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Synergism of preservative system components: Use of the survival curve slope method to demonstrate anti-*Pseudomonas* synergy of methyl paraben and acrylic acid homopolymer/copolymers *in vitro*

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Synopsis

The survival curve slope method allows determination of synergy in multicomponent systems when the slope (i.e., rate of death of the population of test organisms) is a larger negative number than the sum of the slopes of each of the components. This method was used to demonstrate anti-*Pseudomonas* synergy of methyl paraben (MP) and acrylic acid homopolymer/copolymers *in vitro*.

Preservative efficacy testing of nonionic lotions containing 0.2% MP and 0.2% acrylic acid homopolymer/copolymers revealed anti-*Pseudomonas* synergy against *P. aeruginosa, P. putida, P. fluorescens,* and *P. stutzeri.* Addition of 0.1% $CaCl_2$ to the lotion caused significant increases in D-values and eliminated the anti-*Pseudomonas* synergy.

Similar patterns of synergy were observed in lotions containing 0.2% MP + 0.2% carbomer 934, 941 or acrylates/C10-30 alkyl acrylate cross polymer (1342) and in tap water containing 0.2% MP + 0.01% Na_2EDTA. The anti-*Pseudomonas* synergy observed with MP and neutralized acrylic acid homopolymer/copolymers is probably related to chelation of divalent metal ions and similar to permeabilization synergy reported for preservative action by EDTA.

INTRODUCTION

Preservative efficacy testing is performed to determine the type and minimum effective concentrations of preservatives required for products to meet acceptance criteria (1). Testing is needed for each product because the physicochemical composition of a formula may enhance or reduce the antimicrobial effectiveness of preservatives.

When designing the preservative system of a product (2,3), it is desirable to select compounds that enhance the antibacterial action of the preservative system. Synergism is observed when the effect produced by the combination of components is greater than the sum of the effects of each component taken separately. Synergy of antimicrobial preservatives has been reported by several workers (4–7).

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During testing with the linear regression method (8), we noticed that *Pseudomonas aeruginosa* was inactivated more rapidly in nonionic emulsion systems containing carbomer 941 than in products that did not contain this material. By thoroughly studying this system, we developed a method of demonstrating synergy of preservative system components that uses the rates of inactivation of test organisms determined by the linear regression method.

The most desirable outcome of testing antimicrobial preservatives for synergy is finding the combination that will allow the use of fewer and/or reduced concentrations of preservatives in consumer products. The use of a preservative system that has synergistic action is of practical significance because it may help reduce the cost of the product and the irritation or sensitization potential of the formula.

EXPERIMENTAL

TEST ORGANISMS

The strains of *Pseudomonas* used in this study were received directly from the American Type Culture Collection (ATCC) and consisted of *P. aeruginosa* ATCC strains 9027, 9721, 10145, and 27853; *P. cepacia* ATCC strains 13945 and 25416; *P. fluorescens* ATCC 13525; *P. putida* (Biotype A) ATCC 12633; *P. stutzeri* ATCC 17588; and *Pseudomonas* sp. 9230. *P. aeruginosa* 9027 routinely is used in antimicrobial preservative testing (1), and *P. aeruginosa* 27853 is a standardized strain for antibiotic susceptibility testing. Multiple strains of species of *P. aeruginosa* and *P. cepacia* were available, and consequently, *P. aeruginosa* 9027 and *P. cepacia* 13945 were used unless other strains are indicated. *Bacillus cereus* ATCC 11778 was obtained directly from the ATCC. *E. coli* ATCC 8739 was obtained from Hill Top Biolabs, Inc. *Staphylococcus aureus* ATCC 6538 (FDA 209 strain) and *Bacillus* sp. were taken from the Jergens culture collection.

The cultures were maintained by weekly transfer on Tryptic Soy Agar (TSA). All test organisms were grown on TSA with 0.07% lecithin and 0.5% Tween 80 (TSALT) in 150-mm Petri dishes for 24 hr prior to use in preservative efficacy testing. *S. aureus, E. coli, Bacillus* sp., and *B. cereus* were incubated at 37°C. All *Pseudomonas* test organisms were incubated at 30°C for 24 hr in preparation for preservative efficacy testing. All Petri dishes prepared from samples for determination of aerobic plate counts (APCs) were incubated for 48 hr at 37°C (except for those prepared from samples challenged with both *P. cepacia* strains and *P. fluorescens,* which were incubated for 48 hr at 30°C).

TEST SAMPLES

The test samples used in this study included a nonionic o/w lotion (Table I). The lotion was prepared as follows: Parts A, B, and C were heated to 70°C. Part A was added to part C with continuous mixing. Part B was added after 5 min, and mixing was continued as the batch was cooled to ambient temperature. This lotion was selected for studying the effects of emulsion pH, [polyacrylic acid resin (934 or 941) or acrylic acid copolymer (1342), B.F. Goodrich] neutralizing agent [TEA 99% or 85% (Dow Chemical), TEA 99% (Union Carbide), or NaOH], and $CaCl_2$, on antimicrobial activity. The pH readings were adjusted to the stated value (± 0.1 pH unit).

Table I
Nonionic Lotion Formula

Part	Formula components	Weight (%)
A	Mineral oil	7.50
	Glyceryl stearate and PEG 100 stearate	3.50
B	Water	30.00
	Acrylic acid homopolymer/copolymer	0.20
	TEA 99%*	0.30
C	Methyl paraben	0.20
	Water	58.30
		100.00

* pH of lotion adjusted to pH 7.0 (±0.1) by addition of TEA.

A batch of nonionic lotion was prepared and brought up to 99.9% of the final weight by the addition of water after cooling. Approximately 60 min before use in preservative efficacy testing, the lotion with 0.1% $CaCl_2$ was prepared by adding 0.5 g $CaCl_2$ to 499.5 g of the lotion base, with several minutes of mixing. The control was prepared by adding 0.1% filter-sterilized deionized water to the remainder of the batch of lotion, with mixing. The pH of the lotion with 0.1% $CaCl_2$ was pH 5.7 and had a water-thin viscosity. The pH of the control lotion was pH 6.8, and the viscosity of this lotion was not changed noticeably by the addition of water.

Aqueous samples of 0.2% MP, 0.01% Na_2EDTA, 0.2% MP + 0.01% Na_2EDTA, and deionized water (control) were prepared for sterilization time (ST) determinations and were filter-sterilized by passing 5-ml aliquots of each solution through a 0.45-μm filter in a Sweeny-type filter holder. Similarly, aliquots of stock solutions of phenoxyethanol (P) and Nipastat (N), which is a 50:15:10:20 mixture of methyl-, ethyl-, propyl-, and butyl-paraben, were added to sterile saline to give final concentrations of 0, 0.1, 0.5, and 1.0% P, and 0, 0.005, 0.01, 0.05, and 0.10% N.

TEST PROCEDURES

Preservative efficacy tests were performed using saline suspensions from surface growth of each test organism after incubation for 24 hr on TSALT, as described above. A loopful of growth was suspended in 5 ml of saline to give about 10^7 organisms/ml, and 0.1 ml of the saline suspension of each test organism was added to separate 50-ml portions of each test sample in a 100-ml screw-capped bottle. Samples were taken at designated times; APCs were performed using Letheen Broth with 0.01% (v/v) Triton X-100 diluent and TSALT as the recovery system; the Petri dishes were incubated at 37°C for all test organisms (except for both *P. cepacia* strains and *P. fluorescens,* which were incubated at 30°C); and D-values were determined by taking the negative reciprocal of the slope of the survivor curve for each test organism in each test sample, as described in an earlier report (8). All tests were performed at least in duplicate, unless otherwise stated.

STERILIZATION TIMES

A modified preservative efficacy test was used to determine sterilization times (STs) for the test organisms in aqueous samples. The inocula were prepared as above and added to solutions of MP, Na_2EDTA, MP + Na_2EDTA, and deionized water (control). The contents of the tubes were mixed using a Vortex Genie Mixer, and samples were taken at 0, 2, 4, 24, and 48 hr by inserting a sterile swab into the liquid in each tube. A separate Petri dish containing TSALT was streaked with each swab. The Petri dishes were incubated for 48 hr at 30° or 37°C, depending on the test organism, as described above. The Petri dishes were examined for growth of the test organisms, and the ST was determined to be the first time at which test organisms were not recovered from the test solution.

The ST and the concentration of organisms in the inoculum were used to calculate the slope of the survivor curve, correcting for the volume change that occurs when the inoculum is added to the test system. In these studies, the concentration of organisms in test tube samples was 1/100th the concentration in the inoculum because 0.1 ml was added to 10-ml solution in each test tube. D-values were determined by taking the negative reciprocal of the slope of each survivor curve (8). Where no endpoint was reached in the ST experiments, because the test organisms were still alive at 48 hr, the minimum possible ST (MPST) was used. The MPST was defined as a time longer than the last time at which test organisms were recovered (i.e., >48 hr). The MPST and the concentration of the organisms in the inoculum were used to construct a virtual survival curve. The maximum possible slope (MPSlope) of the virtual survivor curve and the corresponding minimum possible D-value (MPD-value) were calculated.

DETERMINATION OF SYNERGY

Synergism was observed when the slope of the survivor curve obtained with the combined components was a larger negative number than the sum of the slopes (or MPSlopes) for each of the components determined separately.

WATER HARDNESS

Duplicate samples of tap water; tap water containing 0.01% Na_2EDTA, adjusted to pH 7.0 by the addition of one drop of TEA; and deionized water were tested for water hardness by the method of Betz Laboratories (9).

A 0.1% 1342 dispersion was prepared by slowly adding 0.1 g 1342 to 99.9 g tap water with vigorous agitation. This dispersion was stirred for 2 hr at room temperature to allow hydration of the 1342. The beakers containing tap water and the 1342 dispersion were covered with aluminum foil and were allowed to stand, undisturbed, at room temperature for 3 days. The dispersion settled to about ⅓ of the liquid level in the beaker after this period. The water layer was decanted to give 1342-treated tap water. Duplicate samples of tap water, 1342-treated tap water, and freshly drawn deionized water were assayed for hardness (9).

STATISTICS

Mean D-values and standard deviation (s) were calculated. Statistically significant differences between mean D-values of duplicate experiments were determined by a two-

tailed t test (10). The Q test was used for rejection of a questionable result when five or more D-value determinations were made using the same test organism and test sample (11).

RESULTS

The survivor curves for *P. aeruginosa* in 0.2% MP adjusted to pH 7.0 with TEA or NaOH, with and without 0.2% 1342, are shown in Figure 1. The system with 1342, MP, and TEA inactivated *P. aeruginosa* so quickly that the APC was 600/ml immediately after inoculation. No organisms were recovered at 2 hr or thereafter in this system. The estimated D-value for *P. aeruginosa* was <0.006 hr, based on the APC of the inoculum and the APC immediately after inoculation. This is indicated by the dashed line in the figure. The system containing MP and 1342 neutralized with NaOH had an initial APC of 4.3×10^5/ml and a D-value of 0.9 hr. Linear regression analysis gave an estimated ST of slightly greater than 5 hr. Solutions of MP and 0.16% NaOH or 0.6% TEA (the amounts of these bases required to adjust the pH to 7.0) did not kill *P. aeruginosa* during the 24-hr test period. This initial experiment was performed once using duplicate Petri dishes.

The results in Table II show the preservative efficacy test results of the nonionic lotion challenged with *S. aureus, B. cereus, E. coli,* and different species of *Pseudomonas.* Most species of *Pseudomonas* were inactivated rapidly, with D-values of ≤ 1.1 hr. The preservative system was much less effective against *S. aureus, B. cereus,* and *E. coli* than against most species of *Pseudomonas* tested. *Pseudomonas* sp., *P. cepacia* 13945, and *P. cepacia* 25416 were more resistant than the other pseudomonads to the anti-*Pseudomonas* action

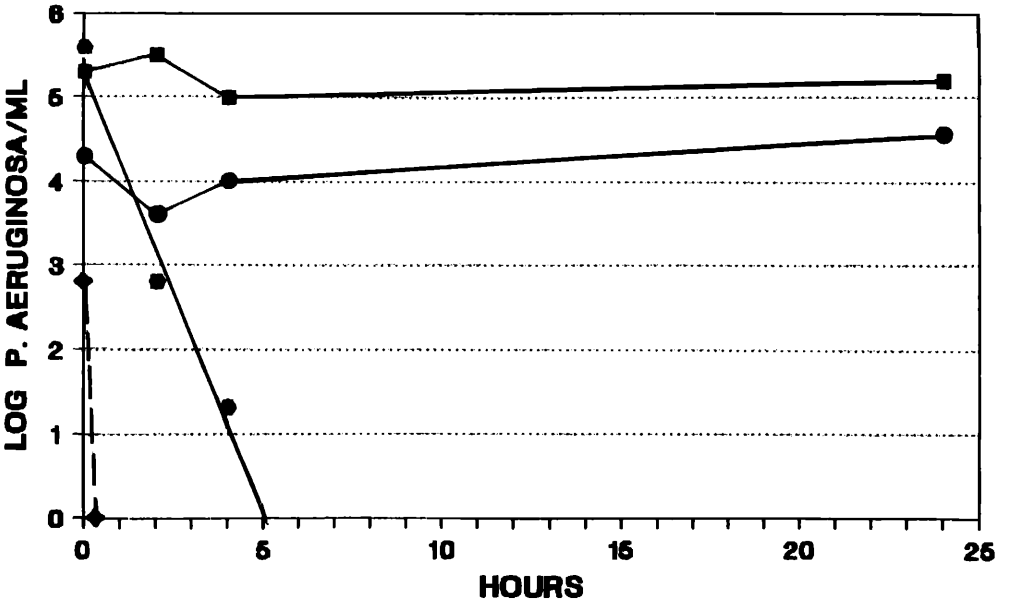

Figure 1. Survivor curves for *P. aeruginosa* 9027 in 0.2% MP adjusted to pH 7.0 with TEA or NaOH, with and without 0.2% 1342. Explanation of symbols: ■ = 0.2% MP with NaOH, ● = 0.2% MP with TEA, ⬣ = 0.2% MP + 0.2% 1342 with NaOH, and ◆ = 0.2% MP + 0.2% 1342 with TEA.

Table II
Preservative Efficacy Test Results Obtained With Nonionic Lotion Containing 0.2% MP and 0.2% 1342 Challenged With *S. aureus, B. cereus, E. coli,* and Several Different Species of *Pseudomonas*

Test organism	D-value
P. aeruginosa 9027	0.9 (0.3)
P. aeruginosa 10145	1.1 (0.1)
P. cepacia 13945	24* (4)
P. cepacia 25416	11* (0)
P. fluorescens 13525	1.1 (0.9)
P. putida 12633	1.0 (0.6)
P. stutzeri 17588	0.5 (0)
Pseudomonas sp. 9230	9.3 (0.9)
S. aureus 6538	26* (0)
B. cereus 11778	7.2 (0.4)
E. coli 8739	78* (28)

Table values are mean D-values in hours, with the standard deviations in parentheses.
* D-values over 10 hr were rounded to the nearest whole number.

of the preservative system, as indicated by the larger D-values obtained with these organisms.

The D-values and slopes of the survivor curves obtained with *P. aeruginosa, P. cepacia, P. fluorescens,* and *P. putida* in the nonionic lotion with MP; with 934, 941, or 1342 and no MP; and with MP and 934, 941, or 1342 are presented in Table III. All lotions were adjusted to pH 7.0 with TEA. The D-values were much smaller (i.e., the rates of death of the test organisms were much faster) in all lotions containing 934, 941, or 1342 with MP than in lotions containing only MP or acrylic acid homopolymer/copolymer. The dramatic anti-*Pseudomonas* effect obtained with MP + 934, in comparison with MP or 934, is illustrated in Figure 2.

In this work, a synergistic effect in multicomponent preservative systems was defined as one in which the slope (i.e., rate of death of the population of test organisms) was a larger negative number in the presence of two or more agents than the sum of the slopes in the presence of each agent by itself. Thus, a synergistic effect was obtained when the slope in the presence of the MP and acrylic acid homopolymer/copolymer was a larger negative number than the sum of the slopes in the presence of MP (without 934, 941, or 1342) and 934, 941, or 1342 (without MP). The test systems that produced synergistic anti-*Pseudomonas* activity are indicated by an asterisk in Table III. All test systems, except the 1342/MP system challenged with *P. cepacia,* exhibited synergistic antibacterial activity. The antibacterial action of MP and 1342 for *P. cepacia* was additive in this test lotion.

The results of preservative efficacy testing of the nonionic lotion containing 0.2% 1342 and 0.2% MP, with and without 0.1% $CaCl_2$, are shown in Table IV. The addition of $CaCl_2$ to the lotion significantly increased the D-values and eliminated the anti-*Pseudomonas* synergy for *P. aeruginosa, P. fluorescens,* and *P. putida.* The $CaCl_2$ significantly decreased the D-values for *P. cepacia* (Table IV). No attempt was made to demonstrate synergy or antagonism in the experiments done with $CaCl_2$ test systems.

Addition of $CaCl_2$ to the lotion decreased the viscosity to a water-thin consistency and decreased the pH from 6.8 to 5.7, but no phase separation was apparent during the

Table III

Synergism of MP and Polyacrylic Acid Homopolymer/Copolymer: Preservative Efficacy Test Results in Nonionic Lotion Containing 0.2% MP; 0.2% 934, 941, or 1342; and 0.2% MP + 0.2% 934, 941, or 1342, Challenged With *P. aeruginosa* 9027, *P. cepacia* 13945, *P. fluorescens* 13525, and *P. putida* 12633

934 Test system:	MP		934		MP + 934	
Test organism	D-value	Slope	D-value	Slope	D-value	Slope
P. aeruginosa	6.3	−0.16	25	−0.04	0.5	−2.0*
P. cepacia	52	−0.02	35	−0.03	11.5	−0.09*
P. fluorescens	31	−0.03	64	−0.02	0.4	−2.5*
P. putida	34	−0.03	9.3	−0.11	0.8	−1.2*
941 Test system:	MP		941		MP + 941	
Test organism	D-value	Slope	D-value	Slope	D-value	Slope
P. aeruginosa	6.3	−0.16	390	−0.003	1.7	−0.59*
P. cepacia	52	−0.02	45	−0.02	17	−0.06*
P. fluoroescens	31	−0.03	400	−0.003	<0.004	>−250*
P. putida	34	−0.03	8	−0.13	1.1	−0.91*
1342 Test system:	MP		1342		MP + 1342	
Test organism	D-value	Slope	D-value	Slope	D-value	Slope
P. aeruginosa	6.3	−0.16	60	−0.02	0.9	−1.1*
P. cepacia	52	−0.02	48	−0.02	24	−0.04
P. fluorescens	31	−0.03	110	−0.01	1.1	−0.9*
P. putida	34	−0.03	16	−0.06	1.0	−1.0*

Table values are D-values in hours and the corresponding slopes of the survivor curves in hours^{-1}.
* Synergistic anti-*Pseudomonas* activity is indicated by a larger negative slope with MP + acrylic acid derivative than with the sum of slopes obtained with MP and polyacrylic acid homopolymer/copolymer.

course of the preservative testing. A 0.1% solution of unneutralized 1342 in tap water had moderate chelating ability. The mean hardness of duplicate tap water samples was 109 (s = 0.71) ppm as $CaCO_3$, and the mean hardness of duplicate 0.1% 1342 solutions was 75 (s = 1.5) ppm as $CaCO_3$. These means were significantly different ($p < 0.01$).

The STs of the test organisms in MP, Na_2EDTA, and MP + Na_2EDTA solutions were determined. The 0.2% MP solutions were not rapidly bacteriocidal because all test organisms (including *S. aureus* and *E. coli*) were viable at 24 hr. *P. cepacia* 13945 and *P. putida* were not recovered at 48 hr (Table V). Similarly, all but two of the pseudomonad test organisms were recovered from the Na_2EDTA solutions at 48 hr. In contrast, the combination of 0.01% Na_2EDTA + 0.2% MP showed marked anti-*Pseudomonas* action, because all test organisms, except *P. cepacia* 13945 and 25416 and *Pseudomonas* sp. 9230, had STs <4 hr. *P. cepacia* 13945 was the only pseudomonad recovered at the 24-hr sampling. *S. aureus* and *E. coli* had STs >48 hr. These results parallel the results obtained in Table II, in which both *P. cepacia* strains and *Pseudomonas* sp. were found to

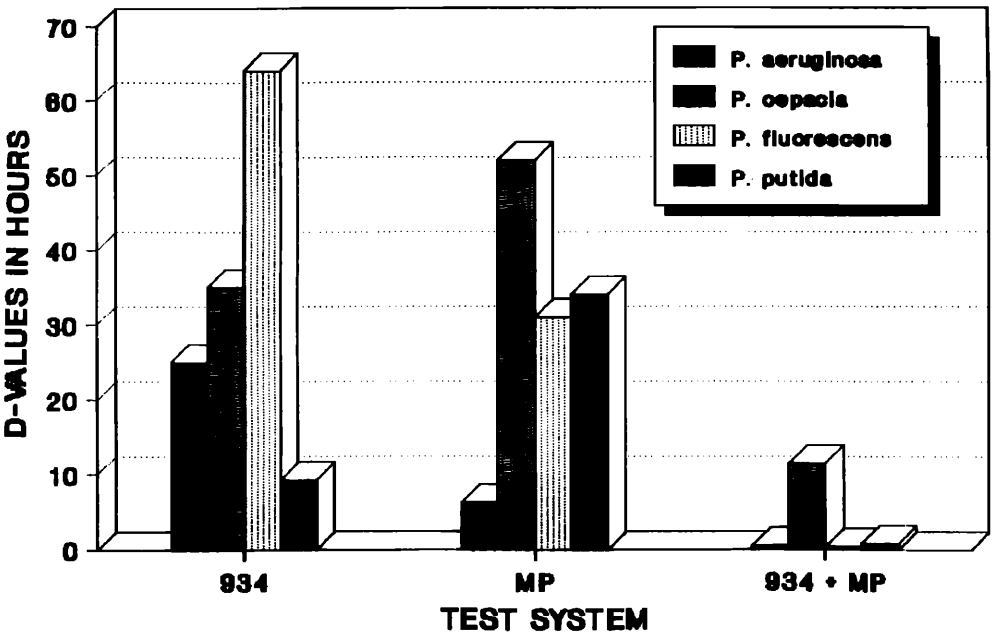

Figure 2. Comparison of D-values for *P. aeruginosa* 9027, *P. cepacia* 13945, *P. fluorescens* 13525, and *P. putida* 12633, determined by preservative efficacy testing of the nonionic lotion containing 0.2% MP, 0.2% 934, or both 0.2% MP and 0.2% 934.

be much more resistant to the preservative system of the lotion containing 0.2% MP + 0.2% 1342. Even though the *P. stutzeri* inoculum contained 1.7×10^7/ml, this organism was not recovered from the initial sampling in the MP + Na_2EDTA solution. Similarly, *P. stutzeri* was inactivated more rapidly than all other test organisms in the lotion containing MP + 1342 (Table II). Hardness analyses of tap water, tap water containing 0.01% Na_2EDTA, and deionized water gave mean hardness values of 137 (s = 5.0), 109 (s = 1.4), and 20 (s = 14) ppm $CaCO_3$, respectively. These means were significantly different ($p < 0.05$).

The STs obtained in Table V were used to calculate survivor curve slopes and D-values and to demonstrate synergy for all *Pseudomonas* test organisms in 0.1% MP + 0.01% Na_2EDTA solutions, except for *P. cepacia* 13945 (Table VI). It was possible to calculate a slope and D-value where STs were known, as in the case for *P. cepacia* 13945 in 0.2%

Table IV

Effect of 0.1% $CaCl_2$ on Preservative Efficacy Test Results of Nonionic Lotion Containing 0.2% MP and 0.2% 1342 Challenged With *P. aeruginosa* 9027, *P. cepacia* 13945, *P. fluorescens* 13525, and *P. putida* 12633

Test organism	Lotion	Lotion with $CaCl_2$
P. aeruginosa	1.8 (0.8)	10* (0)
P. cepacia	90* (16)	13* (0.7)
P. fluorescens	2.0 (0.07)	8.5 (0.4)
P. putida	2.2 (0.28)	11* (1.0)

Table values are mean D-values from duplicate experiments, with standard deviations in parentheses.
* D-values over 10 hr were rounded to the nearest whole number.

Table V
Determination of Sterilization Times of Several *Pseudomonas* Species in MP, Na_2EDTA, and MP + Na_2EDTA Solutions

0.2% MP solutions: Test organism	0 hr	2 hr	4 hr	24 hr	48 hr	ST	MPST
P. aeruginosa 9027	+	+	+	+	+	—	>48
P. aeruginosa 27853	+	+	+	+	+	—	>48
P. aeruginosa 9721	+	+	+	+	+	—	>48
P. aeruginosa 10145	+	+	+	+	+	—	>48
P. cepacia 13945	+	+	+	+	−	48	—
P. cepacia 25416	+	+	+	+	+	—	>48
P. fluorescens 13525	+	+	+	+	+[2]	—	>48
P. putida 12633	+	+	+	+	−	48	—
P. stutzeri 17588	+	+	+	+	+	—	>48
Pseudomonas sp. 9230	+	+	+	+	+	—	>48
S. aureus 6538	+	+	+	+	+	—	>48
E. coli 8739	+	+	+	+	+	—	>48
0.01% Na_2EDTA solutions: Test organism	0 hr	2 hr	4 hr	24 hr	48 hr	ST	MPST
P. aeruginosa 9027	+	+	+	+	+	—	>48
P. aeruginosa 27853	+	+	+	+	+	—	>48
P. aeruginosa 9721	+	+	+	+	+	—	>48
P. aeruginosa 10145	+	+	+	+	+	—	>48
P. cepacia 13945	+	+	+	+	+	—	>48
P. cepacia 25416	+	+	+	+	+	—	>48
P. fluorescens 13525	+	+	+[s]	+	+	—	>48
P. putida 12633	+	+	+[s]	+[2]	−	48	—
P. stutzeri 17588	+	−	+[1]	−	−	24	—
Pseudomonas sp. 9230	+	+	+	+	+	—	>48
S. aureus 6538	+	+	+	+	+	—	>48
E. coli 8739	+	+	+	+	+	—	>48
0.2% MP + 0.01% Na_2EDTA solution: Test organism	0 hr	2 hr	4 hr	24 hr	48 hr	ST	MPST
P. aeruginosa 9027	+	+[s]	−	−	−	4	—
P. aeruginosa 27853	+	+[s]	+[2]	−	−	24	—
P. aeruginosa 9721	+	+[s]	−	−	−	4	—
P. aeruginosa 10145	+	+[1]	−	−	−	4	—
P. cepacia 13945	+	+	+	+	−	48	—
P. cepacia 25416	+	−	−	−	−	2	—
P. fluorescens 13525	+	−	−	−	−	2	—
P. putida 12633	+	−	−	−	−	2	—
P. stutzeri 17588	−	−	−	−	−	0.1*	—
Pseudomonas sp. 9230	+	+	+[1]	−	−	24	—
S. aureus 6538	+	+	+	+	+	—	>48
E. coli 8739	+	+	+	+	+	—	>48
Deionized water (control): Test organism	0 hr	2 hr	4 hr	24 hr	48 hr	ST	MPST
P. aeruginosa 9027	+	+	+	+	+	—	>48
P. aeruginosa 27853	+	+	+	+	+	—	>48
P. aeruginosa 9721	+	+	+	+	+	—	>48
P. aeruginosa 10145	+	+	+	+	+	—	>48

(continued)

Table V (Continued)

Deionized water (control): Test organism	0 hr	2 hr	4 hr	24 hr	48 hr	ST	MPST
P. cepacia 13945	+	+	+	+	+	—	>48
P. cepacia 25416	+	+	+	+	+	—	>48
P. fluorescens 13525	+	+	+	+	+	—	>48
P. putida 12633	+	+	+	+	+	—	>48
P. stutzeri 17588	+	+	+	+	+	—	>48
Pseudomonas sp. 9230	+	+	+	+	+	—	>48
S. aureus 6538	+	+	+	+	+	—	>48
E. coli 8739	+	+	+	+	+	—	>48

Explanation of symbols: +, growth on TSALT; −, no growth on TSALT; numbers in brackets (i.e., [1] and [2]) indicate the number of colonies growing on TSALT; [s] is used to designate scant growth (i.e., 8–15 colonies growing on TSALT). ST, sterilization time in hr. MPST, minimum possible sterilization time in hr.

* *P. stutzeri* was not recovered, so ST was set at 0.1 hr because this is the approximate time required for setting up the series of samples after inoculation.

APC of the saline suspensions inocula:

P. aeruginosa 9027 = 1.1×10^7/ml.
P. aeruginosa 9721 = 1.8×10^7/ml.
P. aeruginosa 27853 = 1.5×10^7/ml.
P. aeruginosa 10145 = 2.0×10^7/ml.
P. cepacia 13945 = 1.7×10^7/ml.
P. cepacia 25416 = 9.9×10^6/ml.
P. fluorescens 13525 = 1.6×10^7/ml.
P. putida 12633 = 1.6×10^7/ml.
P. stutzeri 17588 = 1.7×10^7/ml.
Pseudomonas sp. 9230 = 6.3×10^6/ml.
S. aureus 6538 = 1.4×10^7/ml.
E. coli 8739 = 6.3×10^6/ml.

MP. It was not possible to calculate STs, slopes, and D-values when the organisms were recovered at 48 hr (i.e., ST >48 hr), as for all strains of *P. aeruginosa* in MP or Na_2EDTA. Nevertheless, the survivor curve slope method may be used to determine synergy when the experimentally determined rate of death (slope) is a larger negative number than the sum of the MPSlopes of each of the components. Use of MPSlopes in determining synergy is discussed in greater detail below. No synergy was observed for *S. aureus* or *E. coli* in these experiments.

The results in Table VII show the growth response of *P. aeruginosa* 9027 on TSALT after exposure to various combinations of P and/or N in saline. As one reviews the growth responses of *P. aeruginosa* following exposure to various combinations of P and N, it is apparent that several of the combinations killed the population of *P. aeruginosa* faster than the same concentrations of either N or P used alone. The test organism was not recovered from test systems containing 0.1% N (except for one tube containing both 1% P and 0.1% N, in which one colony developed on TSALT at the 0 hr sampling). *P. aeruginosa* grew on TSALT streaked with 0.1% N; consequently, preservative carryover was not responsible for the lack of growth on TSALT in systems containing the highest concentration of N. Synergy was observed when the slope of the survivor curve was a larger negative number than the sum of the slopes (or MPSlopes) for the components. The systems in which synergism was observed are marked with an asterisk (Table VIII).

Table VI
Use of STs and MPSTs to Determine Antimicrobial Synergy for Several Species of *Pseudomonas* in 0.2% MP, 0.01% Na_2EDTA, and 0.2% MP + 0.01% Na_2EDTA (data from Table V)

0.2% MP solution: Test organism	ST	MPST	D-value	MPD-value	Slope	MPSlope
P. aeruginosa 9027	—	>48	—	>9.5	—	<−0.105
P. aeruginosa 27853	—	>48	—	>9.3	—	<−0.108
P. aeruginosa 9721	—	>48	—	>9.1	—	<−0.109
P. aeruginosa 10145	—	>48	—	>9.1	—	<−0.110
P. cepacia 13945	48	—	9.2	—	−0.109	—
P. cepacia 25416	—	>48	—	>9.6	—	<−0.104
P. fluorescens 13525	—	>48	—	>9.2	—	<−0.108
P. putida 12633	48	—	9.2	—	−0.108	—
P. stutzeri 17588	—	>48	—	>9.2	—	<−0.109
Pseudomonas sp. 9230	—	>48	—	>10	—	<−0.100
S. aureus 6538	—	>48	—	>9.3	—	<−0.107
E. coli 8739	—	>48	—	>10	—	<−0.100
0.01% Na_2EDTA solution: Test organism	ST	MPST	D-value	MPD-value	Slope	MPSlope
P. aeruginosa 9027	—	>48	—	>9.5	—	<−0.105
P. aeruginosa 27853	—	>48	—	>9.3	—	<−0.108
P. aeruginosa 9721	—	>48	—	>9.1	—	<−0.109
P. aeruginosa 10145	—	>48	—	>9.1	—	<−0.110
P. cepacia 13945	48	—	9.2	—	−0.109	—
P. cepacia 25416	—	>48	—	>9.6	—	<−0.104
P. fluorescens 13525	—	>48	—	>9.2	—	<−0.108
P. putida 12633	48	—	9.2	—	−0.108	—
P. stutzeri 17588	24	—	4.6	—	−0.218	—
Pseudomonas sp. 9230	—	>48	—	>10	—	<−0.100
S. aureus 6538	—	>48	—	>9.3	—	<−0.107
E. coli 8739	—	>48	—	>10	—	<−0.100
0.2% MP + 0.01% Na_2EDTA solution: Test organism	ST	MPST	D-value	MPD-value	Slope	MPSlope
P. aeruginosa 9027	4	—	0.8	—	−1.260*	—
P. aeruginosa 27853	4	—	0.8	—	−1.294*	—
P. aeruginosa 9721	24	—	4.6	—	−0.219*	—
P. aeruginosa 10145	4	—	0.8	—	−1.325*	—
P. cepacia 13945	48	—	9.2	—	−0.109	—
P. cepacia 25416	2	—	0.4	—	−2.498*	—
P. fluorescens 13525	2	—	0.5	—	−2.102*	—
P. putida 12633	2	—	0.4	—	−2.602*	—
P. stutzeri 17588	0.1	—	>0.02	—	<−52.304*	—
Pseudomonas sp. 9230	24	—	5.0	—	−0.200	—
S. aureus 6538	—	>48	—	>9.3	—	<−0.107
E. coli 8739	—	>48	—	>10	—	<−0.100

Explanation of symbols: ST, sterilization time in hr; MPST, minimum possible sterilization time in hr; D-value, D-value in hr; MPD-value, minimum possible D-value in hr; Slope, slope of the survivor curve, in hr^{-1}; MPSlope, maximum possible slope of the virtual survivor curve, in hr^{-1}.

* Synergy observed, because the slope of the survivor curve was a larger negative number than the sum of the slopes (or MPSlopes) for the same concentrations of MP and Na_2EDTA taken separately.

Table VII
Determination of Sterilization Times for *P. aeruginosa* 9027 in Saline Containing 0 to 1.0% Phenoxyethanol and 0 to 0.10% Nipastat

Preservative	Growth response at					ST	MPST
	0 hr	2 hr	4 hr	24 hr	48 hr		
Saline control	+/+	+/+	+/+	+/+	+/+	—	>48
0.1% P	+/+	+/+	+/+	+/+	+/+	—	>48
0.5% P	+/+	+/+	+/+	+/+	+/−	—	>48
1.0% P	+/+	+/+	+/−	+/−	−/−	48	—
0.005% N	+/+	+/+	+/+	+/+	+/+	—	>48
0.01% N	+/+	+/+	+/+	+/+	+/+	—	>48
0.05% N	+/+	+/+	+/+	+/+	+/+	—	>48
0.10% N	+/+	−/−	−/−	−/−	−/−	2	—
0.1% P + 0.005% N	+/+	+/+	+/+	+/+	+/+	—	>48
0.1% P + 0.01% N	+/+	+/+	+/+	+/+	+/+	—	>48
0.1% P + 0.05% N	+/+	−/−	−/−	−/−	−/−	2	—
0.1% P + 0.10% N	−/−	−/−	−/−	−/−	−/−	0.1*	—
0.5% P + 0.005% N	+/+	+/+	+/+	+/+	+/−	—	>48
0.5% P + 0.01% N	+/+	+/+	+/+	−/−	−/−	24	—
0.5% P + 0.05% N	+/+	−/−	−/−	−/−	−/−	2	—
0.5% P + 0.10% N	−/−	−/−	−/−	−/−	−/−	0.1*	—
1.0% P + 0.005% N	+/+	+/−	−/−	−/−	−/−	4	—
1.0% P + 0.01% N	+/+	+/−	−/−	−/−	−/−	4	—
1.0% P + 0.05% N	+/+	+/−	−/−	nd**	−/−	4	—
1.0% P + 0.10% N	+/−	−/−	−/−	−/−	−/−	2	—

Table symbols represent growth of *P. aeruginosa* on one (+/−), on both (+/+), or neither (−/−) TSALT plates inoculated from duplicate tubes containing the indicated concentrations of phenoxyethanol (P) or Nipastat (N). Tubes with P and/or N were incubated at room temperature for the times indicated. ST, sterilization time in hr; MPST, minimum possible sterilization time in hr.

* Although *P. aeruginosa* was not recovered in these samples, the ST was set at 0.1 hr because this is the approximate time required for setting up the series of samples after inoculation.

** nd, not done.

The *P. aeruginosa* inoculum APC = 1.7×10^7/ml.

DISCUSSION

This work was initiated when it was realized that the preservative system in adequately preserved nonionic emulsion systems was inactivating *P. aeruginosa* much more rapidly than the other test organisms customarily used in preservative efficacy testing (8). All of these emulsions contained MP. The parabens are known to be active against a wide range of gram-positive bacteria and fungi, but they are less active against gram-negative bacteria, especially the pseudomonads (12). Although 0.2% MP did not kill *P. aeruginosa* in 24 hr, combinations of MP and 1342 caused rapid killing of this test organism (Figure 1). The nonionic lotion with 0.2% MP (Table I) was selected for investigating the cause of this rapid inactivation of *P. aeruginosa* observed in our laboratory.

Pseudomonas has been particularly troublesome for the cosmetic and pharmaceutical industries. *P. aeruginosa, P. cepacia, P. fluorescens, P. putida,* and *P. stutzeri* can survive and grow in deionized water, and they have been isolated from contaminated cosmetics (13–15). *P. aeruginosa* has been recovered from contaminated mascaras and has caused

Table VIII
Use of STs and MPSTs to Determine Antimicrobial Synergy for *P. aeruginosa* 9027 in 0 to 1.0% Phenoxyethanol and 0 to 0.10% Nipastat (data from Table VII)

Preservative	ST	MPST	D-value	MPD-value	Slope	MPSlope
Saline control	—	>48	—	>6.6	—	<−0.151
0.1% P	—	>48	—	>6.6	—	<−0.151
0.5% P	—	>48	—	>6.6	—	<−0.151
1.0% P	48	—	6.6	—	−0.151	—
0.005% N	—	>48	—	>6.6	—	<−0.151
0.01% N	—	>48	—	>6.6	—	<−0.151
0.05% N	—	>48	—	>6.6	—	<−0.151
0.10% N	2	—	0.3	—	−3.615	—
0.1% P + 0.005% N	—	>48	—	>6.6	—	<−0.151
0.1% P + 0.01% N	—	>48	—	>6.6	—	<−0.151
0.1% P + 0.05% N	2	—	0.3	—	−3.615*	—
0.1% P + 0.10% N	0.1	—	−0.01	—	−72.304*	—
0.5% P + 0.005% N	—	>48	—	>6.6	—	<−0.151
0.5% P + 0.01% N	24	—	3.3	—	−0.301	—
0.5% P + 0.05% N	2	—	0.3	—	−3.615*	—
0.5% P + 0.10% N	0.1	—	0.01	—	−72.304*	—
1.0% P + 0.005% N	4	—	0.6	—	−1.808*	—
1.0% P + 0.01% N	4	—	0.6	—	−1.808*	—
1.0% P + 0.05% N	4	—	0.6	—	−1.808*	—
1.0% P + 0.10% N	2	—	0.3	—	—	−3.615

Explanation of symbols: ST, sterilization time in hr; MPST, minimum possible sterilization time in hr; D-value, D-value in hr; MPD-value, minimum possible D-value in hr; Slope, slope of the survivor curve, in hr^{-1}; MPSlope, maximum possible slope of the virtual survivor curve, in hr^{-1}.
* Synergy observed, because the slope of the survivor curve was a larger negative number than the sum of the slopes (or MPSlopes) for the same concentrations of P and N taken separately.

corneal ulcers (16,17). This organism produces several virulence factors that are believed to contribute to its multifactorial pathogenicity and complicate the clinical course of infections (18–23).

P. cepacia has considerable physiological versatility and has broad resistance to antibiotics (24,25). *P. cepacia* 13945 was selected for detailed investigation in this work because, generally, it was more resistant than the other pseudomonads in our culture collection to preservative systems containing MP and acrylic acid homopolymer/copolymers. *P. fluorescens* and *P. putida* were selected for detailed studies here because they are nutritionally versatile and are able to grow on a wide variety of substrates (24,26).

When the lotion was prepared using 0.2% 1342 and 0.2% MP, preservative efficacy testing revealed significant antibacterial activity against most test cultures of *Pseudomonas* (Table II). All fluorescent pseudomonads [*P. aeruginosa, P. fluorescens, P. putida* and *P. stutzeri* (24)] were inactivated rapidly, with D-values ≤1.1 hr. Both *P. cepacia* strains were inactivated more slowly in the preservative system than were the fluorescent pseudomonads. The reasons for the resistance of strains 13945 and 25416 are not known; however, *P. cepacia* is nutritionally versatile and accumulates poly-beta-hydroxybutyrate (PHB) as a carbon reserve (24,27,28). These physiological characteristics may enable *P. cepacia* to be more difficult to inactivate in test systems containing chelating agents and MP than are the fluorescent pseudomonads.

We speculate that PHB may be a classical chelating agent, in the sense described by Marshall *et al.* (29). Intracellular accumulation of PHB by *P. cepacia* (24) may enable this species to retain divalent metal ions as a PHB chelate, which could provide an internal reservoir that may help prevent loss of metal ions to exogenous chelators. This would enable *P. cepacia* to resist more effectively the destabilization caused by external chelating agents (7) than do the fluorescent pseudomonads, which do not accumulate PHB (24). Definitive studies are needed to confirm this.

The data in Table II show that the preservative system in this lotion was less effective for *S. aureus, B. cereus,* and *E. coli* than it was for many of the *Pseudomonas* test cultures. The percentage of sporulation of *Bacillus* sp. was 30–50% at 24 hr (8); however, *B. cereus* produced only a few visible spores in a microscopic field (1000×) when 24-hr TSALT cultures were suspended, stained, and examined microscopically. Experience with *Bacillus* sp. and *B. cereus* 11778 revealed that these organisms produce few (if any) preservative system-resistant spores during 24-hr growth on TSALT at 37°C. These organisms were used to determine the effects of preservative systems on vegetative bacilli.

The data in Table III illustrate anti-*Pseudomonas* synergy because the rate of inactivation (i.e., slope of the survivor curve) of each population of test organisms in MP + 934, 941, or 1342 was a larger negative number than the sum of the rates (slopes) of inactivation in MP and each acrylic acid homopolymer/copolymer taken separately. *P. cepacia* showed an additive effect in lotion containing MP + 1342.

The effect of nonionic lotion pH on the results of preservative efficacy testing with *P. aeruginosa, P. cepacia, P. fluorescens,* and *P. putida* was determined in lotions adjusted to pH 6–9 by adding varying amounts of TEA. No consistent effect of lotion pH on antibacterial activity with these four test organisms was observed. The antibacterial effect of MP is reported to increase with decreasing pH below the pKa of MP (pH 8.17) (30). *P. cepacia* was inactivated more slowly than the other pseudomonads in lotions adjusted to pH 6–9.

Incorporation of $\geq$0.1% 934, 941, or 1342 into the nonionic lotion produced a marked decrease in the D-values for *P. aeruginosa, P. cepacia, P. fluorescens,* and *P. putida,* compared to the D-values obtained in lotions containing no 934, 941, or 1342. We were unable to demonstrate a consistent relationship between the acrylic acid homopolymer/copolymer concentration, from 0.1–0.4%, and the observed rates of death of the test organisms. It is possible that the maximum synergistic action was obtained at $\leq$0.1% polyacrylic acid/acrylic acid copolymer so that higher concentrations produced no further increase in anti-*Pseudomonas* activity.

The addition of 0.1% $CaCl_2$ to the nonionic lotion containing 0.2% 1342 and 0.2% MP produced significant increases in the D-values for the fluorescent pseudomonads (*P. aeruginosa, P. fluorescens,* and *P. putida*) and eliminated the anti-*Pseudomonas* synergy. The opposite effect was observed with *P. cepacia,* because addition of 0.1% $CaCl_2$ produced a significant decrease in D-values for this organism (Table IV). The inhibitory effects of $CaCl_2$ on *P. cepacia* may have been due primarily to the decrease in the pH of this lotion caused by the addition of $CaCl_2$, compared to the control. *P. cepacia* was the only test organism that did not show synergistic anti-*Pseudomonas* activity in the presence of 1342 and MP (Table III). These results reflect the physiological diversity of different species of *Pseudomonas.*

Analyses of tap water and 1342-treated tap water indicated that exposure of the water to the 1342 caused a significant decrease in hardness, expressed as ppm $CaCO_3$. This suggests that 1342 is capable of chelating Ca^{2+} ions. The 0.1% 1342 and 0.01% Na_2EDTA reduced water hardness by similar amounts. This suggests that these compounds have similar chelating abilities for the Ca^{2+} ions.

The ST study revealed little antibacterial activity by either MP or Na_2EDTA alone (Table V). Rapid killing occurred in the presence of MP + Na_2EDTA because no viable organisms were recovered at 4 hr in most test systems. The test organisms found to be more persistent in these tests were, in general, more persistent in lotions containing 1342 and MP (Table II). In some cases, it is believed that differences in results between these two tables may be attributed to differences in APCs of the inocula.

The STs and MPSTs in Table V were used to calculate the slopes and D-values (or MPSlopes and MPD-values) in Table VI. Survivor curve slopes may be determined when the STs and initial inocula of the test organisms are known. For example, *P. aeruginosa* 9027 had an ST of 4 hr in MP + Na_2EDTA, and the APC in the sample was 1.1×10^5/ml. Here, the D-value and slope were 0.8 hr and $-1.26\ hr^{-1}$, respectively. Where STs are not known (i.e., ST >48 hr), the MPD-values and corresponding MPSlopes may be estimated from a virtual survivor curve constructed using the APC of the inoculum and the MPST, as explained above. Here, the MPD-value for *P. aeruginosa* 9027 in MP was calculated to be >9.5 hr and the MPSlope was $< -0.105\ hr^{-1}$. This slope is the negative reciprocal of the MPD-value and represents the fastest possible rate of death of this organism in this test system. If *P. aeruginosa* were being killed at a faster rate, then no organisms would have been recovered at the last sampling (i.e., at 48 hr). The MPSlope for *P. aeruginosa* 9027 in Na_2 EDTA was estimated similarly to be $< -0.105\ hr^{-1}$. Synergy was observed here, because the slope for the system containing MP + Na_2EDTA ($-1.26\ hr^{-1}$) was a larger negative number than the sum of the MPSlopes for MP and for Na_2EDTA ($-0.210\ hr^{-1}$). This procedure was used for each test organism shown in Table VI. The MP + Na_2EDTA system had synergistic anti-*Pseudomonas* activity for all pseudomonads, except for *P. cepacia* 13945. The MPD-values for different strains of *P. aeruginosa* were slightly different due to the slightly different concentrations of organisms in the inocula (Table VI). The estimated STs for *S. aureus* and *E. coli* were >48 hr in all test systems; consequently, it was not possible to establish synergy for these organisms in this experiment.

Numerous workers have reported the enhancement of preservative action by EDTA (3,7,31–34). The potentiation by EDTA is believed to be due to permeabilization synergy, in which one antimicrobial agent (EDTA) assists the passage of the other antimicrobial through the cell wall or membrane (7). We propose that the anti-*Pseudomonas* synergy observed with 934, 941, or 1342 and MP is due, at least in part, to chelation of divalent metal ions and that it is similar to permeabilization synergy reported for the potentiation of preservative action by EDTA (7). Results in support of this are the demonstration that 1342 has chelation activity, the elimination of the synergism observed with the fluorescent pseudomonads by the addition of 0.1% $CaCl_2$ (Table IV), and the similarities in the survival patterns of the various pseudomonads in nonionic lotions with polyacrylic acid or acrylic acid copolymer/MP systems (Table II) and in Na_2EDTA/MP solutions (Table V).

Similar patterns of inactivation were observed in both aqueous and nonionic emulsion

systems. This suggests that the observed synergy is not due to the nonionic emulsions studied here. The effect of 934, 941, or 1342 and MP on the inactivation of *Pseudomonas* was not tested in anionic or cationic emulsion systems or in various surfactant systems such as anionic shampoos and liquid soaps. The reason for this is that 1342 emulsion systems are sensitive to electrolytes, which cause loss of emulsion stability (35).

Adair *et al.* (36) reported that *P. aeruginosa* 9027 underwent lysis following metabolism of di- or tricarboxylic acids and sodium lauryl sulfate, and that lysis was not due to chelation. It is evident that the mechanism reported by these workers is not the same as the mechanism observed in the current work.

The antibacterial synergy of MP and acrylic acid homopolymer/copolymers against most *Pseudomonas* test cultures was not observed with *E. coli* 8739. This gram-negative organism was not inactivated rapidly in nonionic emulsions containing MP and 1342 or in solutions containing MP + Na_2EDTA (Tables II, V, and VI). These findings suggest a mechanism of action that is relatively specific for pseudomonads and not other gram-negative bacteria; however, testing with other strains of *E. coli* and other gram-negative organisms is necessary to confirm this.

The survivor curve slope method of determining synergy of preservative systems has application to both current experimental findings and to data presented in the literature. Application of this method to the ST data of Richards and Hardie (37) revealed synergism for fentichlor/phenylethanol combinations against *E. coli* and *Proteus vulgaris* at 0.0015% or 0.0050% fentichlor + 0.4% phenylethanol, and with 0.0050% fentichlor + 0.4% phenylethanol against *P. aeruginosa*. The survivor curve slope method revealed increased antibacterial activity against *S. aureus;* however, this effect was not synergistic. Our use of the survivor curve slope method corroborated the findings of Richards and Hardie.

The survivor curve slope method of determining synergy was applied to the D-values for *S. aureus,* in various combinations of preservatives in saline, reported by Akers *et al.* (38). Slopes of the survival curves were determined by taking the negative reciprocal of the D-values reported by these workers, and these slopes were used to determine whether mixtures of two preservatives exhibited synergy. Although these workers did not attempt to determine synergy, application of the survival curve slope method to their data for linear analysis of preservatives in saline solutions revealed that systems containing 0.2% phenol + 0.3% m-cresol, 0.2% phenol + 0.2% m-cresol, and 0.2% MP + 1.0% benzyl alcohol exhibited synergy. Akers *et al.* ranked the efficacy of these preservative systems in the top half of the systems tested against *S. aureus.*

The survival curve slope method was used to study synergy in a system reported by Boehm to be synergistic (4). He reported that 0.25% P and 0.09% N were synergistic against *P. pyocyanea.* Since we did not have the same strain as in Boehm's experiments, it was decided to "bracket" the concentrations of P and N used in Boehm's studies. The results in Table VII show the growth response observed with *P. aeruginosa* 9027 in 0 to 1% P and/or 0 to 0.1% N. The STs and MPSTs in Table VII were used to calculate the slopes, MPSlopes, D-values, and MPD-values in Table VIII. Our findings show synergy for concentrations that bracket the synergistic combination reported by Boehm.

The use of kinetic parameters—the slopes of survivor curves obtained by use of the linear regression method—to demonstrate anti-*Pseudomonas* synergy of MP and acrylic acid homopolymer/copolymers *in vitro* has not been reported previously. The survivor

curve slope method may be used to determine synergy when STs are known for the test organisms in systems containing combined preservative system components and in which the inoculum level is known. Isobolograms (5,7,39) are not needed when using this method.

We propose that the synergy with acrylic acid homopolymer/copolymers and MP is due, at least in part, to the chelation of divalent metal ions by the homopolymer/copolymers and that it is similar to the potentiation of preservative action by EDTA. No synergy was demonstrated in systems challenged with *E. coli, S. aureus,* and *B. cereus,* which suggests that the synergy was specific for the pseudomonads. In general, *P. cepacia* was inactivated more slowly than the fluorescent pseudomonads in test systems containing acrylic acid homopolymer/copolymers and MP. It is possible that the primary benefit from the polyacrylic acid/acrylic acid copolymer synergy with MP may be obtained in systems in which EDTA cannot be used or in systems with low ionic strength. Experiments were not performed to determine whether acrylic acid homopolymer/copolymers exert a synergistic effect on MP/EDTA systems, or whether EDTA exerts a potentiating effect on MP/acrylic acid homopolymer/copolymers.

Additional experiments are required to define the range of synergy of MP and other paraben esters with these homopolymer/copolymers and to characterize the mechanism of this synergy with certainty.

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