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# Effect of Surfactants on Antibiotic Resistance

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The effectiveness of surfactants as potentiators of antibiotic activity on several resistant strains of bacteria, selected from clinical sources and laboratory collections, was studied using a tube dilution assay. Bacterial strains included members of the *Enterobacteriaceae* and staphylococci. Cetyltrimethylammonium bromide (CTAB), Tween 80 (Tw80), a mixture of *n*-alkyldimethyl betaines (L14), and alpha-(2,4,5-trichlorophenoxy) propionic acid (TCP) were tested in combination with pencillin G (PenG), methicillin (Met), streptomycin (Sm), polymyxin B (PmB), and chlortetracycline (CTC). Growth response to the drug combinations was compared with the response to each drug alone. CTAB and L14 but not Tw80 or TCP were found to potentiate the activity of CTC on strains of *Escherichia coli*, *Proteus mirabilis*, and *Klebsiella pneumoniae*. Studies on the inhibition of protein synthesis by CTC in cells of a strain of *E. coli* suggested that the surfactants increased the uptake of antibiotic into the cells. CTAB and L14 almost completely sensitized strains of *P. mirabilis*, *Serratia marcescens*, *K. pneumoniae*, and *E. coli* to PmB. With the exception of *K. pneumoniae*, TCP was also effective in potentiating the activity of PmB on the above strains whereas Tw80 showed potentiation only with a strain of *E. coli*. CTAB and L14 but not TCP or Tw80 potentiated the activity of PenG but not Met on strains of staphylococci. Studies of penicillinase in the cells suggested that the surfactants inhibited the formation of this enzyme possibly at the level of induction. None of the surfactants were found to potentiate the activity of Sm.

Antimicrobial drugs can affect bacterial cells in any one of various ways including interference with cell wall synthesis, membrane synthesis, and function, or they may enter the cell and interfere with a vital metabolic process. Drug resistance is observed when a population of cells possesses a physical or biochemical means of counteracting the lethal effect of the antimicrobial agent. Among the various resistance mechanisms known, the role played by the cell membrane or envelope structure is perhaps the least understood. Both the physical and chemical properties of the cell membrane, however, would be expected to play an important role in resistance since a drug must first interact with and in many cases pass through this structure.

The gram-negative bacterial cell envelope consists of an inner cytoplasmic membrane, an intermediate cell wall or peptidoglycan layer, and an outer membrane. An outer membrane like that observed in gram-negative cells is absent in gram-positive bacteria. A role for the outer membrane of gram-negative bacteria as a nonspecific permeability barrier has been suggested since treatment of bacterial cells with ethylenediaminetetraacetic acid releases lipopolysaccharide, an outer membrane component, from the cell envelope with a concomitant increase in permeability to various molecules including antibiotics (12). Related to these observations are studies done with lipopolysaccharide mutants of *Escherichia coli* strains which have also been observed to have altered antibiotic susceptibilities (11, 22). Ethylenediaminetetraacetic acid has been reported to potentiate the activity of antibiotics on antibiotic-resistant bacteria (17, 28). A potentiating effect of surfactants, molecules possessing both hydrophobic and hydrophilic properties, on antibiotic acitivity has also been reported. Thus Tween 80 (Tw80) has been shown to enhance the activity of polymyxin B on a strain of *Pseudomonas aeruginosa* (3) and *E. coli* (2). Benzalkonium chloride was reported to potentiate the activity of chloramphenicol on a strain of *P. aeruginosa* (29) and polyunsaturated fatty acids potentiated the activity of streptomycin on a strain of *Staphylococcus aureus* (16). Scherr and Bechtle (19) made the interesting observation that compounds having plant growth-regulating activity,

such as alpha-(2,4,5-trichlorophenoxy) propionic acid (TCP), demonstrated potentiation for antibiotics, including polymyxin B, on microorgansims. A marked inhibition of penicillinase induction in *S. aureus* by anionic surfactants of the aliphatic sulfate type has been reported by Kaminski (10).

These studies suggest that surfactants, acting as membrane perturbants, might promote easier accessibility of antibiotics to their target sites on or within the bacterial cell. We therefore decided to evaluate the effectiveness of different classes of surfactants as potentiators of the inhibitory activity of tetracyclines, streptomycin, polymyxins, and penicillins using several antibiotic-resistant strains of bacteria. Surfactants included cetyltrimethylammonium bromide (CTAB), *n*-alkyldimethyl betaine (L14), Tw80, and TCP. These represent cationic, amphoteric, nonionic, and anionic surfactants, respectively. Possible mechanisms of potentiation were also investigated. Such a study could further define the importance of the bacterial cell membrane or envelope in antibiotic resistance.

Potentiation, as used here, refers to a combination of drugs which increase each other's activity so that the effect is greater than that expected if each one acted alone.

## MATERIALS AND METHODS

**Bacterial strains.** Bacterial strains were initially classified as being resistant or susceptible to an antibiotic according to the disk diffusion method of Bauer et al. (1).

*E. coli* Sc8190 and Sc8280 have been described elsewhere (4). These are related antibiotic-susceptible and -resistant strains, respectively. *E. coli* Sc8280 harbors resistance factor 222 (R factor 222) which is characterized by sulfonamide-, streptomycin-, chloramphenicol-, and tetracycline-resistant traits. *E. coli*/Tc was a laboratory strain resistant to tetracyclines and streptomycin. *E. coli* Sc8599 and Sc8600 were obtained from Edward Meyers (Squibb Institute for Medical Research, Princeton, N.J.). *E. coli* Sc8600 was a polymyxin-resistant strain developed from Sc8599.

*Klebsiella pneumoniae*/pm was originally a clinical isolate resistant to tetracyclines and polymyxins and maintained in the laboratory in a lyophylized state.

*Proteus mirabilis* pm5 and pm5/R$^+$ were obtained from James Punch (Department of Microbiology, Virginia Commonwealth University, Richmond, Va.). These are related strains which are both resistant to the polymyxins. *P. mirabilis* pm5/R$^+$ also harbors R factor 222 described above. *P. mirabilis* 190A-1 and *Serratia marcescens* 164E3 and 164E4 were obtained from Roy Cleeland (Hoffmann-LaRoche, Nutley, N.J.). *P. mirabilis* 190A-1 and *S. marcescens* 164E3 were resistant to streptomycin and the polymyxins. *S. marcescens* 164E4 was a streptomycin-susceptible variant of 164E3 which was also found to be susceptible to the polymyxins. *S. marcescens* DeJohn was a clinical isolate obtained at New York Hospital and was resistant to streptomycin and the polymyxins.

*Staphylococcus* NYH was a New York Hospital clinical isolate which was resistant to penicillin G and was penicillinase (EC 3.5.2.6) inducible. *S. aureus* Meuse R was obtained from Leon D. Sabath (Harvard Medical School, Boston, Mass.). This strain was a clinical isolate resistant to methicillin and was penicillinase inducible.

All of the above strains were maintained both as lyophilized cultures and on nutrient agar slants.

**Medium, antibiotics, and surfactants.** All experiments were done using synthetic broth AOAC (Difco Laboratories, Detroit, Mich.). For the growth of staphylococci, the AOAC medium was supplemented with 1.0 $\mu$g of biotin per liter.

Potassium penicillin G, polymyxin B, chlortetracycline hydrochloride, tetracycline hydrochloride, and streptomycin sulfate were obtained from Calbiochem. Los Angeles, Calif. Methicillin was a gift to Bristol Laboratories, Syracuse, N.Y.

CTAB and TCP were obtained from Sigma Chemical Co., St. Louis, Mo. Lonzaine 14 (L14) is an *n*-alkyl mixture of dimethyl betaines (*n* = C14, 50%; C12, 40%, C16, 10%) and was a gift of the Lonza Co., Fair Lawn, N.J. Tw80 was obtained from Nutritional Biochemicals, Cleveland, Ohio.

**Growth studies.** A standard tube dilution assay was developed to investigate the effectiveness of antibiotic-surfactant combinations as growth inhibitors of the antibiotic-resistant strains. Growth studies were carried out in tubes (12 by 75 mm). Stock solutions of inhibitors were diluted into sterile medium and various amounts of these dilutions were added to the assay tubes. Sterile medium was added to each tube to obtain a final volume of 2.0 ml. Stock solutions of polymyxin B, penicillin G, methicillin, streptomycin, and Tw80 were prepared in medium just prior to use; chlortetracycline and CTAB were used as 2.0-mg/ml solutions in water; L14 was used as a 0.31% solution in water; TCP was diluted into sterile medium from a 10.0-mg/ml solution in sodium hydroxide.

Inocula were prepared by inoculating 20 ml of medium with a 16- to 18-h broth culture and incubating at 37 C in a New Brunswick Scientific incubator-shaker until growth reached late logarithmic phase as determined by optical density readings on a Coleman Jr. spectrophotometer at a wave length of 600 nm ($OD_{600}$). The culture was chilled in an ice bath and diluted with medium so that 0.1 ml of culture yielded an initial $OD_{600}$ of 0.02 per assay tube. Assay tubes were incubated in a rack tilted at a 45° angle in the shaker-incubator. The spectrophotometer was adapted to fit the assay tubes so that turbidimetric measurements of the growth response to various drugs could be easily done. In some experiments, inocula were prepared as described but in the presence of noninhibitory concentrations of surfactant and/or antibiotic. This was done to determine either the effect of surfactant on induction of antibiotic resist-

ance or if pretreatment of cells with surfactant would sensitize the cells to an antibiotic.

Effectiveness of inhibitor was determined by measuring differences in OD at a fixed incubation time between a culture with no drug and one with drug and calculating percentage of inhibiton. The time of incubation for each assay varied with the bacterial strain and was that time when growth of the control culture reached late logarithmic phase (3 to 6 h). Percentage of inhibition was defined as the ratio of the OD of the control culture minus the OD of the inhibited culture to the OD of the control culture minus the OD at the start of incubation. This ratio multiplied by 100 gave the percentage of inhibition.

For initial screening of the effect of the various surfactant-antibiotic combinations, a noninhibitory concentration of surfactant was tested in combination with at least two subinhibitory concentrations of antibiotic. An inhibition of growth by the surfactant-antibiotic combination greater than that by the antibiotic alone was taken to indicate potentiation.

To further evaluate those drug combinations which suggested potentiation, a modification of the method of Elion et al. (5) was used. The response (percentage of inhibition) of duplicate cultures to increasing concentrations of antibiotic and surfactant, both alone and in combination, was determined. Dose-response curves were drawn by plotting the percentage of inhibition against the $\log_{10}$ of the drug concentration. The resulting curves were found to give a straight line relationship between 20 and 80% inhibition. From these curves, the effective drug dose which inhibited growth rate by 50% ($ED_{50}$) was measured. The ratio of the $ED_{50}$ for the combined drugs to the $ED_{50}$ of each drug alone in the inhibitory mixture is the fractional inhibitory concentration. If the sum of the fractional inhibitory concentration of each drug in a combination is equal to 1.0, an additive effect is suggested; if the sum is less than 1.0, potentiation is suggested; and if it is greater than 1.0, there is antagonism.

**Measurement of protein synthesis.** Protein synthesis was measured as the rate of incorporation of uniformly labeled [$^{14}$C]leucine (New England Nuclear, Boston, Mass.; 280 mCi/ml) into the hot trichloroacetic acid-insoluble cell fraction. The uptake tubes contained medium, logarithmic phase cells to give an $OD_{600}$ of 0.04, inhibitors, and [$^{14}$C]leucine (0.2 $\mu$Ci/ml) in a total volume of 6.0 ml. Tubes were preincubated at 37 C in a water bath without shaking for 10 min prior to addition of label. Uptake of label was measured at 37 C by transferring, at various time intervals, 1.0-ml samples of each culture to tubes containing an equal volume of 10% trichloroacetic acid. These tubes were heated in a boiling water bath for 5 min and the contents were filtered through 0.45-$\mu$m membrane filters (Millipore Corp.) followed by washing the filters with 5% trichloroacetic acid. The filters were dried under a heat lamp and transferred to scintillation vials containing 10 ml of 0.4% Packard premix P {98% 2,5-diphenyloxazole, 2% *p*-bis-[2]-(5-phenyloxazole)benzene} in toluene. Radioactivity was measured using a Packard model 2002 liquid scintillation spectrometer.

**Penicillinase assay.** Enzyme activity was assayed according to the acidimetric method described by Rubin and Smith (18). One unit of penicillinase activity was that amount of enzyme which hydrolyzed 1 $\mu$mol of penicillin G in 1 h (18). Specific activity was expressed as units of enzyme activity per milligram of protein.

**Protein determination.** Protein was determined according to the method of Lowry et al. (13) using bovine serum albumin as a standard.

**Induction of penicillinase.** Staphylococcal cells were subcultured to an $OD_{600}$ of 0.2 on a New Brunswick shaker-incubator at 37 C at which time the cultures were diluted with an equal volume of sterile medium containing penicillin G to give a final concentration of 100 $\mu$g/ml. In some flasks, CTAB was included in the diluent. Incubation was continued for 2 h. The cultures were chilled in an ice bath, centrifuged at 20,000 $\times$ *g* at 5 C, washed once with 2 mM potassium phosphate buffer, pH 7.6, and resuspended in buffer. Cells were brokin by sonication in an ice bath for 10 min with an MSE ultrasonic disentergrator. The sonicates were clarified by centrifugation as described above and assayed for penicillinase activity and protein as described.

## RESULTS

**Comparative surfactant susceptibility of antibiotic-susceptible and -resistant strains.** Results presented in Table 1 show that the polymyxin-resistant *E. coli* Sc8600 strain was more susceptible to the surfactants than the parent Sc8599 strain. The R factor 222 containing *E. coli* Sc8280 strain did not appear to differ in surfactant susceptibility from the Sc8190 strain whereas *P. mirabilis* pm5/R$^+$ was found to be less resistant to the surfactants and to polymyxin B than strain pm5. Cross-resistance between polymyxin B and surfactants was evident with the *S. marcescens* strains. Tw80 was not found to inhibit the growth of any of the bacterial strains up to the highest concentration tested (5.0 mg/ml).

**Growth response to antibiotic-surfactant combinations.** Figure 1 is an example of the type of growth response observed with combinations of a noninhibitory concentration of surfactant with subinhibitory concentrations of antibiotic. In this case, the potentiating effect of CTAB on chlortetracycline activity is evident. The growth response of other antibiotic-resistant gram-negative strains indicated that CTAB and L14 but not TCP or Tw80 potentiate the activity of chlortetracycline; CTAB, L14, and TCP but not Tw80 potentiate the activity of polymyxin B; none of the surfactants potentiate streptomycin or penicillin G activity. Growth

TABLE 1. *A comparison of the susceptibility of related antibiotic-susceptible and -resistant strains to surfactants, chlortetracycline (CTC), and polymyxin B (PmB)*[a]

| Strain | $ED_{50}$ (μg/ml)[b] | | | | |
|---|---|---|---|---|---|
| | Surfactant | | | Antibiotic | |
| | L14 | CTAB | TCP | CTC | PmB |
| *Escherichia coli* | | | | | |
| Sc8190 | 270.0 | 6.8 | 880 | 0.2 | S[c] |
| Sc8280 | 320.0 | 7.2 | 690 | 9.4 | S |
| Sc8599 | 450.0 | 12.5 | 500 | S | 1.5 |
| Sc8600 | 35.5 | 5.3 | 325 | S | 198.0 |
| *Proteus mirabilis* | | | | | |
| pm5 | 310.0 | 23.0 | 850 | 1.6 | 6,200.0 |
| pm5/R$^+$ | 109.0 | 13.5 | 400 | 3.4 | 1,800.0 |
| *Serratia marcescens* | | | | | |
| 164E4 | 88.0 | 6.4 | 590 | R[c] | 6.4 |
| 164E3 | 620.0 | 27.0 | 650 | R | 1,600.0 |

[a] See text for details of the bioassay procedure.
[b] That concentration of inhibitor which inhibited growth by 50% of the control.
[c] (S) Susceptible and (R) resistant as determined by the method of Bauer et al. (1).

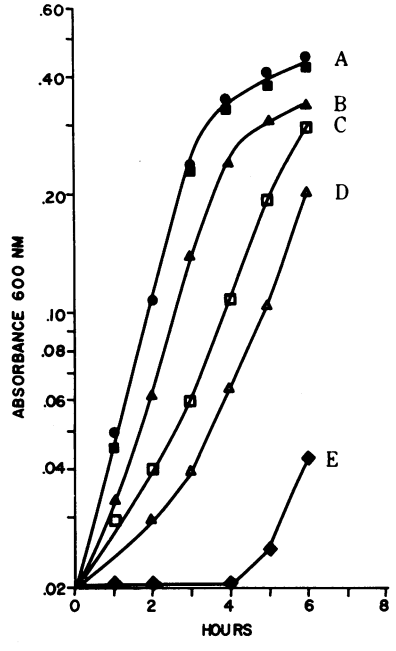



FIG. 1. *Growth response of E. coli Sc8280 in the presence of CTAB and/or chlortetracycline (CTC). Cells were cultured in 2.0 ml of medium as described. Symbols: ●, no drug additions, curve A; ■, 5.0 μg of CTAB/ml, curve A; ▲, 10.0 μg of CTC/ml, curve B; □, 20.0 μg of CTC/ml, curve C; (Δ) 5.0 μg of CTAB + 10.0 μg of CTC/ml, curve D, ◆, 5.0 μg of CTAB + 20.0 μg of CTC/ml, curve E.*

response of the staphylococci to antibiotic-surfactant combinations indicated that penicillin G activity but not methicillin activity was enhanced by CTAB and L14 but not TCP or Tw80.

**Tetracycline-surfactant combinations.** Results presented in Table 2 demonstrated that CTAB and L14, at concentrations which by themselves had little or no effect on bacterial growth rate, decreased the $ED_{50}$ for chlortetracycline about two- to threefold for all four resistant gram-negative strains.

Subculture of the *E. coli* Tc and Sc8280 strains in the presence of 1.0 μg of chlortetracycline per ml was found to increase the antibiotic resistance level 2.3-fold and 4.3-fold, respectively. No effect of CTAB on this induction could be demonstrated. When chlortetracycline-induced cultures were challenged with chlortetracycline in the presence of 5.0 μg of CTAB per ml, susceptibility to chlortetracycline was found to be similar to that of uninduced cultures challenged without CTAB present (Table 2).

The effect of CTAB on the inhibition of protein synthesis in *E. coli* Tc cultures by tetracycline hydrochloride is presented in Fig. 2. It can be seen that incorporation of label into the trichloroacetic acid-insoluble material in the presence of CTAB alone was similar to that of the control culture. Tetracycline was observed to initially inhibit protein synthesis for

TABLE 2. *Effect of CTAB and L12 on the susceptibility of tetracycline-resistant strains to chlortetracycline (CTC)*[a]

| Strain | Surfactant[b] | Surfactant (μg/ml) | CTC $ED_{50}$ (μg/ml) | Relative susceptibility[c] |
|---|---|---|---|---|
| *Escherichia coli* Tc | None | 0.0 | 11.5 | 1.0 |
| | CTAB (0.69) | 5.0 | 4.0 | 2.9 |
| | L14 (0.60) | 50.0 | 7.4 | 1.6 |
| *E. coli* Sc8280 | None | 0.0 | 9.4 | 1.0 |
| | CTAB (0.69) | 5.0 | 3.8 | 2.5 |
| | L14 (0.33) | 77.5 | 4.3 | 2.2 |
| *Proteus mirabilis* pm5/R$^+$ | None | 0.0 | 3.4 | 1.0 |
| | CTAB (0.71) | 10.0 | 2.0 | 1.7 |
| | L14 (0.71) | 77.5 | 1.1 | 3.1 |
| *Klebsiella pneumoniae*/pm | None | 0.0 | 6.0 | 1.0 |
| | CTAB (0.67) | 10.0 | 3.3 | 1.8 |
| | L14 (0.41) | 310.0 | 2.4 | 2.5 |

[a] The standard bioassay was used as described.

[b] The numbers in parenthesis represent the fractional inhibitory concentration of the surfactant. These concentrations of surfactants by themselves had little or no effect on growth rate.

[c] The ratio of the $ED_{50}$ of CTC alone to the $ED_{50}$ of CTC plus surfactant.

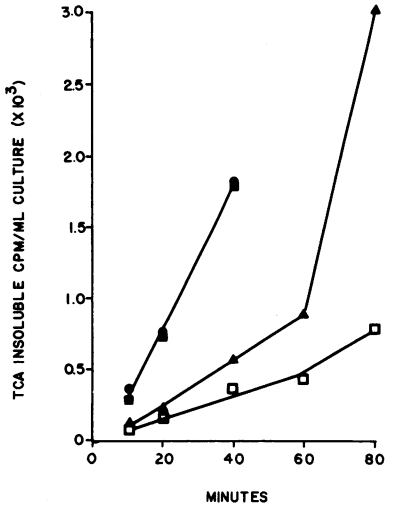
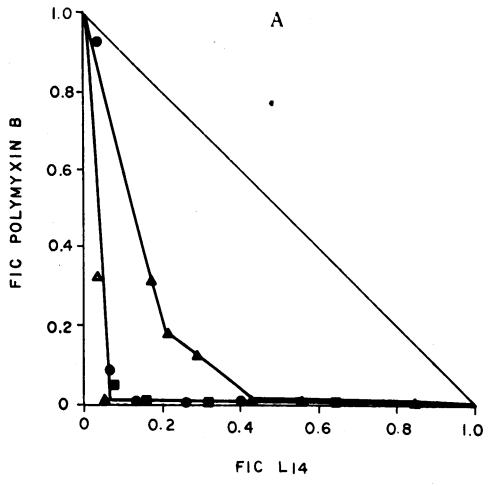


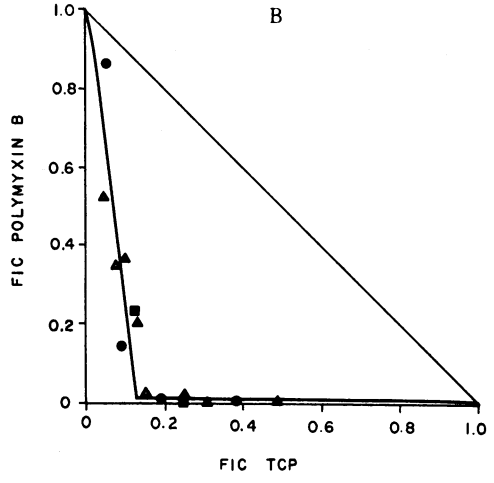

FIG. 2. *Incroporation of [$^{14}$C]leucine into the hot trichloroacetic acid-insoluble fraction of E. coli Tc cells cultured with and without CTAB and/or tetracycline·hydrochloride (TC). The procedure is as described. Symbols: ●, no drug additions; ■, 5.0 μg of CTAB/ml; ▲, 20.0 μg of TC/ml; □, 5.0 μg of CTAB + 20.0 μg of TC/ml.*

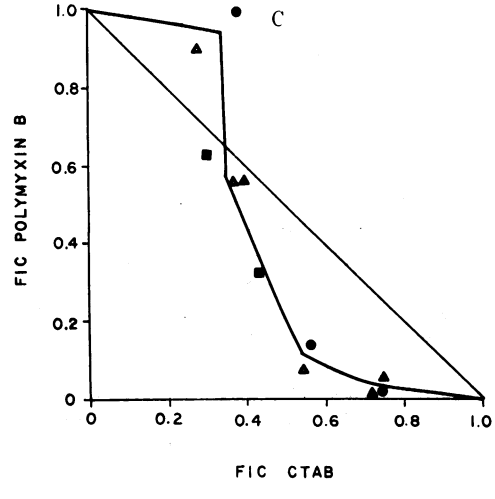
60 min after which time incorporation of label occurred at a rate similar to that of the control, whereas the combined effect of CTAB and tetracycline was to further inhibit protein synthesis with little recovery by 80-min incubation time.

**Polymyxin B-surfactant combinations.** Effects of drug combinations on strains of *Proteus* and *Serratia* are presented in Fig. 3 as isobolograms. It can be seen that, except for the combination of L14 and polymyxin B with *P. mirabilis* pm5/R$^+$, each isobol is the same for a given drug combination regardless of the strain tested. CTAB, L14, and TCP were found to greatly potentiate the activity of polymyxin B as demonstrated by the large deviation of the curves from the summation line. Maximal effective concentrations of L14 and polymyxin B were 6.5 and 1.0% of the $ED_{50}$ for each drug, respectively, except for *P. mirabilis* pm5/R$^+$ in which case these values were 21 and 19.5%, respectively. For TCP and polymyxin B the values were 12.5 and 1.5%, respectively, and for CTAB and polymyxin B, 59 and 10%, respectively.

Results on the effectiveness of polymyxin B-surfactant combinations against *P. mirabilis* 190A-1, *K. pneumoniae*/pm, and *E. coli* Sc8600 are presented in Table 3. Combination of CTAB, or L14, with polymyxin B indicated potentiation when tested against the *Proteus* and *Klebsiella* strains since the sums of the fractional inhibitory concentrations are less than one. TCP was not effective in potentiating the activity of polymyxin B against *K. pneumoniae*/pm (data not shown) but did potentiate antibiotic activity against *P. mirabilis* 190A-1 and *E. coli* Sc8600. CTAB and L14 did not appear to be as effective in enhancing the activity of polymyxin B against *E. coli* Sc8600, although susceptibility to the antibiotic was increased two- to fourfold. Tw80 was found to




potentiate polymyxin B activity against this strain.

To further define the combined action of surfactants and polymyxin B on resistant cells, the effect of subculture in the presence of L14 or polymyxin B on susceptibility to one or the other drug was studied. Both *P. mirabilis* 190A-1 and *S. marcescens* 164E3 were subcultured in the presence of either L14 or polymyxin B, which in combination would completely inhibit the growth of these organisms. These subcultures were used as inocula for the standard bioassay as described. The results indicated that pretreatment with L14 or polymyxin B did not alter susceptibility to polymyxin B or L14, respectively.

**Penicillin-surfactant combinations.** Results presented in Table 4 show that both CTAB and L14 potentiated the activity of penicillin G on *Staphylococcus* NYH and *S. aureus* Meuse R. CTAB, at the fractional inhibitory concentration of 0.6, was a better potentiator than L14 tested at the same fractional inhibitory concentration.

The effect of CTAB on the synthesis of penicillinase by *S. aureus* Meuse R cells is presented in Table 5. It can be seen that the addition of 100 μg of penicillin G/ml of medium increased the specific activity of penicillinase about 10-fold over the constitutive enzyme level. When increasing amounts of CTAB were present with penicillin G, the amount of penicillinase synthesized per milligram of cell protein was progressively inhibited. Specific activity decreased to 24% of that in the culture without CTAB. CTAB, up to a concentration of 100 μg/ml, was not found to affect penicillinase activity when added to the enzyme reaction mixture.

## DISCUSSION

The importance of the bacterial cell membrane or envelope in contributing to the resistance of microorganisms to the toxic effects of antimicrobial agents is still a matter of conjecture. Recent scientific investigations now suggest that the phenomenon of antimicrobial resistance is not as simple as once imagined and

FIG. 3. *Isobolograms of the combined growth inhibitory effects of polymyxin B and (A) L14, (B) trichlorophenoxypropionic acid (TCP), and (C) CTAB on Proteus and Serratia strains. The standard broth dilution assay was used to determine $ED_{50}$ values and fractional inhibitory concentrations (FIC) were calculated as described. Symbols: ■, P. mirabilis pm5; ▲, P. mirabilis pm5/R+; ●, S. marcescens 164E3; △, S. marcescens DeJohn.*

TABLE 3. *Inhibition of growth of Proteus mirabilis 190A-1, Klebsiella pneumoniae/pm, and Escherichia coli Sc8600 by combinations of surfactants (SAA) and polymyxin B (PmB)*[a]

| Strain | SAA | $ED_{50}$ (μg/ml)[b] | | Fractional inhibitory concentration | | | Relative susceptibility[d] |
|---|---|---|---|---|---|---|---|
| | | SAA | PmB | SAA | PmB | Sum | |
| *P. mirabilis* 190A-1 | None | 0.0 | >4,000.0 | 0.0 | 1.0 | 1.0 | 1.0 |
| | CTAB | 11.0 | 250.0 | 0.69 | <0.06 | <0.75 | >16.0 |
| | L14 | 50.0 | 9.4 | 0.44 | <0.002 | <0.44 | >426.0 |
| | TCP | 125.0 | 110.0 | 0.71 | <0.03 | <0.74 | >36.0 |
| *K. pneumoniae* pm | None | 0.0 | >4,000.0 | 0.0 | 1.0 | 1.0 | 1.0 |
| | CTAB | 10.0 | 3.2 | 0.67 | <0.0004 | <0.67 | >1,250.0 |
| | L14 | 310.0 | 10.4 | 0.41 | <0.003 | <0.41 | >385.0 |
| *E. coli* Sc8600 | None | 0.0 | 198.0 | 0.0 | 1.0 | 1.0 | 1.0 |
| | CTAB | 2.5 | 100.0 | 0.47 | 0.51 | 0.98 | 2.0 |
| | L14 | 25.0 | 45.0 | 0.70 | 0.28 | 0.93 | 4.4 |
| | TCP | 100.0 | 14.0 | 0.20 | 0.07 | 0.27 | 142.0 |
| | Tw80 | 5,000.0 | 52.0 | | 0.26 | | 3.8 |

[a] The standard bioassay was used as described.
[b] That concentration of each drug which inhibited growth by 50%.
[c] The ratio of the $ED_{50}$ for the combined drugs to the $ED_{50}$ of each drug when used alone.
[d] The ratio of the $ED_{50}$ of PmB alone to the $ED_{50}$ of PmB plus surfactant.

TABLE 4. *Inhibition of growth of Staphylococcus NYH and Staphylococcus aureus Meuse R by combinations of surfactants (SAA) and penicillin G (PenG)*[a]

| Strain | SAA | $ED_{50}$ (μg/ml) | | Fractional inhibitory concentration | | | Relative susceptibility |
|---|---|---|---|---|---|---|---|
| | | SAA | PenG | SAA | PenG | Sum | |
| NYH | CTAB | 0.0 | 129.0 | 0.0 | 1.0 | 1.0 | 1.0 |
| | | 0.2 | 62.0 | 0.3 | 0.48 | 0.78 | 2.1 |
| | | 0.4 | 10.0 | 0.61 | 0.08 | 0.69 | 12.9 |
| | L14 | 0.0 | 129.0 | 0.0 | 1.0 | 1.0 | 1.0 |
| | | 4.0 | 75.0 | 0.31 | 0.58 | 0.89 | 1.7 |
| | | 8.0 | 17.5 | 0.62 | 0.14 | 0.76 | 7.4 |
| Meuse R | CTAB | 0.0 | 295.0 | 0.0 | 1.0 | 1.0 | 1.0 |
| | | 0.7 | 15.8 | 0.58 | 0.05 | 0.63 | 18.7 |
| | L14 | 0.0 | 295.0 | 0.0 | 1.0 | 1.0 | 1.0 |
| | | 10.0 | 92.0 | 0.59 | 0.31 | 0.90 | 3.2 |

[a] The method, abbreviations, and definitions are described in Table 3.

that in many instances several factors may interact each contributing to overall resistance. Experimental evidence presented here suggests that, at least for some antibiotics, cell membranes or envelopes are one of the contributory factors.

Although in this study surfactants were found to potentiate the toxic effects of chlortetracycline, penicillin G, and polymyxin B on resistant bacterial strains, this phenomenon is not a general one but depends on the bacterial strain and the type of antibiotic.

The enhancement of the inhibitory effect of chlortetracycline by CTAB and L14 but not TCP and Tw80 suggests the involvement of the quaternary ammonium group of these compounds. Uptake of tetracyclines by bacterial cells has been shown to be an energy-dependent process (6) and resistance to these antibiotics is associated with decreased accumulation of the drug (6, 24). Higher levels of antibiotic resistance can be induced with low levels of antibiotic (6). It is possible, therefore, that CTAB and L14 potentiate the activity of chlortetracycline

TABLE 5. *The effect of CTAB on the synthesis of penicillinase in Staphylococcus aureus Meuse R*[a]

| Culture additions (μg/ml) | | Inhibition of growth (%) | Penicillinase sp act[b] | Relative activity[c] |
|---|---|---|---|---|
| PenG | CTAB | | | |
| 0.0 | 0.0 | 0.0 | 0.39 | 0.09 |
| 100.0 | 0.0 | 33.0 | 4.18 | 1.00 |
| 100.0 | 0.5 | 37.0 | 3.33 | 0.80 |
| 100.0 | 1.0 | 52.0 | 2.31 | 0.55 |
| 100.0 | 2.0 | 59.0 | 1.00 | 0.24 |
| 100.0 | 4.0 | 93.0 | 1.28 | 0.31 |

[a] See text for experimental procedure. PenG, Penicillin G.

[b] Specific activity is expressed as units/milligram of protein. One unit of enzyme activity was that amount of enzyme which hydrolyzed 1.0 μmol of PenG in 1 h.

[c] The ratio of enzyme specific activity in induced cells to that in uninduced or induced cells with CTAB present.

either by interfering with the induction mechanism or by overcoming the antibiotic exclusion mechanism. Since it could not be demonstrated that CTAB interfered with the induction mechanism, it appears that the antibiotic exclusion mechanism is affected. In support of this view, it could be demonstrated that the inhibition of protein synthesis in *E. coli* cells by tetracycline was greater in a cell culture treated with both CTAB and tetracycline than with tetracycline alone (Fig. 2). This suggests that, in the presence of CTAB and presumably L14, more antibiotic is taken up by the resistant cells.

Franklin and Foster (7) have recently reported that ethylenediaminetetraacetic acid-osmotic shock or ethylenediaminetetraacetic acid treatment alone of tetracycline-resistant cells of *E. coli* initially increased the inhibition of protein synthesis by tetracyclines followed by recovery of resistance. Similar treatment of susceptible cells showed no such effect. Presumably, resistance components are associated with the outer membrane of the cell envelope since such treatment is known to deplete cells of components of this membrane (12). With respect to this, it is interesting that Voss (27) has obtained evidence which indicates that cationic surfactants, when added to a suspension of *E. coli* cells, cause the release of cell surface antigens into the surrounding medium. It is therefore possible that CTAB and L14 disrupt the outer cell membrane leading to increased adsorption and transport of the antibiotic into the cell.

A recent report by Ulitzer (25) on the transport of β-galactosides into permeaseless *E. coli* cells after treatment with noninhibitory concentrations of CTAB may be significant in interpreting the phenomenon of potentiation of chlortetracycline activity. Although these mutant cells were unable to hydrolyze a β-galactoside because of a defective transport system, CTAB-treated cells were able to do so (25). Sodium dodecyl sulfate, benzalkonium chloride, Triton X-100, and Tw80 had no effect on transport (25). It was suggested that a subunit of the carrier protein is somehow unmasked by the action of CTAB on the outer layer of the cell membrane.

In contrast to their effects on susceptibility to chlortetracycline, CTAB, L14, and TCP were observed to almost completely sensitize strains of *Proteus* and *Serratia* to polymyxin B (Fig. 3). *K. pneumoniae*/pm differed from the above mentioned strains in that TCP did not sensitize this strain to polymyxin B. This could be due to the large capsule which is characteristic of such strains. Tw80 was found to sensitize only the polymyxin-resistant strain *E. coli* Sc8600 to polymyxin B (Table 3). This would suggest that the mechanism of resistance in these cells differs in some unknown way from the other strains tested. This effect of Tw80 is similar to the results of Bliss and Warth (2) who reported an increase in the sensitivity of a susceptible strain of *E. coli* to polymyxin B in the presence of this surfactant. According to Brown and Winsley (3), Tw80 also potentiates the activity of polymyxin B against *P. aeruginosa*. It was suggested that Tw80 alters the outer wall structure, allowing easier entry by this antibiotic.

Since the polymyxins are compounds which have the properties of cationic surfactants, resistance to this antibiotic might be expected to confer cross-resistance to other surfactants since these compounds have a common target site, the cell membrane, and presumably a common mechanism of resistance. This was found to be the case with a resistant strain of *Serratia* (Table 1). It was unexpected, however, to find that the polymyxin-resistant *E. coli* Sc8600 strain was more susceptible to CTAB, L14, and TCP than its parent strain (Table 1). This, together with the fact that CTAB and L14 were not very effective potentiating agents with this strain, again suggests that the mechanism of polymyxin resistance is different from the other polymyxin-resistant strains.

The presence of R factor 222 in the *P. mirabilis* strain was also seen to affect susceptibility to the surfactants and polymyxin B. In this instance, *P. mirabilis* pm5/R$^+$ was found to be concomitantly less resistant to polymyxin B and to CTAB, L14, and TCP than its parent

strain (Table 1). Also, the maximum effective concentrations of L14 and polymyxin B differed from those of the parent (Fig. 3). It is interesting that the same R factor in *E. coli* Sc8280 did not alter the susceptibility of this strain to these surfactants. Surface alterations which occur in cells that contain R factors are not known, but this factor apparently affects *P. mirabilis* differently than *E. coli* with respect to susceptibility to surfactants.

Sud and Feingold (21) have reported that pretreatment of *P. mirabilis* cells with polymyxin B sensitizes them to sodium deoxycholate, whereas the reverse situation does not occur. This did not appear to be the case with the surfactants used in the present study and the results indicated that both surfactant and antibiotic must be present at the same time to exert a potentiating effect.

Studies of *P. mirabilis* by others have provided evidence which suggests that polymyxin-susceptible target sites are present in these organisms but are inaccessible to the antibiotic (20, 23). The best evidence for this is that L-forms were 400-fold more susceptible to this antibiotic, suggesting that the outer envelope structures play a role in the intrinsic resistance characteristic of these strains (23). Since *S. marcescens* is also intrinsically resistant to the polymyxins, it is a strong possibility that its outer envelope structures play a similar role in resistance. *K. pneumoniae* and *E. coli* are intrinsically susceptible to the polymyxins and it would be interesting to determine if L-forms of the resistant strains are susceptible to polymyxin B. The simplest mechanism, therefore, to explain the potentiation by surfactant-polymyxin B combinations would be partial loss of outer membrane components or alteration of the outer cell membrane in the resistant strains by these surfactants leading to easier accessibility of the antibiotic to its susceptible target site(s).

Marked enhancement of polymyxin B activity by TCP is most interesting because this compound does not possess the long hydrocarbon chain present in CTAB or L14. Scherr and Bechtle (19) originally reported that compounds such as TCP demonstrated in vitro potentiating activity for a number of antibiotics against strains of *Sarcina lutea*, *S. aureus*, and *Mycobacterium tuberculosis*. Its effect on bacteria is not known, but derivatives of phenoxypropionic acid such as 2(*p*-chlorophenoxy)-2-methyl propionate have been under investigation in recent years because of their use as agents in the therapy of hyperlipidemia (26). Experimental evidence suggests that these drugs act as inhibitors of acetyl coenzyme A carboxylase which catalyzes the first committed step in the synthesis of fatty acids by carboxylation of acetyl coenzyme A to form malonyl coenzyme A (14). Since fatty acids are integral parts of the cell envelope components, inhibition of fatty acid synthesis by TCP would be expected to affect membrane function. It could therefore be profitable to study the effect of TCP, as well as CTAB and L14, on cellular metabolic functions, particularly lipid synthesis, to determine if such affects are sufficent to explain the potentiation phenomenon.

Both CTAB and L14 were found to potentiate the activity of penicillin G against two strains of staphylococci whereas TCP and Tw80 had no effect. None of the surfactants were found to potentiate the activity of penicillin G against a strain of *E. coli* suggesting that the permeability barrier to this antibiotic was not altered. This is contrary to the effect of EDTA which has been reported to damage the permeability barrier to penicillin G in a strain of *E. coli* (8).

It is suspected that the potentiating effect of CTAB and L14 on penicillin G activity against staphylococci is related to penicillinase for the following reasons: (i) neither CTAB nor L14 affected the susceptibility of *S. aureus* Meuse R to methicillin, a penicillinase-resistant penicillin, whereas susceptibility to penicillin G was increased up to 19-fold (Table 4); (ii) both strains were inducible penicillinase producers. A study of the effect of CTAB on the specific activity of penicillinase (Table 5) strongly suggested that this surfactant, and presumably L14, interfers with the formation of this enzyme. No direct effect of CTAB on enzyme activity was observed in vitro.

The manner in which CTAB and L14 would affect the synthesis of penicillinase cannot be known without further experimentation. The surfactants might interfere with the induction mechanism although the experimental conditions did not allow us to distinguish between this and interference with enzyme synthesis. Imsande (9) has proposed a working model for the regulation of penicillinase synthesis. According to this model, the inducer (penicillin) is thought to interact with a site on an antirepressor molecule to change its conformation to one which can actively bind to and inactivate a repressor molecule. Inactivation of the repressor molecule permits transcription of the penicillinase structural gene and subsequently enzyme synthesis occurs. Although the repressor substance appears to be located in the cell cyto-

plasm, it is possible that the antirepressor is bound to the cell membrane (9). CTAB or L14 may therefore alter the microenvironment of the antirepressor molecule, or bind to the molecule, so that the inducer cannot interact. One could predict from this hypothesis that a mutant strain of staphylococci which is constitutive for penicillinase synthesis would not show an inhibition of enzyme synthesis or potentiation of antibiotic activity by surfactant.

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