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A potentiating effect of EDTA on the bactericidal activity of lower concentrations of ethanol

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Summary

The bactericidal activity of 10 and 5% ethanol alone and in the presence of EDTA against *Ps. aeruginosa*, *E. coli* and *Staph. aureus* has been studied using the extinction-time and the death-curve methods. EDTA at various concentrations was found to potentiate the bactericidal activity of ethanol. This potentiation increased with increase in the EDTA concentration from 0.05 to 0.3% against *Ps. aeruginosa* and *Staph. aureus*. Similar results were obtained with *E. coli* in EDTA concentrations of 0.05–0.2% but not with 0.3% which showed the least potentiating effect.

Introduction

EDTA has been shown to potentiate the activity of a number of preservatives (MacGregor and Elliker, 1958; Nezval, 1964; Brown and Richards, 1965; Reybrouk and Van de Voorde, 1969; Adair et al., 1971; Russell, 1971; Richards and McBride, 1972). Brown (1968), however, reported that it caused a reduction in the activity of solutions of phenylmercuric nitrate. In a review by Wilkinson (1975), it was reported that high kills were achieved with low concentrations of EDTA alone against several Gram-negative bacteria including *Ps. aeruginosa* although the test inocula were not generally sterilized. Since the antimicrobial activity of low concentrations of ethanol is usually limited (Morton, 1971), this work was designed to investigate the effect of EDTA on the bactericidal activity of such concentrations.

Materials and Methods

Nutrient broth no. 2, nutrient agar, absolute ethanol and ethylenediaminetetraacetic acid potassium salt were purchased from Merck. The organisms were

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Pseudomonas aeruginosa NCTC 6750, *Escherichia coli* NCTC 9001 and *Staphylococcus aureus* NCTC 3761.

Maintenance of microorganisms and preparation of the inoculum

The bacterial cultures were maintained by weekly subcultures on nutrient agar slopes. After incubation for 24 h at 37°C, the slopes were stored at 5°C. Prior to each experiment, each organism was activated by subculturing on 3 successive days. The last 24 h culture was harvested by washing the surface growth of each slope with 5 ml of sterile distilled water and the resultant suspension which contained about 10^9 viable cells · ml^{-1} used on the same day for the evaluation process described below.

Determination of bactericidal activity by the extinction-time and the death-curve methods

Reaction mixtures with final volumes of 10 ml were prepared to contain various concentrations of ethanol, EDTA or their combinations in sterile distilled water as indicated in Table 1 and the test organisms in concentrations of about $1-5 \times 10^7$ viable cells · ml^{-1} and kept at 25 ± 1°C. At specified time intervals (Table 1), a loopful from each reaction mixture was transferred by means of a standard 4-mm loop into 10 ml of the recovery medium (nutrient broth no. 2) in duplicates. The recovery broth tubes were then incubated at 37°C for 48 h to determine the corresponding extinction-times. The extinction-time was considered as the shortest time at which there was no growth in the two recovery tubes.

Alternatively, for the determination of the bactericidal activity by the death-curve method, 1 ml samples were withdrawn from the reaction mixtures at specified time

TABLE 1

EXTINCTION-TIME OF VARIOUS CONCENTRATIONS OF EDTA, ETHANOL AND THEIR COMBINATIONS USING 3 DIFFERENT BACTERIA AS TEST ORGANISMS

Reaction mixtures		Extinction time (h)		
EDTA (%) +	Ethanol (%)	*Ps. aeruginosa*	*E. coli*	*Staph. aureus*
0	10	> 24	24	24
0.05	0	> 24	> 24	> 24
0.10	0	> 24	> 24	> 24
0.20	0	> 24	> 24	> 24
0.30	0	> 24	> 24	> 24
0.05	5	> 24	> 24	> 24
0.10	5	> 24	> 24	> 24
0.20	5	24	> 24	> 24
0.30	5	22	> 24	14
0.05	10	4	6	12
0.10	10	3	6	12
0.20	10	3	6	6
0.30	10	2	8	4

intervals (Figs. 1–4) and serially diluted 10-fold in sterile distilled water. The dilution brings the concentrations of the antimicrobial agents to non-inhibitory levels and also allows for appropriate dilution for counting the number of surviving organisms. The latter was determined by the surface viable count technique of Miles and Misra (1938), using agar plates. Then drops per plate were taken for each time interval. The resultant colonies were counted after incubation of the plates at 37°C for 24–48 h and the mean counts of 10 drops for each time interval were used to calculate the original count in the reaction mixtures. The log number of survivors per ml was plotted against time to obtain the corresponding death-curves in Figs. 1–4.

In each experiment a control was included containing the test organism in sterile distilled water without the addition of any antimicrobial agent.

Results

Extinction-time method

EDTA at all the concentrations used (0.05–0.3%) did not achieve sterility within 24 h with all the test organisms. On the other hand the highest concentration of ethanol (10%), killed within 24 h the added inocula of *E. coli* and *Staph. aureus* but did not kill *Ps. aeruginosa* (Table 1).

When 5% ethanol was combined with either 0.05, 0.1 or 0.2% EDTA, the extinction-time was 22 h or more with all the test bacteria. The combination of 5% ethanol and 0.3% EDTA also gave similar results with *E. coli* and *Ps. aeruginosa* while a shorter extinction-time of 14 h was obtained with *Staph. aureus* (Table 1).

All the combinations of 10% ethanol and EDTA showed marked bactericidal activity against all the test organisms. The corresponding extinction-times were in the range of 2–4 h, 6–8 h and 4–12 h for *Ps. aeruginosa, E. coli* and *Staph. aureus*, respectively (Table 1).

Death-curve method

All the concentrations of ethanol, EDTA and their combinations did exert, but to varying degrees, some lethal action on the test organisms as shown in Figs. 1–4. The various concentrations of EDTA when combined with 10% ethanol exerted pronounced killing effects which in all cases were higher than the effect of any EDTA combination with 5% ethanol.

With *Ps. aeruginosa* (Fig. 1), ethanol at the highest concentration (10%) did not exert any appreciable effect, whereas 0.3% EDTA alone produced a slight decrease in the viable cell count. In general, at the same alcohol concentration the death rates of the combinations increased with increase in the concentration of EDTA; the highest killing effect being produced by the 0.3% EDTA and 10% ethanol combination. The death rate was initially very high — up to 15 min — but decreased thereafter. The death-curves for the other combinations followed similar pattern with change to the lower rate starting after 45 min contact time.

With *E. coli* (Fig. 2), ethanol (5 and 10%), the different concentrations of EDTA and all the combinations of EDTA and 5% ethanol showed extremely low death

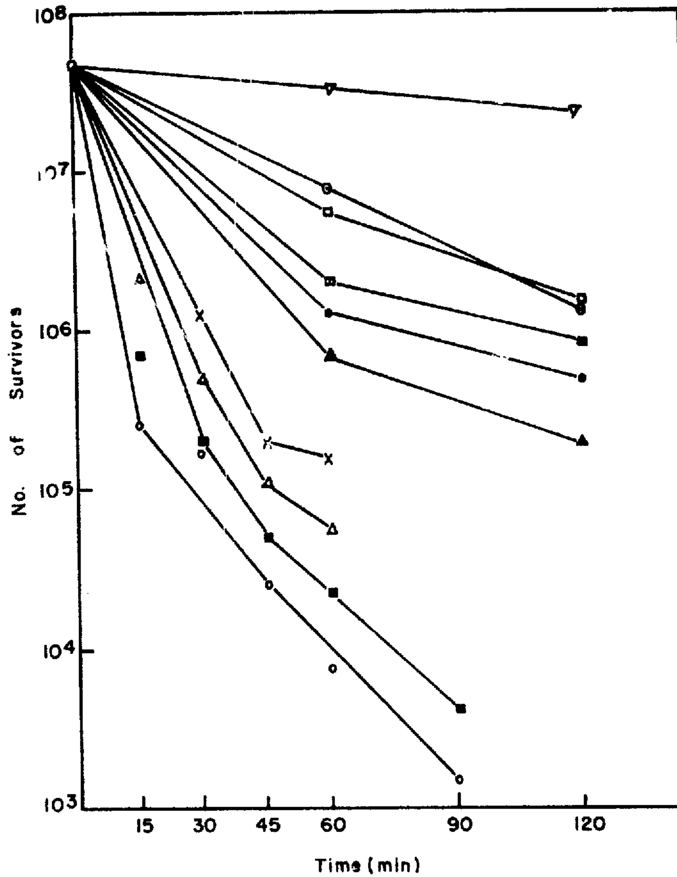

Fig. 1. Death-curves showing the bactericidal activity of various concentrations of EDTA and ethanol in combinations using *Ps. aeruginosa* as test organism. Key: ▽——▽, 10% ethanol; ⊡——⊡, 0.1% EDTA + 5% ethanol; ×——×, 0.05% EDTA + 10% ethanol; ○——○, 0.3% EDTA + 10% ethanol; □——□, 0.05% EDTA + 5% ethanol; ●——●, 0.2% EDTA + 5% ethanol; △——△, 0.1% EDTA + 10% ethanol; ⊙——⊙, 0.3% EDTA; ▲——▲, 0.3% EDTA + 5% ethanol; ■——■, 0.2% EDTA + 10% ethanol.

rates in comparison with combinations containing 10% ethanol. Of all the combinations of EDTA and 10% ethanol, that which contained the highest concentration of EDTA (0.3%) exerted the lowest killing effect. The death rate exhibited by the combinations containing lower concentrations of EDTA (0.2, 0.1 and 0.05%), however, increased with increase in EDTA concentration. The results obtained with the combinations containing 5% ethanol showed increased activity with increase in the concentration of EDTA with no point of inflexion; but was in all cases lower than the lethal activity of 10% ethanol only (Fig. 2).

Figs. 3 and 4 show the death-curves obtained with the various concentrations of EDTA combined with 10% or 5% ethanol, respectively, using *Staph. aureus* as test organism. The killing effect increased with increase in the concentration of EDTA for all combinations. The combination containing 10% ethanol and 0.3% EDTA exhibited, relatively, the highest bactericidal effect with a uniform and constant

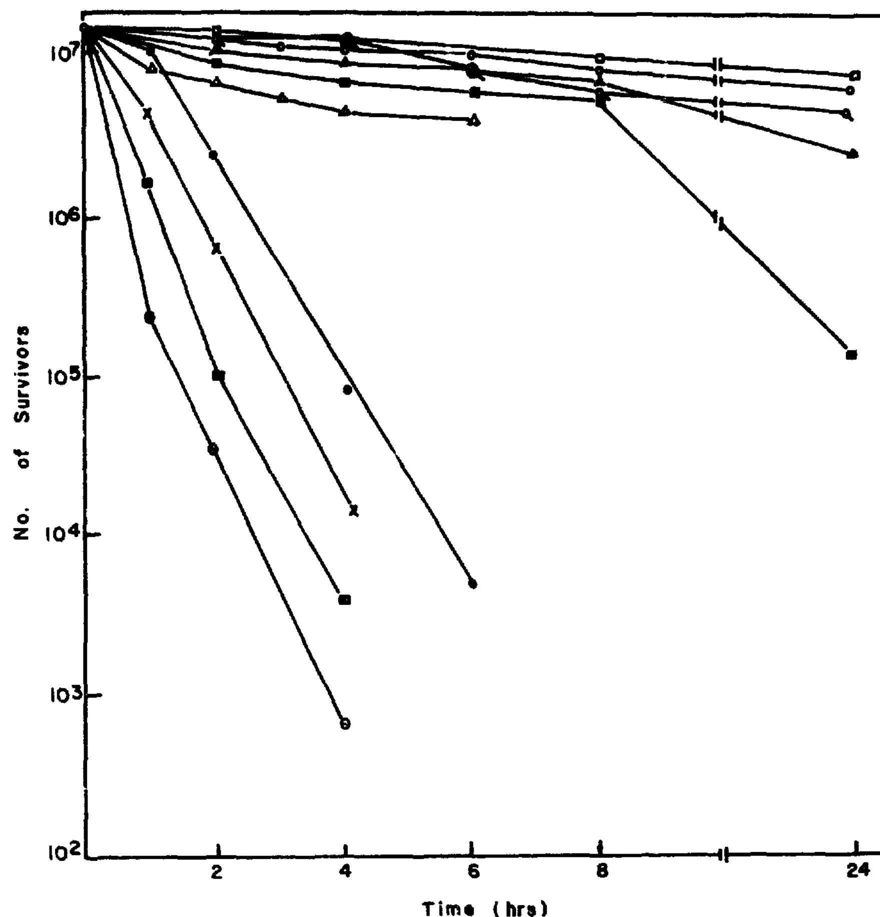

Fig. 2. Death-curves showing the bactericidal activity of various concentrations of EDTA and ethanol in combination using *E. coli* as test organism. Key: O——O, 0.3% EDTA; ▲——▲, 0.2% EDTA + 5% ethanol; ●——●, 0.3% EDTA + 10% ethanol; □——□, 0.05% EDTA + 5% ethanol; ■——■, 0.3% EDTA + 5% ethanol; ×——×, 0.05% EDTA + 10% ethanol; O——O, 0.1% EDTA + 5% ethanol; △——△, 10% ethanol; ⊡——⊡, 0.1% EDTA + 10% ethanol; ⊙——⊙, 0.2% EDTA + 10% ethanol.

death rate throughout the period of test, whereas the 0.2, 0.1 and 0.05% EDTA combinations showed an initial fast death rate during the first hour before decreasing. The 10% ethanol curve showed an initial high bactericidal activity during the first 2 h followed by a decrease in the death rate. The 10% ethanol and 0.05% EDTA combination, however, exerted a steady death rate which was lower than that of 10% ethanol alone during the first 2 h followed by a higher rate thereafter.

Discussion

The marked increase in the bactericidal activity of ethanol by EDTA is probably due to the sensitizing action of EDTA on the organisms. EDTA is known to sensitize Gram-negative organisms especially *Ps. aeruginosa* to the action of several antimi-

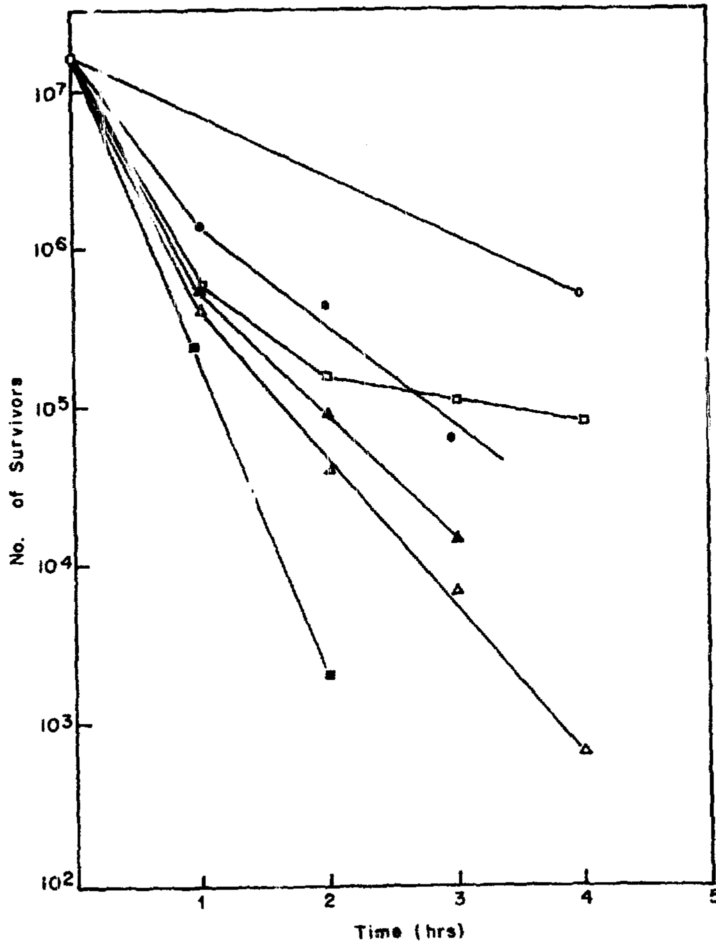

Fig. 3. Death-curves showing the bactericidal activity of 0.3% EDTA, 10% ethanol and combinations of various concentrations of EDTA (0.3, 0.2, 0.1, 0.05%) with ethanol using *Staph. aureus* as test organism. Key: ○———○, 0.3% EDTA; ▲———▲, 0.1% EDTA + 10% ethanol; □———□, 10% ethanol; △———△, 0.2% EDTA + 10% ethanol; ●———●, 0.5% EDTA + 10% ethanol; ■———■, 0.3% EDTA + 10% ethanol.

crobial agents (Repaske, 1956; MacGregor and Elliker, 1958). The effect of magnesium limitation on the EDTA-sensitivity of *Ps. aeruginosa* (Brown and Melling, 1969), and the apparently unusual capacity of this organism for Mg ions (Webb, 1966), also tend to implicate magnesium as the metal involved. The increased activity of ethanol with the increase in the EDTA concentration may probably be due to its greater chelating capacity. The marked difference between the lethal effect of the combinations containing 10% and 5% ethanol is due to the fact that the bactericidal activity of ethanol increases with increase in concentration (Morton, 1971).

EDTA has been shown to affect the bacterial cell wall and this effect was associated with the release of large molecular weight lipopolysaccharides from the cell wall of *Ps. aeruginosa* and *E. coli* (Gray and Wilkinson, 1965; Leive, 1965). Asbell and Eagon (1966) postulated that divalent ions are essential for the integrity

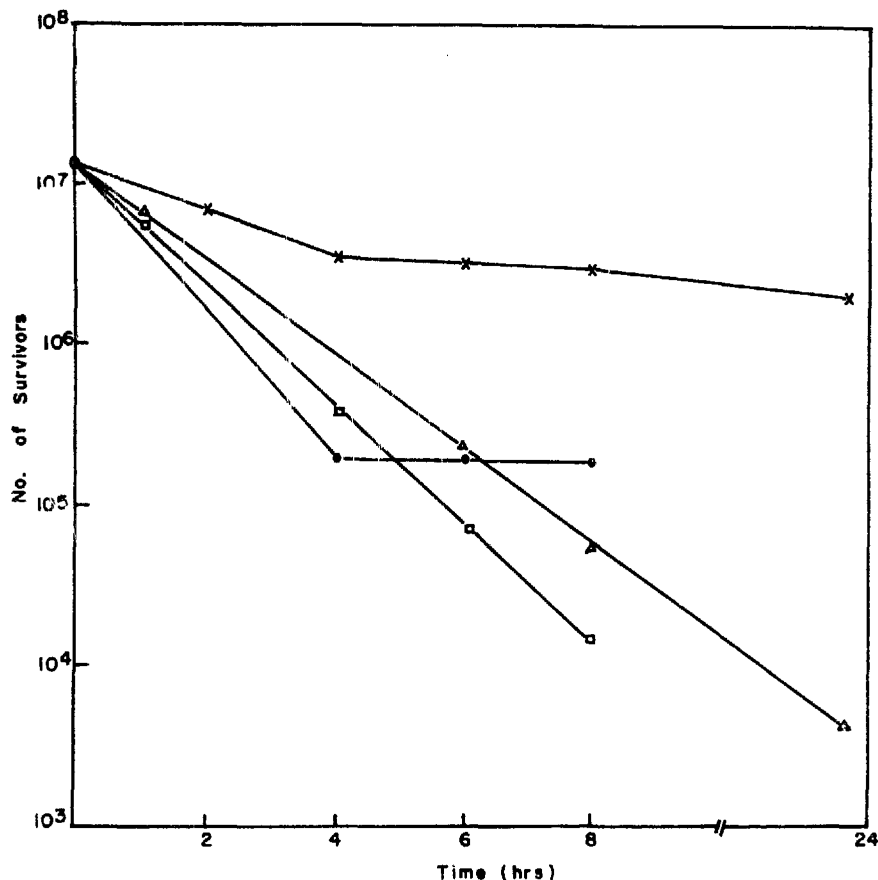

Fig. 4. Death-curves showing the bactericidal activity of combinations of EDTA (0.3, 0.2, 0.1, 0.05% and 5% ethanol using *Staph. aureus* as test organism. Key: ×———×, 0.05% EDTA + 5% ethanol; □———□, 0.3% EDTA + 5% ethanol; △———△, 0.2% EDTA + 5% ethanol; ●———●, 0.1% EDTA + 5% ethanol.

of the lipopolysaccharide layer of the cell wall of Gram-negative bacteria because lipopolysaccharide units are cross-linked by these ions. Therefore, the apparent antagonism exhibited by the 10% ethanol and 0.3% EDTA combination with *E. coli* may be due to the fact that the amount of lipopolysaccharides released as a result of the chelation of Mg ions by 0.3% EDTA was so high as to reduce the activity of ethanol to some extent. Although EDTA does not induce lysis of the Gram-positive *Staph. aureus* (Repaske, 1956 and 1958), the results obtained indicate that it markedly potentiates the action of 10% ethanol. Ethanol for maximum activity might require the absence of some divalent ions or their presence at minimal concentrations, a condition which is achievable in the presence of the chelating agent EDTA.

Our results show that the addition of EDTA enhances markedly the bactericidal effect of lower concentrations of ethanol against *Ps. aeruginosa, E. coli* and *Staph. aureus*. The highest bactericidal activity against all these organisms was obtained

using 0.2% EDTA and 10% ethanol, a finding which could prove useful in some pharmaceutical and cosmetic preparations and possibly in skin sterilization.

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