ or NaCl, and mobility was determined by moving-boundary electrophoresis. Mobilities of the suspended cells, in (μ/sec)/(v/cm), were calculated as 1.18 in $CaCl_2$ and 3.30 in NaCl. Calcium ion is evidently more effective than sodium ion in reducing the net negative charge on the cell, and presumably in increasing adsorption of the C_{12}ABS anion in that manner.

As Hotchkiss (1946) showed, bactericidal activity of surfactants is accompanied by a

TABLE 3. *Adsorption of $C_{12}ABS^{35}$ by bacterial cell suspensions in 15 min at 37 C*

Organism	Added $C_{12}ABS^{35}$	$CaCl_2$	$C_{12}ABS^{35}$ adsorbed by cells
	ppm	*M*	%
Staphylococcus aureus	8 (2.2×10^{-5} M)	—	2.0
	8	2×10^{-4}	1.9
	8	8×10^{-3}	5.9
	8	8×10^{-2}	10
Escherichia coli	9 (2.9×10^{-5} M)	—	4.3
	9	2.25×10^{-4}	6.9
	9	9×10^{-3}	13
	9	9×10^{-2}	18

TABLE 4. *Release of nitrogen compounds from Staphylococcus aureus after exposure to $C_{12}ABS$ and $CaCl_2$ for 15 min at 37 C*

$C_{12}ABS$	$CaCl_2$	Survival	N in supernatant
ppm	*M*	%	*μg/ml*
—	—	100	14
25	—	90	29
25	5×10^{-3}	1.3	58

leakage of the constituents of the cell into the environment. Table 4 shows that the increased kill of *S. aureus* by $C_{12}ABS$ in the presence of $CaCl_2$ is accompanied by release of nitrogen compounds into the solution.

DISCUSSION

Relatively little work on the adsorption of electrolytes and surfactants at bacterial surfaces has been reported. Adsorption of cations at the negatively charged cell surface was indicated by the electrophoretic studies of Winslow, Falk, and Caulfield (1923); Ca ions seemed more readily adsorbed than Na ions. McCalla (1940) studied the adsorption of 10^{-3} M and lower concentrations of H ions and of metal, alkali, and alkaline earth cations by *E. coli*, and demonstrated preferential adsorption of Ca, Mg, Ba, Mn, and Hg; more strongly adsorbed ions were able to replace those less strongly adsorbed. The possibility of adsorption at sites other than the surface of the cell wall is indicated by the work of Harris, Eisenstark, and Dragsdorf (1954), who obtained evidence that the site of adsorption of mercuric ions by *E. coli*, *Salmonella pullorum*, and *Azotobacter agile* is inside the cell wall, at the level of the cytoplasmic membrane.

There is also electrophoretic evidence of the adsorption of surfactants on the cell surface. Dyar and Ordal (1946) demonstrated that the negative charge of several species is increased by the anionic surfactant sodium tetradecyl sulfate, and decreased by the cationic surfactant cetyl pyridinium chloride. This work, and that of Dyar (1948), implicated the surface lipides of the cell as the constituents primarily responsible for the adsorption of anionic surfactants. In contrast, Loveday and James (1957) found that the electrophoretic mobility of *Aerobacter aerogenes* is decreased at low concentrations of phenol or sodium dodecyl sulfate and increased at higher concentrations; the increased mobility of the cells at the higher concentrations of phenol or dodecyl sulfate was attributed to adsorption of a second layer of ions by van der Waals forces, with the polar groups directed outward. The concentration of phenol or surfactant causing 100% kill appeared to be that which gave complete formation of the first layer on the surface of the cell.

If the action of surfactants is not directly on the cell wall, the difference in susceptibility of gram-positive and gram-negative species may be due to an inability of the surfactant ion to penetrate the more complex wall of the gram-negative cell, although adsorption on the cell wall has been shown to occur. The data given here support the assumption that cations are adsorbed on the negatively charged cell wall, or at the cytoplasmic membrane (Harris et al., 1954), and promote the adsorption of surfactant anions. These appear to exert their bactericidal effect by altering the permeability of the cytoplasmic membrane (Hotchkiss, 1946; Newton, 1960). There is direct evidence (Salton, 1957;

Gilby and Few, 1957, 1960) that ionic surfactants can disrupt the cytoplasmic membrane; in this way, they are able to cause the lysis of protoplasts.

There is no *a priori* reason to expect a close correspondence experimentally between bactericidal action and adsorption of the surfactant; e.g., Table 3 shows no increased adsorption by *S. aureus* of 8 ppm of $C_{12}ABS^{35}$ in the presence of 10^{-4} M $CaCl_2$, although $CaCl_2$ concentrations as low as 10^{-5} M cause decreased survival in the presence of 25 ppm of $C_{12}ABS$ (Table 1). Only some fraction of the total surfactant adsorbed need penetrate to the cytoplasmic membrane to cause death of the cell.

The well-known observation that polyvalent cations reduce the antibacterial activity of cationic surfactants may be mentioned here; reduction in activity may be due to exclusion of the surfactant from the bacterial surface.

Thus, the role of the more effective cations in promoting the bactericidal activity of anionic surfactants against *S. aureus* appears to be in increasing adsorption of the surfactant at the cell wall; penetration of the anion to the cytoplasmic membrane then results in disorganization of the membrane, loss of intracellular metabolites, and death of the cell without distinct morphological changes. In this manner, the bactericidal activity of some anionic surfactants may be increased many-fold.

Acknowledgments

The capable assistance of J. D. Kennedy, H. W. Lampe, and W. L. Gagen in various phases of the work is gratefully acknowledged.

Literature Cited

Dyar, M. T. 1948. Electrokinetical studies on bacterial surfaces. II. Studies on surface lipids, amphoteric material, and some other surface properties. J. Bacteriol. **56**:821–834.

Dyar, M. T., and E. J. Ordal. 1946. Electrokinetic studies on bacterial surfaces. I. The effects of surface-active agents on the electrophoretic mobilities of bacteria. J. Bacteriol. **51**:149–167.

Gilby, A. R., and A. V. Few. 1957. Reactivity of ionic detergents with *Micrococcus lysodeikticus*. Nature **179**:422–423.

Gilby, A. R., and A. V. Few. 1960. Lysis of protoplasts of *Micrococcus lysodeikticus* by ionic detergents. J. Gen. Microbiol. **23**:19–26.

Harris, J. O., A. Eisenstark, and R. D. Dragsdorf. 1954. A study of the location of adsorbed mercuric ions in Escherichia coli. J. Bacteriol. **68**:745–748.

Hotchkiss, R. D. 1946. The nature of the bactericidal action of surface-active agents. Ann. N.Y. Acad. Sci. **46**:479–492.

Loveday, D. E. E., and A. M. James. 1957. Relationship between the concentration of anionic surface-active agents and the electrophoretic mobility and viability of *Aerobacter aerogenes*. Nature **180**:1121–1122.

McCalla, T. M. 1940. Cation adsorption by bacteria. J. Bacteriol. **40**:23–32.

Newton, B. A. 1960. The mechanism of the bactericidal action of surface-active compounds: a summary. J. Appl. Bacteriol. **23**:345–349.

Salton, M. R. J. 1957. The action of lytic agents on the surface structures of the bacterial cell. Proc. Intern. Congr. Surface Activity, 2nd, London **4**:245–253.

Winslow, C.-E. A., I. S. Falk, and M. F. Caulfield. 1923. Electrophoresis of bacteria as influenced by hydrogen-ion concentration and the presence of sodium and calcium salts. J. Gen. Physiol. **6**:177–200.