Effects of Organic Cations on the Gram-negative Cell Wall and Their Bactericidal Activity with Ethylenediamine- tetra-acetate and Surface Active Agents

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SUMMARY

When Gram-negative cell walls were damaged by lysozyme and ethylenediaminetetra-acetic acid (EDTA) in tris buffer, tris played an active role through its action as an organic cation. Other organic cations such as aliphatic amines and quaternary ammonium compounds were more effective than tris in modifying the cell wall and making it permeable to other solutes. The most effective organic cations had one C₁₂ to C₁₆ alkyl chain. At pH 10, EDTA 100 µg./ml and *N,N*-dimethyldodecylamine (DDA) 2.5 µg./ml. modified the cell wall of *Escherichia coli* so as to permit a more than 99.99 % kill by 20 µg./ml. of a zwitterionic surface active agent, 2-hydroxy-3-(dimethylhexadecylammonio) propane-1-sulphonate (HAPS), in 10 min. at 37°. At these concentrations, the individual compounds were bactericidally ineffective. Four other Gram-negative species were shown to be similarly susceptible to killing by this system. Together, EDTA and DDA appeared to remove surface components of the Gram-negative cell wall. Alone, DDA and other organic cations removed somatic antigens from the cell wall.

INTRODUCTION

Gram-negative bacteria are generally susceptible to the bactericidal action of cationic surface active agents, but are usually unaffected by those anionic and zwitterionic agents which are active against Gram-positive organisms. It was suggested by Voss (1963) that the greater resistance of Gram-negative species may be due to the greater complexity of their cell walls, which exclude the agent from the interior of the cell.

The Gram-negative cell wall may be so modified by treatment with EDTA in tris buffer at pH 8 as to permit conversion of the cell by lysozyme to osmotically fragile rods (Voss, 1964) or 'osmoplasts' (Asbell & Eagon, 1966*a*). In studies on *Escherichia coli* modified by treatment with EDTA and tris buffer, it was observed that such organisms may be killed by treatment with zwitterionic agents to which the organisms are normally resistant. As in the case of lysis by lysozyme, killing by the agent indicates changes induced in the cell wall by EDTA and tris buffer. Further study revealed that tris is not a physiologically inert buffer, but plays a specific role as an organic cation in modifying the permeability of the cell wall; other organic cations are much more effective than tris. Elucidation of the role of the organic cation forms the substance of this report.

METHODS

Organisms. The test organisms were *Escherichia coli* (ATCC 10536), *E. freundii*, *Pseudomonas aeruginosa*, *Proteus mirabilis*, and *Salmonella typhi*; *S. typhi* TY2V and 0901 and *E. freundii* (BALLERUP 107) were obtained through the courtesy of the late Dr P. R. Edwards (Communicable Disease Center, Public Health Service, Atlanta, Ga.), *S. typhi* 9992V and 12839 were obtained from the American Type Culture Collection. *Escherichia coli* was obtained as ATCC 10536 and used in the earlier part of this work; after the observation that this culture produced acid but no gas from glucose or lactose, it was replaced with a fresh, gas-forming culture of ATCC 10536 from the American Type Culture Collection with no discernible difference in results.

Test compounds. EDTA was used as the disodium salt; solutions were adjusted to pH 8.0 before sterilization. The zwitterionic agent 2-hydroxy-3-(dimethylhexadecylammonio)-propane-1-sulphonate (HAPS), was prepared and purified in our laboratories, and sterilized as a 0.1% solution in 0.85% NaCl; it is much less soluble in water alone. *N,N*-dimethyldodecylamine (DDA) was prepared by distillation of 'dimethyl coconut amine' (Armour). Some amines were derived from coconut or soybean oil, and thus contain a mixture of alkyl chains. Other compounds which were not available commercially were synthesized in our laboratories. Fatty amines were dissolved in ethanol, and diluted with water before use; at values up to about pH 10, they are present principally as cations.

Bactericidal tests. All organisms except *Proteus mirabilis* were grown in brain heart infusion broth (Difco) for 24 hr at 37°. Plate counts were made on brain heart infusion agar incubated at 37° for 24 hr; *P. mirabilis* was grown in nutrient broth, and plated on nutrient agar overlaid with plain agar to inhibit spreading. Exposure to bactericidal agents was done as described earlier (Voss, 1963) for 10 min. at 37° with suspensions of about 60–120 million washed organisms/ml.

Lysis. Changes in optical density of suspensions of organisms at room temperature were measured with a Coleman Junior spectrophotometer at 660 m μ .

Precipitin tests. *Escherichia coli* was harvested from ten 150 mm. plates, washed, and suspended in 400 ml. water. These suspensions contained 3–4 mg. dry wt. organisms/ml. Portions were treated for 10 min. at 37° with DDA 10 and 50 μ g./ml. or with EDTA, HAPS, dodecyltrimethylammonium bromide (DTAB), or alkyl dimethylbenzylammonium chloride (Roccal), all 50 μ g./ml. in the presence or absence of Na₂CO₃ 100 μ g./ml. Organisms were removed by centrifugation for 20 min. at 5000 g. The supernatant fluids were tested for antigenic activity in double diffusion tests in agar, using as the source of antibody a 1/5 dilution of rabbit antiserum prepared against whole *E. coli* organisms.

RESULTS

Activity of cations other than tris

Tris was more effective than other buffers (e.g. phosphate buffer) in promoting the bactericidal activity of a mixture of EDTA and HAPS. The corresponding nitro compound, tris(hydroxymethyl) nitromethane, was inactive, indicating that it is the free amino group of tris buffer which affects the cell wall. Table 1 shows the results of a comparison of other amines and related compounds for activity in promoting the bactericidal action of EDTA and HAPS against *Escherichia coli* at concentrations at

Table 1. *Survival of Escherichia coli in systems containing EDTA (100 µg./ml.) + HAPS (20 µg./ml) + amines or other compounds*

Test compound	Concn. ($\times 10^{-4}$ M)	Survival (%)	
		In complete systems	With test compound alone
Tris(2-Amino-2-hydroxymethyl-propane-1,3-diol)	500	3.0	.
	250	15	.
Tris(hydroxymethyl)nitromethane	500	74	.
Ammonia	500	71	.
Methylamine	500	16	.
Ethylamine	500	2.9	.
	250	17	.
Diethanolamine	250	0.72	.
	125	8.9	.
3-Aminopropanol	500	0.11	.
	250	5.4	.
	125	16	.
<i>n</i> -Propylamine	500	0.06	.
	250	0.40	.
	125	4.3	.
<i>n</i> -Butylamine	250	0.26	.
	125	0.82	.
<i>sec</i> -Butylamine	250	0.15	.
	125	0.87	.
Dibutylamine	125	~ 0.02	.
	62.5	4.0	.
<i>n</i> -Hexylamine	125	0.005	.
	62.5	0.007	.
	31.2	0.07	.
<i>n</i> -Octylamine	7.8	~ 0.001*	.
	3.9	~ 0.002*	.
<i>n</i> -Decylamine	15.6	0.001*	.
<i>n</i> -Dodecylamine	0.244	0.0028*	26
	0.122	0.021*	.
<i>n</i> -Hexadecylamine	0.244	0.037*	1.3*
	0.122	0.0049*	36*
<i>N</i> -Methyldodecylamine	0.244	0.0014*	16*
<i>N,N</i> -Dimethyldodecylamine (DDA)	0.244	~ 0.00006*	0.20*
	0.122	0.0042*	72*
	0.061	0.021*	.
<i>N,N</i> -Dimethylhexadecylamine	0.244	0.015*	2.2*
	0.122	> 0.4*	12*
Didecylamine	0.244	> 0.5*	70*
Dicoco amine†	0.244	0.055*	.
	0.122	0.089*	.
<i>N</i> -Methyl dicoco amine†	0.244	> 1*	80*
<i>N</i> -Methyl disoya amine‡	0.244	> 0.9*	.
Coco-1,3-propylene diamine†	0.244	0.00028*	5.1*
	0.122	0.0064*	40*
Dodecyl piperidine	0.244	~ 0.00002*	.
	0.122	0.00038*	4.8*
<i>N,N</i> -Dimethyldodecylamine oxide	0.244	> 0.9*	.
Coco ethanolamine†	0.244	24*	.
Coco amide†	0.244	18*	.

* In the presence of 100 µg./ml. sodium triphosphate.

† Derived from coconut oil fatty acids consisting of about 65% lauric acid, 25% myristic acid, and 10% palmitic acid.

‡ Derived from soybean oil fatty acids consisting of about 12% palmitic acid 25% oleic acid, and 53% linoleic acid.

which the latter two compounds together were ineffective. A number of amines of low molecular weight, more or less closely related to tris, were found to possess similar activity. As the length of the alkyl chain increased, the bactericidal activity of the mixture increased markedly. Concentrations of the amine buffers were decreased as activity increased; therefore sodium triphosphate ($\text{Na}_5\text{P}_3\text{O}_{10}$) 100 $\mu\text{g./ml.}$ was added to maintain at pH 8.5–9.0. Maximum activity was reached with amines containing alkyl chains of 12–16 C atoms. The further substitution on the N atom of one or two methyl groups had little effect. Amines with two alkyl chains were less active. Such

Table 2. *Per cent survival of Escherichia coli in systems containing EDTA (100 $\mu\text{g./ml.}$) + Na_2CO_3 (100 $\mu\text{g./ml.}$) + HAPS (20 $\mu\text{g./ml.}$), and organic cations or related compounds*

Test compound	Concentration ($\mu\text{g./ml.}$)	Survival (%)	
		Complete system	Test compound + Na_2CO_3 only
<i>N,N</i> -Dimethyldodecylamine (DDA)	2.5	0.0074	15
Decyl methyl sulfoxide	5	> 6	.
Hexadecyl methyl sulfoxide	5	> 6	.
Dodecyl methyl sulphone	5	> 6	.
Dodecyl methyl sulphide	5	> 6	.
Dodecyldimethylsulphoxonium methosulphate	2.5	0.0081	38
Dodecyldimethylsulphonium iodide	2.5	0.0033	68
3-Tridecylpyridine	2.5	> 6	.
Dodecyltrimethylammonium bromide	2.5	0.018	32
Cetyltrimethylammonium bromide	2.5	0.067	88
Cetylpyridinium chloride	2.5	0.019	78
Alkyldimethylbenzylammonium chloride (Roccal)	2.5	0.19	0.10
2-Dodecyl-1,3-bis-(trimethylammonio)-propane dibromide	2.5	0.00074	43
Dimethyldodecyl-3-(trimethylammonio)-propylammonium dibromide	2.5	0.00095	59
1-Dodecyl-2-imino-imidazolidine	2	0.135	30
1,2-Bis-(dimethylamino)-dodecane	2.5	0.0017	70
2-Chlorodimethyldodecylamine	2.5	0.11	54

related compounds as coconut alkyl ethanolamine or the amides of coconut fatty acids were ineffective, while other compounds such as coco-1,3-propylene diamine and dodecyl piperidine were highly active. The increasing activity of the aliphatic amines with EDTA and HAPS was not due to the increasing bactericidal activity of the amines alone as the length of the alkyl chain grew; the active amines effectively killed *E. coli*, in the presence of EDTA and HAPS, at concentrations at which the amines alone possessed only slight bactericidal activity.

A further series of organic cations and related compounds, including DDA, is compared in Table 2. In this case, the compounds were tested in the presence of EDTA 100 $\mu\text{g./ml.}$, HAPS, 20 $\mu\text{g./ml.}$ and Na_2CO_3 100 $\mu\text{g./ml.}$ to buffer the system at pH 10. Na_2CO_3 was used instead of sodium triphosphate as a buffer to avoid combining the chelating effects of EDTA and triphosphate. Four non-ionic alkyl sulphur compounds were ineffective in increasing bactericidal activity, but two cationic sulphonium and sulfoxonium compounds were highly effective, as were a number of quaternary ammonium compounds and additional amines. It is evident that there was no specific requirement for a positively charged N atom. A compound with an alkyl chain of C_{12}

or longer and a positively charged group at one end was sufficient to give high bactericidal activity in the presence of EDTA, HAPS and an alkaline buffer.

Action of the bactericidal system

For further work, DDA was chosen as the organic cation; at a concentration of 2.5 $\mu\text{g./ml.}$, it showed only very limited bactericidal activity against *Escherichia coli* at pH 10. EDTA was used at 100 $\mu\text{g./ml.}$, at which concentration it was present in excess; decrease to 10 $\mu\text{g./ml.}$ caused no significant loss of activity. At 20 $\mu\text{g./ml.}$ and above, HAPS alone was not appreciably bactericidal for *E. coli*; however, increasing the concentration in the presence of the other components of the system increased killing, and decreasing the concentration decreased the killing. The concentration of Na_2CO_3 was not critical. A number of alkaline buffers gave similar results in the range pH 9–10.

EDTA could be replaced by a number of other chelating agents; however, only *N*-hydroxyethylethylenediaminetriacetic acid (Versenol), diethylenetriaminepentaacetic acid (Versenex 80), and 1,2-diaminocyclohexane-*N,N'*-tetra-acetic acid were as effective as EDTA.

Table 3. *Effect of omission of single components of the bactericidal system on kill of Escherichia coli*

EDTA ($\mu\text{g./ml.}$)	DDA ($\mu\text{g./ml.}$)	Na_2CO_3 ($\mu\text{g./ml.}$)	HAPS ($\mu\text{g./ml.}$)	Survival (%)
100	2.5	100	20	0.00040
100	0	100	20	42
0	2.5	100	20	18
100	2.5	0	20	0.012
100	2.5	100	0	34

HAPS, which is regarded as the actual lethal agent in the bactericidal system through its presumed effect on the cytoplasmic membrane (see Hotchkiss, 1946; Salton, 1951), could be replaced by other zwitterionic agents such as betaines, or by cationic quaternary ammonium compounds. Many of the latter are themselves highly bactericidal, and perhaps fulfil the function of organic cation and surface active agent simultaneously. Anionic and non-ionic surface active agents were ineffective as replacements for HAPS.

The fact that each component of the bactericidal system containing EDTA, DDA, HAPS and Na_2CO_3 was required for full activity is indicated by the data in Table 3. Omission of EDTA, DDA, or HAPS caused a sharp decrease in the killing of *Escherichia coli*; lack of Na_2CO_3 caused a much smaller decrease.

The bactericidal action of the components of the EDTA + DDA + Na_2CO_3 + HAPS system against *Escherichia coli* was studied in greater detail by exposing the organisms to one or two components under the conditions of the bactericidal test, removing the organisms by centrifugation, and then exposing them to the remainder of the system. The results of this study (Table 4) implied that EDTA and DDA acted jointly to exert a non-lethal effect on the cell wall, which thus became more permeable to HAPS. The surface active agent penetrates the wall and causes death of the organism, presumably by damaging the cytoplasmic membrane. The role of Na_2CO_3 was relatively non-specific, though it appeared to increase the effect of DDA more than that of EDTA or HAPS. Although killing might be influenced by carry-over of test compounds

from the initial to the final exposure, it seems clear that the initial effect of EDTA and especially of DDA was required *before* HAPS could kill the organism. This conclusion is supported by the survival curve shown in Fig. 1. It is assumed that the low rate of killing for the first minute or two represented the time required for EDTA and DDA to affect the cell wall.

Table 4. *Role of the components of the bactericidal system in killing Escherichia coli*

Concentrations of reagents: EDTA (100 $\mu\text{g./ml.}$), DDA (2.5 $\mu\text{g./ml.}$), Na_2CO_3 (100 $\mu\text{g./ml.}$), HAPS (20 $\mu\text{g./ml.}$).

Initial and final exposures: 10 min. at 37° C.

Initial exposure to	Final exposure to	Final pH value	Survival (%)
EDTA	DDA + Na_2CO_3 + HAPS	9.9	114 49
DDA	EDTA + Na_2CO_3 + HAPS	9.9	80 0.49
EDTA + DDA	Na_2CO_3 + HAPS	9.9	118 0.015
EDTA + DDA + Na_2CO_3	HAPS	8.0	34 0.0013
EDTA + Na_2CO_3	DDA + HAPS	7.6	79 8.1
DDA + Na_2CO_3	EDTA + HAPS	7.6	54 < 0.015
HAPS	EDTA + DDA + Na_2CO_3	9.9	87 2.8
HAPS + Na_2CO_3	EDTA + DDA	7.4	60 75
EDTA + HAPS	DDA + Na_2CO_3	10.0	84 1.8
DDA + HAPS	EDTA + Na_2CO_3	9.9	92 45
EDTA + Na_2CO_3 + HAPS	DDA	7.9	27 9.2
DDA + Na_2CO_3 + HAPS	EDTA	6.8	16 12

Six consecutive exposures of 12 isolates of *Escherichia coli* to the action of the EDTA + DDA + Na_2CO_3 + HAPS system did not produce any evidence that the survivors had mutated to a state of greater resistance to the bactericidal action.

Because of the great superiority of other alkyl cations over tris buffer in promoting the bactericidal activity of HAPS in the presence of EDTA, the efficiency of DDA in inducing lysis of *Escherichia coli* by lysozyme was compared with that of tris buffer. As shown in Table 5, 0.21 mg. DDA was more effective than 2.4 mg. tris, and about as effective as 12 mg. tris, in promoting lysis as measured by the decrease in optical density at 660 $m\mu$.

A basic assumption throughout this work was that the surface active substance was the actual lethal agent, and that its action on the cytoplasmic membrane would be accompanied by a leakage of N and P from the cell. To test this, organisms at twice

the usual concentration were exposed to the EDTA + DDA + Na_2CO_3 + HAPS system for 10 min. at 37° . The N and P content of the supernatant fluid after centrifugation was compared with that of a control suspension of organisms. It was found that $4.5 \mu\text{g. N/ml.}$ and $0.8 \mu\text{g. P/ml.}$ were released on killing the organisms. Ultraviolet absorption curves showed a peak at $260 \text{ m}\mu$ in the supernatant fluid of killed organisms, with an optical density of $0.2\text{--}0.3$. Calculations based on adenine ribonucleotide as a model system indicated that this quantity of nucleic acid would yield about $0.4\text{--}0.6 \mu\text{g. P/ml.}$ A major proportion of the P released may be accounted for as nucleic acid, as would

Fig. 1. Survival of *Escherichia coli* on exposure at 37° to EDTA ($100 \mu\text{g./ml.}$) + DDA ($2.5 \mu\text{g./ml.}$) + Na_2CO_3 ($100 \mu\text{g./ml.}$) + HAPS ($20 \mu\text{g./ml.}$).

Table 5. Lysis of *Escherichia coli* by lysozyme, EDTA, and DDA or tris buffer*

EDTA (mg.)	DDA (mg.)	tris (mg.)	Na_2CO_3 (mg.)	lysozyme (mg.)	pH value	Decrease in special density ($660 \text{ m}\mu$) at 30 min.	Viscosity† at 30 min.
1.0	0.21	.	0.2	0.1	7.8	0.21	++
1.0	0.021	.	0.2	0.1	7.6	0.08	—
1.0	.	48	.	0.1	8.1	0.30	++++
1.0	.	12	.	0.1	8.1	0.20	++++
1.0	.	2.4	.	0.1	7.5	0.04	—
1.0	0.21	.	0.2	.	8.0	0.12	—
1.0	.	48	.	.	8.1	0.11	++

* 6 ml. washed bacterial suspension in 8 ml. total volume.

† —, no perceptible increase in viscosity; + + + +, highly viscous.

be expected from the increased viscosity which appeared on lysis of the suspensions. This viscosity was decreased rapidly by adding a trace of deoxyribonuclease.

That the bactericidal activity of the EDTA + DDA + Na_2CO_3 + HAPS system was not restricted to *Escherichia coli* is shown by the data in Table 6. Four other Gram-

negative species were also killed by this system. At the concentrations used, none of the individual components were actively bactericidal. It is of interest that possession of an outermost layer of Vi antigen by some strains of *Salmonella typhi* and by the V dissociant of *E. freundii* appeared to confer some slight resistance to the killing effect.

Table 6. *Bactericidal activity of a mixture of EDTA (100 µg./ml.) + DDA (2.5 µg./ml.) + Na₂CO₃ (100 µg./ml.) + HAPS (20 µg./ml.) against organisms other than Escherichia coli, and the effect of Vi antigen on survival*

Test organism	Vi antigen	% Survival
<i>Proteus mirabilis</i>	.	0.17
<i>Pseudomonas aeruginosa</i>	.	0.0048
<i>Escherichia freundii</i> (BALLERUP 107W)	—	0.0045
<i>E. freundii</i> (BALLERUP 107V)	+	0.034
<i>Salmonella typhi</i> 0901	—	0.005*
<i>S. typhi</i> TY2V	+	0.013*
<i>S. typhi</i> 9992V	+	0.0082*
<i>S. typhi</i> 12839	+	0.14*

* With DDA (1 µg./ml.), at which concentration DDA alone was not actively bactericidal against *S. typhi*.

Removal of somatic antigens by organic cations

It seemed probable that EDTA and DDA removed some surface components of the cell wall, thus rendering it more permeable to HAPS. To test this, washed suspensions of *Escherichia coli* were treated for 10 min. at 37° with dodecyltrimethylammonium bromide (DTAB), alkyl dimethylbenzylammonium chloride (Roccal), DDA, EDTA or HAPS (at 10 or 50 µg./ml.), in the presence or absence of Na₂CO₃ 100 µg./ml. The content of somatic antigens in these extracts was compared by the double diffusion method in agar, using rabbit antisera prepared against whole organisms. Comparison of the zones of precipitation showed that antigens were removed by DDA 50 µg./ml. and even more effectively by the other two cationic agents, DTAB and Roccal. Extraction by DDA 10 µg./ml. was less effective, but still evident. Evidence of reaction consisted primarily of a broad, diffuse zone of precipitate; in some cases minor bands of precipitate also indicated the presence of two slower-moving antigenic components. Na₂CO₃ had no perceptible effect on the extraction of antigens by the cations; Na₂CO₃, EDTA and HAPS were essentially ineffective in removing antigens from the cell wall.

DISCUSSION

Although Weidel, Frank & Martin (1960) concluded that the outermost layer of the cell wall of *Escherichia coli* is lipoprotein, the somatic antigens which are responsible for specific agglutination of Gram-negative bacteria are known to be polysaccharide or lipopolysaccharide. Shands (1965), by using ferritin-labelled antibody, demonstrated extension of somatic antigen for some distance beyond the cell wall of *E. coli* and *Salmonella typhimurium*. It is probably an over-simplification to regard the outermost layer of the multilayered cell envelope as consisting solely of lipoprotein or lipopolysaccharide. From biochemical and immunological evidence, both appear to be present.

Martin (1963) suggested that the complex wall of Gram-negative organisms affords more protection to the organism than does the simpler Gram-positive wall. Loss or decrease of somatic antigens at the cell surface decreases the resistance of Gram-

negative organisms to normal serum or to EDTA and lysozyme (Herzberg & Green, 1964; Osawa & Muschel, 1964; Wardlaw, 1963). Similarly, results given here indicate that absence of Vi antigen on *Escherichia freundii* and *Salmonella typhi* organisms is associated with greater susceptibility of the organisms. Thus, the demonstrated removal of somatic antigens from *E. coli* by DDA and other organic cations would be expected to increase the susceptibility of the organisms to deleterious agents.

Work with several species has shown that EDTA causes loss of lipid, lipoprotein or lipopolysaccharide from the cell wall (Colobert, 1958; Gray & Wilkinson, 1965; Leive, 1965). Repaske (1958) indicated that EDTA removed inorganic cations from cell walls. The importance of calcium and other divalent cations in the cell walls of various Gram-negative species (Humphrey & Vincent, 1962; Asbell & Eagon, 1966*a, b*) appears to be due to its function as a salt bridge in binding macromolecules on the surface of the cell wall.

Removal of surface components of the Gram-negative cell wall by organic cations and EDTA, or disorganization of the structure of the wall, may make it susceptible to the action of lysozyme and permeable to other solutes. When these solutes are surface active compounds which can now penetrate the cell wall, they may cause death of the cell by damaging the cytoplasmic membrane (Hotchkiss, 1946; Salton, 1951; Gilby & Few, 1960). In the system studied here, the lethal effect of HAPS on *Escherichia coli* is an indicator of changes induced by EDTA and DDA in the cell wall in the same manner as lysis by lysozyme indicates damage by EDTA and tris buffer (Repaske, 1958).

There is a growing realization that tris buffer actively modifies the effect of EDTA on the cell wall. Goldschmidt & Wyss (1966) showed that tris and other amines might assist the rupture of *Azotobacter* cysts by EDTA, but attributed the activity of the amines to the formation of complexes with EDTA. Asbell & Eagon (1966*a, b*) observed that tris buffer enhanced the lethal effect of EDTA on *Pseudomonas aeruginosa*. However, many workers have used EDTA in tris buffer without recognizing that the latter specifically affects the cell wall. For example, Nossal & Heppel (1966) exposed *Escherichia coli* to sucrose + EDTA in tris buffer preparatory to osmotic shock for the release of degradative enzymes, and Wolin (1966) lysed *Vibrio succinogenes* with EDTA in tris buffer. The work reported here suggests that a number of organic cations, including tris, specifically affect the Gram-negative cell wall by removing surface components.

It may be speculated that chelating agents and organic cations act together to cause non-lethal damage to the walls of Gram-negative organisms by breaking salt bridges and by salt formation with anionic polymers (lipoproteins, lipopolysaccharides) at the cell surface. Removal of these constituents of the cell wall renders the wall more permeable to other compounds, which can then penetrate to the interior of the cell. The penetration by a zwitterionic agent, such as HAPS, results in disorganization of the cytoplasmic membrane and death of the organism. The modified cell wall is still impermeable to some other compounds, such as anionic surfactants.

Organic cations containing an alkyl chain with about 12–16 C atoms may be used at low concentrations as a tool for dissection of the Gram-negative cell wall and for the preparation of partially purified extracts of somatic antigens. The O and Vi antigens of *Salmonella typhi* have been extracted by treatment of heavy suspensions of organisms with low concentrations of DDA (unpublished). With this organism, EDTA too is

effective in removing somatic antigens under similar conditions. Perhaps antigens suitable for use as vaccines could be prepared from a number of Gram-negative species in this fashion.

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REFERENCES

- ASBELL, M. A. & EAGON, R. G. (1966*a*). The role of multivalent cations in the organization and structure of bacterial cell walls. *Biochem. biophys. Res. Commun.* **22**, 664.
- ASBELL, M. A. & EAGON, R. G. (1966*b*). Role of multivalent cations in the organization, structure, and assembly of the cell wall of *Pseudomonas aeruginosa*. *J. Bact.* **92**, 380.
- COLOBERT, L. (1958). Étude de la lyse de Salmonelles pathogènes provoquée par le lysozyme, après délipidation partielle de la paroi externe. *Annls Inst. Pasteur, Paris* **95**, 156.
- GILBY, A. R. & FEW, A. V. (1960). Lysis of protoplasts of *Micrococcus lysodeikticus* by ionic detergents. *J. gen. Microbiol.* **23**, 19.
- GOLDSCHMIDT, M. C. & WYSS, O. (1966). Chelation effects on *Azotobacter* cells and cysts. *J. Bact.* **91**, 120.
- GRAY, G. W. & WILKINSON, S. G. (1965). The effect of ethylenediaminetetraacetic acid on the cell walls of some Gram-negative bacteria. *J. gen. Microbiol.* **39**, 385.
- HERZBERG, M. & GREEN, J. H. (1964). Composition and characteristics of cell walls of smooth strains of *Salmonella typhimurium* and derived rough variants. *J. gen. Microbiol.* **35**, 421.
- HOTCHKISS, R. D. (1946). The nature of the bactericidal action of surface active agents. *Ann. N.Y. Acad. Sci.* **46**, 479.
- HUMPHREY, B. & VINCENT, J. M. (1962). Calcium in cell walls of *Rhizobium trifolii*. *J. gen. Microbiol.* **29**, 557.
- LEIVE, L. (1965). Release of lipopolysaccharide by EDTA treatment of *Escherichia coli*. *Biochem. biophys. Res. Commun.* **21**, 290.
- MARTIN, H. H. (1963). Bacterial protoplasts—a review. *J. theoret. Biol.* **5**, 1.
- NOSSAL, N. G. & HEPPEL, L. A. (1966). The release of enzymes by osmotic shock from *Escherichia coli* in exponential phase. *J. biol. Chem.* **241**, 3055.
- OSAWA, E. & MUSCHEL, L. H. (1964). Studies relating to the serum resistance of certain Gram-negative bacteria. *J. exp. Med.* **119**, 41.
- REPASKE, R. (1958). Lysis of Gram-negative organisms and the role of Versene. *Biochim. biophys. Acta* **30**, 225.
- SALTON, M. R. J. (1951). The adsorption of cetyltrimethylammonium bromide by bacteria, its action in releasing cellular constituents and its bactericidal effects. *J. gen. Microbiol.* **5**, 391.
- SHANDS, J. W. (1965). Localization of somatic antigen on Gram-negative bacteria by electron microscopy. *J. Bact.* **90**, 266.
- VOSS, J. G. (1963). Effect of inorganic cations on bactericidal activity of anionic surfactants. *J. Bact.* **86**, 207.
- VOSS, J. G. (1964). Lysozyme lysis of Gram-negative bacteria without production of spheroplasts. *J. gen. Microbiol.* **35**, 313.
- WARDLAW, A. C. (1963). The complement-dependent bacteriolytic action of normal human serum. II. Cell wall composition of sensitive and resistant strains. *Can. J. Microbiol.* **9**, 41.
- WEIDEL, W., FRANK, H., & MARTIN, H. H. (1960). The rigid layer of the cell wall of *Escherichia coli* strain B. *J. gen. Microbiol.* **22**, 158.
- WOLIN, M. J. (1966). Lysis of *Vibrio succinogenes* by ethylenediaminetetraacetic acid or lysozyme. *J. Bact.* **91**, 1781.