magnesium salt of the other two carboxylic acids compared to the calcium salt again suggests that qualitative trends reported for alkali halides or alkaline earth metal salts are not seen in organic carboxylic acid salts.

In view of these results, the equations (5) predicting only qualitatively the solubilities of a series of inorganic salts cannot be used for organic salts. The solubility of several salts of the organic compound of interest should be determined experimentally, and this parameter should be considered in the selection of the most suitable drug entity for further development.

REFERENCES

(1) T. Higuchi, M. Gupta, and L. W. Busse, *J. Am. Pharm. Assoc., Sci. Ed.*, **42**, 157 (1953).

(2) R. Anderson, *Aust. J. Pharm.*, **42**, 919 (1961).

(3) F. S. Hom and J. Autian, *J. Am. Pharm. Assoc., Sci. Ed.*, **45**, 608 (1956).

(4) S. F. Kramer and G. L. Flynn, *J. Pharm. Sci.*, **61**, 1896 (1972).

(5) "Treatise on Analytical Chemistry," I. M. Kolthoff, P. J. Elving, and E. B. Sandell, Eds., The Interscience Encyclopedia, Interscience, New York, N.Y., 1959.

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Preservation of Solubilized and Emulsified Systems I: Correlation of Mathematically Predicted Preservative Availability with Antimicrobial Activity

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Abstract □ Mathematical models were investigated for the distribution and antimicrobial activity of chlorocresol in solubilized and emulsified systems stabilized with a nonionic surfactant. The concentration of free preservative in the solubilized systems was described adequately by an equation widely used to describe the binding of small molecules to macromolecules. For the emulsions, this equation was combined with an expression for the partitioning of the preservative between the oil and water phases. It was confirmed that short-term antimicrobial activity can be related to the free (unbound) preservative concentration in the aqueous phase and that preservative solubilized within the surfactant micelles or partitioned into the oil phase does not contribute to short-term preservation.

Keyphrases □ Chlorocresol—in solubilized and emulsified systems, mathematical model relating phase distribution to antimicrobial activity □ Models, mathematical—relating phase distribution to antimicrobial activity, chlorocresol in solubilized and emulsified systems □ Antimicrobial activity—chlorocresol in solubilized and emulsified systems, related to phase distribution with mathematical model □ Distribution, phase—chlorocresol in solubilized and emulsified systems, related to antimicrobial activity with mathematical model

The ability of preservatives to prevent microbial spoilage of solubilized and emulsified products is normally assessed by empirical tests involving inoculation of the finished product and examination during prolonged storage. Such methods are laborious, time consuming, and usually qualitative.

Mathematical models have been developed that predict the preservative concentration required in a surfactant solution (1–3) or an emulsion (2–9). These models were based on the assumption that antimicrobial activity is a function of the free (unbound or nonmicellar) preservative concentration in the aqueous phase.

Attempts to correlate the predictions made by mathematical equations with the observed antimicrobial activity of preservatives in solubilized systems generally confirmed that the antimicrobial action is largely a function of the free preservative concentration and that preservative associated with the surfactant micelles has little or no activity (10–14).

Although much work has been done on the distribution and antimicrobial activity of preservatives in oil–water systems (15–21), few studies have been made in oil-in-water emulsions stabilized with nonionic surfactants (5, 6). In simple oil-in-water dispersions, the antimicrobial activity appears to depend mainly on the preservative concentration in the aqueous phase while preservative partitioned into the oil phase is biologically inactive. However, Bean *et al.* (18, 20) suggested that preservative adsorbed at the oil–water interface also contributes to the overall activity; an increase in the oil–water ratio results in an increase in interfacial area and, therefore, should enhance the antimicrobial activity. Since the interfacial area in oil-in-water emulsions is much larger than in simple oil-in-water dispersions, antimicrobial activity might increase enormously with an increase in the oil–water ratio.

In this study, a physicochemical and microbiological evaluation of two mathematical models, one for surfactant solutions and the other for emulsions, was made to test their utility. The influence on antimicrobial activity of factors such as surfactant concentration, oil–water ratio, and interfacial area was given particular attention.

THEORETICAL

Garrett (3) suggested that the binding of preservatives with surfactant macromolecules can be quantified using expressions similar to those established for the protein binding of drugs.

Kazmi and Mitchell (1) compared various methods of expressing the interaction of a number of commonly used preservatives with the nonionic surfactant cetomacrogol and found that protein binding equations such

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as Eq. 1 could be used to describe the binding data:

$$r = \frac{nK[D_f]}{1 + K[D_f]} \quad \text{(Eq. 1)}$$

where r is the molar ratio of bound preservative to total surfactant, $[D_b]/[M]$; n is the number of independent binding sites on the surfactant molecules; K is the association constant for the binding of a molecule of preservative to one of these sites; and $[D_f]$ is the concentration of free preservative.

Equation 1 can be rearranged as:

$$\frac{r}{[D_f]} = nK - rK \quad \text{(Eq. 2)}$$

According to this simple model, a Scatchard plot (22) of $r/[D_f]$ against r should give a straight line from which the binding constants n and K can be obtained. However, Scatchard plots for the preservative–surfactant systems studied were nonlinear (1).

Failure to obtain a straight line is common in protein binding studies and has been attributed to factors including heterogeneity of binding sites, interaction between sites, and initiation of confirmation changes (23). Hence, the binding constants were determined from the slope of Scatchard plots in the concentration range of free preservative appropriate for antimicrobial activity (1). The total preservative concentration, $[D_t]$, necessary to provide this concentration of free preservative in the aqueous phase of the solubilized system was than calculated using a rearrangement of Eq. 1:

$$[D_t] = [D_f]\left[1 + \frac{nK[M]}{1 + K[D_f]}\right] \quad \text{(Eq. 3)}$$

Expansion of Eq. 1 according to the theory of multiple equilibria (23) with the assumption of two sets of independent binding sites was shown later (2, 9, 24) to characterize the interaction over a wide range of surfactant and preservative concentrations:

$$r = \frac{n_1K_1[D_f]}{1 + K_1[D_f]} + \frac{n_2K_2[D_f]}{1 + K_2[D_f]} \quad \text{(Eq. 4)}$$

where Set 1 has n_1 sites and an association constant K_1 and Set 2 has n_2 sites and an association constant K_2. The molar binding ratio, r, and the equilibrium concentration of free preservative, $[D_f]$, were determined experimentally for various values of $[D_t]$ and $[M]$, and the binding constants were computed from Eq. 4 using a nonlinear regression program to curve fit r as a function of $[D_f]$ (Langmuir-type plot) and $r/[D_f]$ as a function of r (Scatchard plot). Rearrangement of Eq. 4 gives Eq. 5, from which the total preservative concentration can be calculated for any value of $[D_f]$ or $[M]$ (9):

$$[D_t] = [D_f]\left[1 + \frac{n_1K_1[M]}{1 + K_1[D_f]} + \frac{n_2K_2[M]}{1 + K_2[D_f]}\right] \quad \text{(Eq. 5)}$$

The interaction of a preservative with a mixture of two surfactants also can be predicted from the binding constants that characterize the interaction with each surfactant comprising the mixture (2). Nevertheless, as pointed out elsewhere (9), a fit of the experimental data to Eq. 4 does not prove that the surfactant–preservative interaction occurs by any particular mechanism. Although the equations quantify the interaction and permit predictions to be made over a wide range of surfactant and preservative concentrations, caution should be exercised in ascribing a physical mechanism to the interaction based on the numerical values of n and K. Indeed, Schellman *et al.* (25), in discussing the results of their protein binding studies, emphasized the point that only the product nK has theoretical significance without knowledge of the heterogeneity of the binding sites.

As discussed previously (1), since the interaction occurs between the solute and surfactant micelles rather than monomer surfactant molecules, $[M]$ in Eqs. 1–5 should be the concentration of micelles, n the number of binding sites per micelle, and K the association constant for reaction with the micelles. From a practical viewpoint, however, it is more convenient to express $[M]$ in terms of the total surfactant concentration.

Additional terms can be included in Eq. 5 to give Eq. 6, from which the total concentration of preservative, $[D_t]$, can be calculated for emulsions:

$$[D_t] = [D_f]\left[\frac{1 + n_1K_1[M]}{1 + K_1[D_f]} + \frac{n_2K_2[M]}{1 + K_2[D_f]} + K_w{}^oq\right] \Big/ (q + 1) \quad \text{(Eq. 6)}$$

where $K_w{}^o$ is the oil–water partition coefficient and q is the oil–water ratio. In Eq. 6, the term $[M]$ is strictly the concentration of surfactant available in the aqueous phase for interaction with the preservative and not the total concentration.

In a complex dispersion, the total surfactant concentration will not be available for this interaction. For example, some surfactant will be adsorbed at the oil–water interface, some surfactant may partition into the oil phase, and some surfactant will exist in monomer (nonmicellar) form. Each of these factors will reduce the amount of surfactant available for binding with the preservative and may affect the oil–water partition coefficient. The monomer surfactant concentration will be approximately equal to the critical micelle concentration, which for a nonionic surfactant such as cetomacrogol is sufficiently low (26) to be neglected at the surfactant concentrations used in this work.

Garrett (3) suggested that an ultracentrifuge technique be used to determine the "operative partition coefficient" in the actual emulsion system and the equilibrium concentration of surfactant in the aqueous phase as a function of the total surfactant concentration. However, studies using a three-chamber dialysis cell (7), which permitted direct estimation of the distribution of a preservative and surfactant between the oil, micellar, and aqueous phases of an emulsion, demonstrated excellent agreement between observed and predicted values of $[D_b]$ when the initial (*i.e.*, total) surfactant concentration and $K_w{}^o$, obtained by simple partition between the oil and water phases, were substituted into the equation.

In this work, the validity of Eqs. 5 and 6 was first confirmed by physicochemical means. Then the equations were used to calculate the total preservative concentration required in various solubilized and emulsified dispersions to provide a given free preservative concentration in the aqueous phase. The antimicrobial activities of the various dispersions containing different concentrations of total preservative but the same free concentration were then compared by following the death rate of *Escherichia coli* by viable counting using a membrane filtration technique.

EXPERIMENTAL

Materials—Mineral oil (light liquid paraffin BPC[1]), trypticase soy broth[2], trypticase soy agar[2], peptone[3], and reagent grade sodium chloride[4] were used as received. Chlorocresol, cetomacrogol 1000 BPC[5], and silicone rubber membrane were as described previously (24). Deionized glass-distilled water was used throughout, and all studies were carried out at 25°.

Interactions of Chlorocresol with Cetomacrogol—The interaction of chlorocresol with cetomacrogol was studied by equilibrium dialysis, using silicone rubber as the dialysis membrane. The chlorocresol was analyzed spectrophotometrically at 280 nm, and the binding constants (n and K) were computed as described previously (9, 24).

Distribution of Chlorocresol between Mineral Oil and Water and in Mineral Oil Emulsions Stabilized with Cetomacrogol—The experimental procedures for the determination of distribution coefficients and the dialysis technique for studying the distribution of a preservative in emulsions were described previously (7).

Microbiological Procedures—*Sterilization of Experimental Solutions*—Trypticase soy broth and trypticase soy agar were rehydrated and sterilized according to the specifications of the manufacturer. Deionized glass-distilled water, 0.85% saline, and peptone water (peptone, 1.0 g; sodium chloride, 8.5 g, and distilled water to 1 liter) were sterilized by autoclaving at 121° for 15 min. Aqueous chlorocresol solutions and cetomacrogol solutions, with and without chlorocresol, were sterilized by passing the solutions through sterile 0.45-μm filters[6]. Mineral oil was sterilized by dry heat at 160° for 1 hr.

Preparation of Sterile Mineral Oil Emulsions—Sterile mineral oil and cetomacrogol solutions, with or without chlorocresol, were mixed in various ratios and passed through sterile hand homogenizers at least five times to ensure the formation of stable emulsions. All operations were carried out aseptically under a laminar flow hood[7].

For emulsions containing chlorocresol, the mineral oil and cetomacrogol solutions were mixed prior to homogenization and agitated in a

[1] British Drug Houses, Poole, England.
[2] B.V.L., Division of Bioquest.
[3] Difco Laboratories.
[4] Mallinckrodt Chemical Works.
[5] Texofor A1P, Glovers Chemical Ltd., Leeds, England; $CH_3(CH_2)_m = (OCH_2CH_2)_n$ OH, where m may be 15 or 17 and n may be 20–24. The molecular weight was taken as 1300.
[6] HA, Millipore Corp.
[7] Bioquest Biological Cabinet, Division of Bioquest.

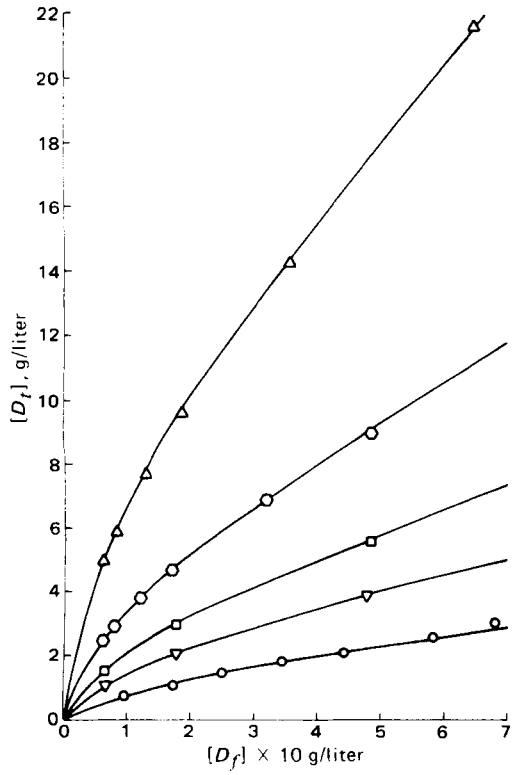

Figure 1—*Variation of free chlorocresol concentration,* $[D_f]$, *with total chlorocresol concentration,* $[D_t]$, *for the interaction of chlorocresol with cetomacrogol. Key [cetomacrogol concentrations (percent)]:* ○, *1.0;* ▽, *2.0;* □, *3.0;* ○, *5.0; and* △, *10.0. The points are experimental; the curves were calculated using Eq. 5 where* $n_1 = 0.697$, $n_2 = 136$, $K_1 = 1.87 \times 10^3$ *liters/mole, and* $K_2 = 2.09$ *liters/mole.*

water bath maintained at 25° for about 3 days to allow the preservative to equilibrate between the various phases of the emulsion.

Organism—Replicate slants of *E. coli* (ATCC 8739) were used in all experiments. The slants were prepared from a single colony and stored in a refrigerator at 4°.

Bacterial Cultures—An aliquot (5 ml) of trypticase soy broth was inoculated from a fresh slant, and the culture was allowed to grow for 12 hr at 37° in an incubator. A 0.2-ml sample of this culture was transferred to 50 ml of fresh trypticase soy broth and incubated at 37° for 12 hr. This final *E. coli* suspension was used to prepare a series of dilutions in trypticase soy broth for the construction of a standard curve of absorbance at 550 nm *versus* the number of viable organisms counted using a pour-plate technique. The purity of the culture was checked using Gram's staining procedures and examination of the growth on MacConkey agar.

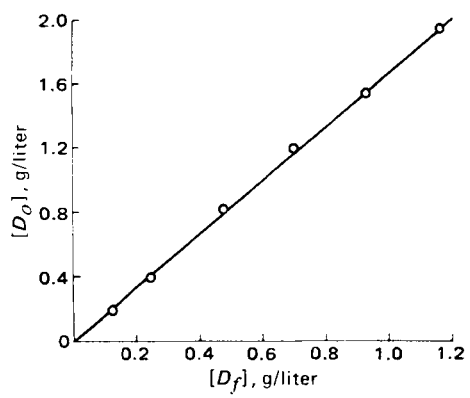

Figure 2—*Concentration of preservative in oil,* $[D_o]$, *as a function of free preservative in the aqueous phase,* $[D_f]$, *for the distribution of chlorocresol between mineral oil and water. The points are experimental; the curve was fitted using least-squares method. The slope =* $1.67 = K_w^o$.

Table I—Comparison of Required with Observed Chlorocresol Concentrations in Mineral Oil Emulsions Stabilized with Cetomacrogol[a]

Oil–Water Ratio	$M \times 10^3$ $[D_t]$ Calculated from Eq. 6	$[D_f]$ Required	$[D_f]$[b] Observed
0.2	27.15	2.45	2.45
0.5	22.54	2.45	2.32
1.0	17.93	2.45	2.35

[a] Cetomacrogol concentration = 23.08×10^{-3} *M*. [b] Mean of three observations.

Preparation of Standard E. coli *Suspension*—An aliquot of the culture was diluted with trypticase soy broth, and the absorbance was measured at 550 nm. From the absorbance and the dilution factor, the concentration of *E. coli* in the culture was determined from the standard curve. The culture was diluted with 0.85% saline to produce the desired number of organisms per milliliter of suspension.

Viable Counting Using Pour-Plate Technique—Culture samples were diluted with an appropriate volume of sterilized 0.85% saline to produce a final count of 50–200 colonies/plate. From these dilutions, 1-ml aliquots were pipetted into each of three petri plates; then 12 ml of sterile trypticase soy agar, kept at 45° in a water bath, was poured into each plate. The plates were rotated to disperse the organisms and incubated for 48 hr at 37°. The colonies were counted using a colony counter[8].

Viable Counting Using Membrane Filtration Technique—A six-place sterility test manifold unit with 47-mm hydrophobic-edge filters[9] (0.45 μm) was used. Details of the filtration assembly, sterilization, and operation are given elsewhere (27). All operations were performed under a laminar flow hood.

Samples were diluted with an appropriate volume of sterile normal saline to produce 100–200 colonies/filter. From these dilutions, 1-ml aliquots were pipetted into funnels containing approximately 10 ml of peptone water, and vacuum was applied immediately to draw the sample through the filter. The filter was washed five times with 25-ml portions of peptone water, and vacuum was applied immediately each time. After filtration, the filter was transferred to trypticase soy agar plates and incubated at 37° for 24 hr; then the colonies were counted. For chlorocresol-treated *E. coli*, the plates were incubated for an additional 12 hr, and colonies were recounted to check for the emergence of new colonies.

Bactericidal Activity of Chlorocresol in Water, Aqueous Solutions of Cetomacrogol, and Mineral Oil Emulsions Stabilized with Cetomacrogol—The aqueous solutions, surfactant solutions, and freshly homogenized emulsions containing chlorocresol were agitated in a water bath maintained at 25° to equilibrate the preservative between the various phases. After about 3 hr, 41.5 ml of each dispersion was inoculated with 0.5 ml of standard *E. coli* culture to give 10^6 organisms/ml. Changes in the preservative concentration, surfactant concentration, and oil–water ratio due to the addition of 0.5 ml of the culture were considered in the experiment design. The solutions were sampled at given time intervals, and viable counts were made using the membrane filtration technique. Controls, consisting of water, aqueous cetomacrogol solutions, and mineral oil emulsions without chlorocresol, were run with each experiment to check the viability of the organism.

RESULTS AND DISCUSSION

Interaction of Chlorocresol with Cetomacrogol—Figure 1 shows the total preservative concentration, $[D_t]$, plotted as a function of free preservative concentration, $[D_f]$, for the interaction of chlorocresol with various cetomacrogol concentrations. The experimentally determined values and the curves calculated by substitution of the binding constants into Eq. 5 agree closely. Hence, using Eq. 5, the total concentration of preservative required in a given surfactant solution can be calculated to provide any desired concentration of free preservative.

Distribution of Chlorocresol between Mineral Oil and Water—Figure 2 shows the chlorocresol concentration in the oil phase, $[D_o]$, plotted as function of the concentration in the aqueous phase, $[D_f]$. A linear relationship between $[D_o]$ and $[D_f]$ indicates that the distribution of chlorocresol between mineral oil and water obeys the simple partition law. The slope of the line gives the partition coefficient, K_w^o. The value

[8] Fisher.
[9] HAEG, Millipore Filter Corp.

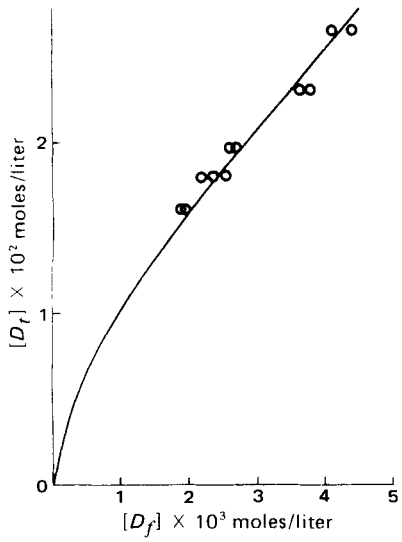

Figure 3—*Variation of free chlorocresol concentration,* $[D_f]$, *in the aqueous phase of an emulsion with total chlorocresol concentration,* $[D_t]$, *for an oil-in-water emulsion containing 50% (v/v) mineral oil emulsified with 3% (w/v) cetomacrogol. The points are experimental; the curve was calculated from Eq. 6.*

of $K_w{}^o$ obtained was 1.67, compared with the value of 1.53 reported previously (20).

Distribution of Chlorocresol in Mineral Oil Emulsions Stabilized with Cetomacrogol—Figure 3 is a plot of the total preservative concentration, $[D_t]$, *versus* the free preservative concentration, $[D_f]$, for the distribution of chlorocresol in mineral oil emulsions of a fixed oil–water ratio. The experimentally determined values and the curve calculated from the experimental value of $K_w{}^o$ and the preservative–surfactant binding constants (n and K) using Eq. 6 agree reasonably well. Close agreement also was found between $[D_f]$ predicted using Eq. 6 and the observed values for the distribution of chlorocresol in mineral oil emulsions of other oil–water ratios (Table I). These results, together with earlier work (7) on the distribution of benzoic acid in both mineral oil and peanut oil emulsions, confirm the validity of Eq. 6. Therefore, the total concentration of preservative required to provide the desired concentration of free preservative in the aqueous phase of an emulsion can be calculated with Eq. 6.

Comparison between Membrane Filtration and Pour-Plate Techniques—The death rate of microorganisms in a given solution can be studied by counting the number of surviving organisms as a function of time. Pour-plate or roll tube methods are often used for the viable counting of microorganisms. These techniques involve dilution of the sample in normal saline or one-fourth strength Ringer's solution and subsequent plating on agar. To follow the death rate of microorganisms in systems containing preservative, large dilutions are required to reduce the preservative concentration, which would otherwise inhibit the microorganism growth upon plating. This dilution is a serious limitation, especially when the death rate is followed to 100% mortality. Since large dilutions are not possible at high mortality levels, the chances of carryover of preservative to the growth medium are great.

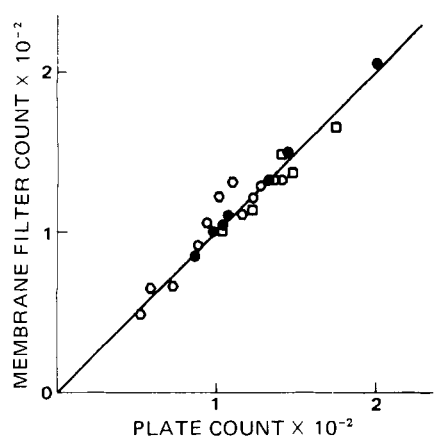

Figure 4—*Comparison between filtration and pour-plate techniques for counting* E. coli. *Different symbols represent separate experiments. The correlation coefficient = 0.95.*

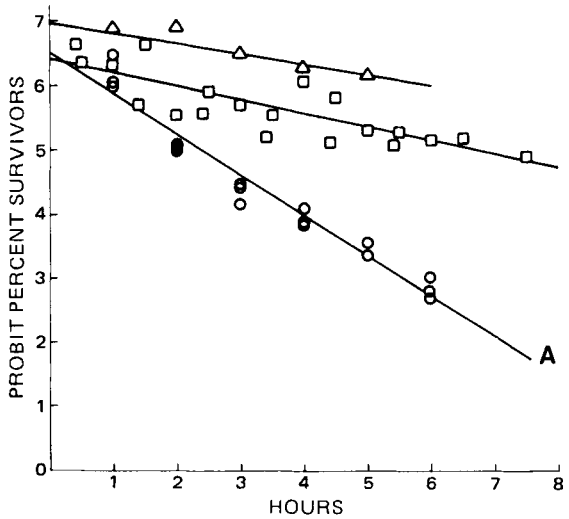

Figure 5—*Probit percent survivors as a function of time for the bactericidal activity of chlorocresol in water against* E. coli. *Key [chlorocresol concentration (percent)]: △, 0.02; □, 0.03; and ○, 0.035.*

The membrane filtration method avoids these problems by rinsing of the test filter with sterile fluid after sample filtration. The test filter is a special type, having a hydrophobic rim approximately 3 mm wide. This rim prevents intrusion of the sample fluid into the area under the sealing edge of the filter holder. All growth-inhibiting residuals are within immediate reach of the rinse fluid and are diluted to the point where they have no discernible effect on culturing. Thus, it is possible to follow the death rate to any desired level of mortality without limiting the sample size and without danger of residual preservative inhibiting microorganism growth.

Figure 4 shows that there was a good correlation between the membrane filtration method and the pour-plate technique for counting *E. coli.*

Bactericidal Activity of Chlorocresol in Water—Figure 5 is a plot of the probit percent survivors *versus* time for the bactericidal activity of chlorocresol in water against *E. coli.* The symbols represent experimental data, while lines were fitted using a desk calculator[10] programmed for linear regression analysis. Increasing the chlorocresol concentration from 0.02 to 0.03% made little change in the *E. coli* death rate. However, at 0.035% chlorocresol, there was a marked increase in the death rate. Since 0.035% chlorocresol produced 99.9% mortality within 6–8 hr, a time suitable for the experimental design, this concentration was selected as a reference standard to which the bactericidal activity of chlorocresol in aqueous cetomacrogol solutions and mineral oil emulsions could be compared. The results of the control experiments are shown in Fig. 6A. The viability of *E. coli* in water remained almost unchanged during the 8 hr.

Bactericidal Activity of Chlorocresol in Aqueous Solutions of Cetomacrogol—Figure 7 shows probit percent survivors plotted as a function of time for the bactericidal activity of chlorocresol in water (curve A) and aqueous solutions of cetomacrogol (curves B′, C′, and D′) against *E. coli.* The aqueous solution contained 0.035% chlorocresol, and surfactant solutions contained sufficient total preservative, as calculated from Eq. 5, to provide a free concentration of 0.035% chlorocresol in the aqueous phase. The symbols represent experimental data, and the lines were fitted using a linear model, Eq. 7 (28), and a computer program (29):

$$Y = \beta_0 + \beta_1 x_1 + \beta_2 x_2 + \beta_3 x_3 + \beta_4 t + \beta_5 x_1 t + \beta_6 x_2 t + \beta_7 x_3 t + \epsilon \quad \text{(Eq. 7)}$$

where β_0 is the intercept of curve A; β_1, β_2, and β_3 are the differences between the intercepts of curves B′ and A, C′ and A, and D′ and A, respectively; β_4 is the slope of curve A; β_5, β_6, and β_7 are the differences in slope between curves B′ and A, C′ and A, and D′ and A, respectively; x's are the

[10] Wang 600.

Table II—Analysis of Survivor-Time Curves for the Antimicrobial Activity of Chlorocresol in Solubilized and Emulsified Systems Compared with an Aqueous Solution, All Containing the Same Initial Concentration of Free Preservative

Parameter from Eq. 7[a]	Parameter Estimate: Solubilized Systems (Fig. 7)	Parameter Estimate: Emulsions (Fig. 8)
β_0	6.50	6.50
β_1	0.09	−0.12
β_2	−0.13	−0.13
β_3	−0.03	0.07
β_4	−0.63	−0.63
β_5	0.18[b]	0.13[c]
β_6	0.31[d]	0.24[d]
β_7	0.28[d]	0.24[d]
$(\beta_5 - \beta_6)$	−0.13[c]	0.11
$(\beta_5 - \beta_7)$	−0.10	0.11
$(\beta_6 - \beta_7)$	0.03	0.00

[a] See text for definitions. [b] $p < 0.0005$. [c] $p < 0.05$. [d] $p < 0.0001$.

dummy variables where $x_1 = 1$ if curve B′ and otherwise is zero, $x_2 = 1$ if curve C′ and otherwise is zero, and $x_3 = 1$ if curve D′ and otherwise is zero; ϵ is a random variable; and t is time.

Table II summarizes the statistical analysis of the intercepts and slopes of the curves in Fig. 7. A Z test (30) was used to test the significance of the model parameters. Differences between the intercepts of surfactant curves B′, C′, and D′ and the intercept of curve A (water) were not significant, while differences between the slopes of curves B′, C′, and D′ with respect to curve A were significant. However, the differences between the slopes of the surfactant curves B′ and D′ $(\beta_5 - \beta_7)$ and of C′ and D′ $(\beta_6 - \beta_7)$ were not significant, although the difference between the slopes of curves B′ and C′ $(\beta_5 - \beta_6)$ was significant.

Comparison with Fig. 5 shows that the three surfactant curves fall between the values of the slopes for 0.030 and 0.035% aqueous chlorocresol solutions; *i.e.*, a difference in $[D_f]$ of 0.005% produced a marked change in the slope. This concentration difference should be contrasted with the large differences between $[D_t]$ for each surfactant concentration, *i.e.*, B′, 0.1743%; C′, 0.4528%; and D′, 0.7324%. The slope of the probit–survivor curve is very sensitive to changes in $[D_f]$ in the concentration range chosen for these experiments (Fig. 5). Therefore, any factor producing a slight change in $[D_f]$ will significantly affect the slope of the survivor–time curve.

The difference between the slopes of curves B′ and C′ in Fig. 7 suggests that increasing the cetomacrogol concentration may decrease bactericidal activity. This decrease could be due to a stimulation of microbial growth (31–35) or protection of the organism by the nonionic surfactant (36–40).

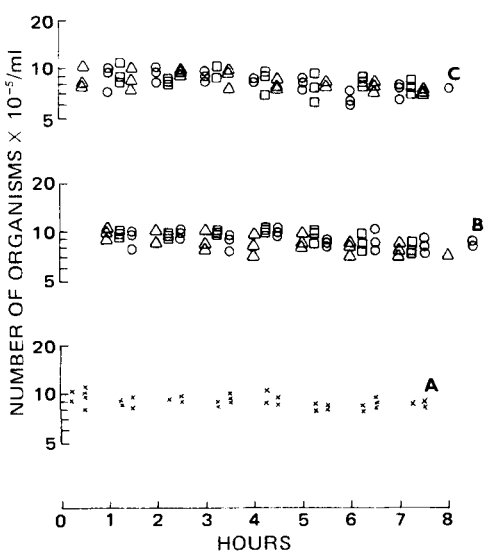

Figure 6—*Semilogarithmic plot of the number of organisms per milliliter as a function of time for the survival of* E. coli. *Key: A, water; B, aqueous cetomacrogol solutions with concentrations of cetomacrogol as in Fig. 1; and C, mineral oil emulsions with values of* q *and* M *as in Fig. 8.*

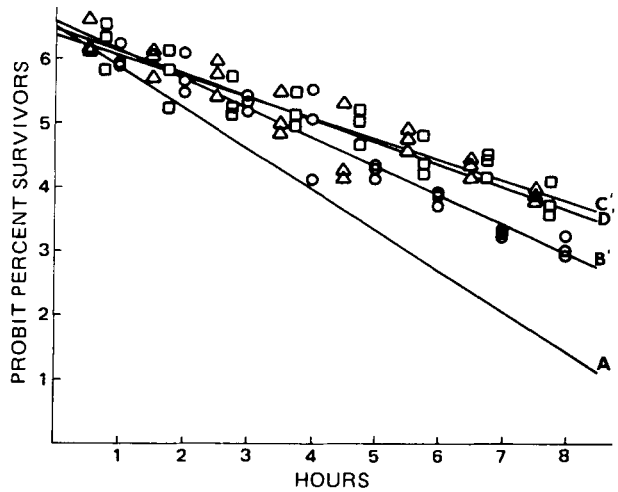

Figure 7—*Probit percent survivors as a function of time for the bactericidal activity of chlorocresol in aqueous cetomacrogol solutions against* E. coli. *Key [cetomacrogol concentration (percent)]: A, 0.0 (from Fig. 5); B, ○, 1.0; C, □, 3.0; and D, △, 5.0. Key [total preservative concentration, $[D_t]$ (percent)]: A, 0.0350; B, 0.1743; C, 0.4528; and D, 0.7314. The initial free preservative concentration, $[D_f]$, = 0.035%. The points are experimental; the lines were fitted using Eq. 7.*

However, the absence of statistically significant differences between the slopes of curves B′ and D′ and curves C′ and D′ indicates that increasing the surfactant concentration is not important, provided $[D_f]$ is the same for each solution. The antimicrobial activity of chlorocresol in each surfactant solution was less than that of the solution in water. Therefore, under the experimental conditions, capacity defined as the ability of the system to resist changes in $[D_f]$ is not important[11]. Moreover, the statistically significant differences between the slopes of curve A (water) and the surfactant curves B′, C′, and D′ indicate, apart from any effect due to the capacity factor, that it is more appropriate to compare the antimicrobial activity of one surfactant solution of preservative with another rather than with that of a solution of the preservative in water.

Bactericidal Activity of Chlorocresol in Mineral Oil Emulsions Stabilized with Cetomacrogol—A realistic test of the theory that preservative activity in emulsions depends on $[D_f]$ is to compare the viability of a given microorganism in various emulsions with the same $[D_f]$ but varying $[D_t]$ values. An equal biological response in emulsions with differing oil–water ratios would then indicate that the antimicrobial activity is essentially a function of $[D_f]$ and that the preservative in the oil phase is biologically inactive. This approach was used in the design of the emulsion experiments in a manner analogous to that used for solubilized systems.

Figure 8 shows probit percent survivors plotted as a function of time for the bactericidal activity of chlorocresol in water (curve A) and mineral oil emulsions of varying oil–water ratios (curves B, C, and D) against *E. coli*. The symbols represent experimental data; the lines were fitted using the linear model, Eq. 7. The initial value of $[D_f]$ was the same for all curves, although the total preservative concentration, $[D_t]$, was varied.

The results of control experiments are shown in Fig. 6C. The viability of *E. coli* in mineral oil emulsions of a varying oil–water ratio, q, remained almost unchanged during the 8-hr interval.

A statistical analysis of the slopes of the curves in Fig. 8 was performed using the method described for Fig. 7 (Table II). Differences between the intercepts of the emulsion curves B, C, and D and the intercept of curve A for aqueous solutions of chlorocresol (β_1, β_2, and β_3, respectively) were not significant. Differences between the slopes of curves B, C, and D and the slope of curve A (β_5, β_6, and β_7, respectively) were significant. However, the differences between the slopes of the emulsion curves $(\beta_5 - \beta_6)$, $(\beta_5 - \beta_7)$, and $(\beta_6 - \beta_7)$ were not significant. This result confirms the suggestion that the antimicrobial activity of chlorocresol in mineral oil emulsions is essentially a function of $[D_f]$.

Additional support for this suggestion comes from a statistical comparison of the survivor–time curves for emulsions B, C, and D (Fig. 8) with the solubilized systems B′, C′, and D′ (Fig. 7) summarized in Table III. None of the intercepts of the emulsion curves differed significantly from

[11] See Part II of this series for a full discussion of this concept.

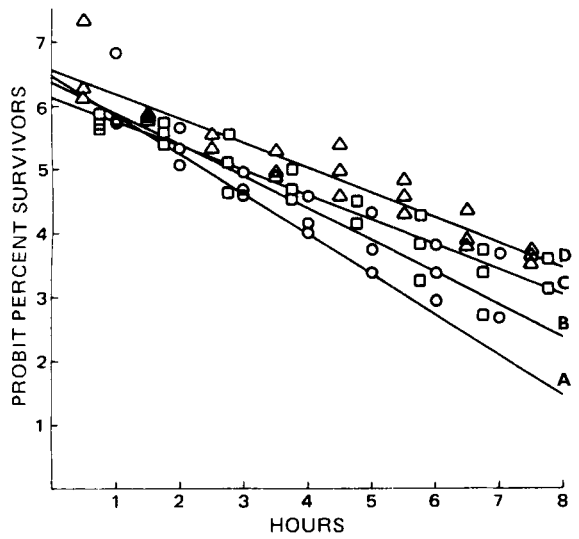

Figure 8—*Probit percent survivors of* E. coli *as a function of time for the bactericidal activity of chlorocresol in water (curve A from Fig. 5) and mineral oil emulsions stabilized with 3.0% (w/v) cetomacrogol. Key (oil–water ratio, q): B, ○, 1.0; C, □, 0.5; and D, △, 0.2. Key [total preservative concentration, $[D_t]$ (percent)]: A, 0.035; B, 0.2557; C, 0.3214; and D, 0.3871. The initial free preservative concentration, $[D_f]$, = 0.035%. The points are experimental; the lines were fitted using Eq. 7.*

the intercepts of the solubilized systems, except for the intercept of emulsion C which differed from that of solubilized system B′. More importantly, differences between the slopes of the survivor–time curves for the emulsions when compared with each solubilized system were not significant, other than the curve for emulsion B which differed in slope from that of solubilized systems C′ and D′.

The preservative activity of the solubilized and emulsified systems was similar, for the short-term bactericidal activity studied, when the dispersions initially contained an equal concentration of free preservative. This was true despite a wide range in the total preservative concentrations, differences in surfactant concentrations, the presence or absence of an oil phase, and variations in the percentage of the oil phase where present. In addition, the results provide striking indirect evidence of the validity of Eqs. 5 and 6 for calculating the total preservative concentration.

The statistically significant differences between the slopes of curve A (water) and emulsion systems B, C, and D again indicate, as was found for solubilized systems, that an emulsion should be compared with other emulsions (Table II) or solubilized systems (Table III) rather than with water (Table II). Although solubilized and emulsified dispersions with the same $[D_f]$ are equitoxic, the present results indicate that they do not have the same activity as a solution of the preservative in water with the same $[D_f]$.

Table III—Comparison of Survivor–Time Curves for the Antimicrobial Activity of Chlorocresol in Emulsified and Solubilized Systems, Each Containing the Same Initial Concentration of Free Preservative

Parameter from Eq. 7[a]	Parameter Estimate		
	B′	C′	D′
β_0	6.59	6.36	6.47
β_1	−0.22	0.01	−0.09
β_2	−0.45[b]	−0.22	−0.32
β_3	−0.02	0.20	0.10
β_4	−0.45	−0.32	−0.34
β_5	−0.05	−0.18[c]	−0.15[c]
β_6	0.06	−0.07	−0.05
β_7	0.06	−0.07	−0.05

[a] Where β_0 = intercepts of curves B′, C′, and D′ (Fig. 7, solubilized systems); β_1 = differences between intercepts of emulsion curve B (Fig. 8) and curves B′, C′, and D′, respectively; β_2 = differences between intercepts of emulsion curve C (Fig. 8) and curves B′, C′, and D′, respectively; β_3 = differences between intercepts of emulsion curve D (Fig. 8) and curves B′, C′, and D′, respectively; β_4 = slopes of curves B′, C′, and D′; β_5 = differences in slope between emulsion curve B and curves B′, C′, and D′, respectively; β_6 = differences in slope between emulsion curve C and curves B′, C′, and D′, respectively; and β_7 = differences in slope between emulsion curve D and curves B′, C′, and D′, respectively. [b] $p < 0.05$. [c] $p < 0.0005$.

The choice of a suitable value of $[D_f]$ depends on whether inhibition or bactericidal activity is desired. Minimum inhibitory concentrations are normally determined in a nutrient growth medium and, as discussed elsewhere (41), the nutritive value of the aqueous phase of a solubilized or an emulsified system is unlikely to be as great as that of nutrient broth or other growth media. Hence, if a minimum inhibitory concentration is used for $[D_f]$, the total concentration calculated from Eq. 5 or 6 should, in most cases, provide free preservative in excess of the minimum concentration necessary to inhibit growth. On the other hand, death or some specified percentage mortality within a finite time is probably a more desirable objective in preservation than simply growth inhibition. Whichever criterion is adopted, substitution of the selected value of $[D_f]$, together with the appropriate constants, into Eq. 5 or 6 will give the total preservative concentration required.

REFERENCES

(1) S. J. A. Kazmi and A. G. Mitchell, *J. Pharm. Pharmacol.*, **23,** 482 (1971).

(2) S. J. A. Kazmi and A. G. Mitchell, *Can. J. Pharm. Sci.*, **11,** 10, (1976).

(3) E. R. Garrett, *J. Pharm. Pharmacol.*, **18,** 589 (1966).

(4) R. A. Anderson and C. E. Chow, *J. Soc. Cosmet. Chem.*, **18,** 207 (1967).

(5) H. S. Bean, G. H. Konning, and S. A. Malcolm, *J. Pharm. Pharmacol., Suppl.*, **21,** 173S (1969).

(6) N. K. Patel and J. M. Romanowski, *J. Pharm. Sci.*, **59,** 372 (1970).

(7) S. J. A. Kazmi and A. G. Mitchell, *ibid.*, **60,** 1422 (1971).

(8) G. H. Konning, *Can. J. Pharm. Sci.*, **9,** 103 (1974).

(9) A. G. Mitchell and S. J. A. Kazmi, *ibid.*, **10,** 67 (1975).

(10) F. D. Pisano and H. B. Kostenbauder, *J. Am. Pharm. Assoc., Sci. Ed.*, **48,** 310 (1959).

(11) S. M. Blaug and S. S. Ahsan, *J. Pharm. Sci.*, **50,** 138 (1961).

(12) A. G. Mitchell, *J. Pharm. Pharmacol.*, **16,** 533 (1964).

(13) W. P. Evans and S. F. Dunbar, "Surface Activity and Microbial Cell," Society of Chemical Industry, London, England, 1965, p. 169.

(14) N. K. Patel, *Can. J. Pharm. Sci.*, **2,** 77 (1967).

(15) F. Atkins, *Manuf. Chem.*, **21,** 51 (1950).

(16) E. R. Garrett and O. R. Woods, *J. Am. Pharm. Assoc., Sci. Ed.*, **42,** 736 (1953).

(17) H. W. Hibbott and J. Monks, *J. Soc. Cosmet. Chem.*, **12,** 2 (1961).

(18) H. S. Bean, J. P. Richards, and J. Thomas, *J. Boll. Chim. Farm.*, **101,** 339 (1962).

(19) H. S. Bean and S. M. Heman-Ackah, *J. Pharm. Pharmacol., Suppl.*, **16,** 58T (1964).

(20) H. S. Bean, S. M. Heman-Ackah, and J. Thomas, *J. Soc. Cosmet. Chem.*, **16,** 15 (1965).

(21) H. S. Bean and S. M. Heman-Ackah, *J. Pharm. Pharmacol., Suppl.*, **17,** 142S (1965).

(22) G. Scatchard, *Ann. N.Y. Acad. Sci.*, **51,** 660 (1949).

(23) J. Steinhardt and J. A. Reynolds, "Multiple Equilibria in Proteins," Academic, New York, N.Y., 1969.

(24) S. J. A. Kazmi and A. G. Mitchell, *J. Pharm. Sci.*, **62,** 1299 (1973).

(25) J. S. Schellman, R. Lumry, and L. T. Sammuels, *J. Am. Chem. Soc.*, **76,** 2808 (1954).

(26) A. G. Mitchell and L. S. C. Wan, *J. Pharm. Sci.*, **53,** 1467 (1964).

(27) "Sterility Testing," MRP-4, Millipore Filter Corp., Bedford, Mass., 1968.

(28) W. Medenhall, "Introduction to Linear Models and the Design and Analysis of Experiments," Duxbury Press, Wadsworth Publishing, Belmont, Calif., 1968, p. 48.

(29) A. J. Barr and J. H. Goodnight, "Statistical Analysis System," Department of Statistics, North Carolina State University, Raleigh, N.C., 1972.

(30) W. Medenhall, "Introduction to Linear Models and the Design and Analysis of Experiments," Duxbury Press, Wadsworth Publishing, Belmont, Calif., 1968, p. 21.

(31) R. J. Dubos and B. D. Davies, *J. Exp. Med.*, **83,** 409 (1946).

(32) W. L. Williams, H. P. Broquist, and E. E. Snell, *J. Biol. Chem.*, **170,** 619 (1947).

(33) A. Bolle and A. Mirimanoff, *J. Pharm. Pharmacol.*, **2,** 685 (1950).

(34) M. G. de Navarre and H. E. Bailey, *J. Soc. Cosmet. Chem.*, **7**, 427 (1956).
(35) M. G. de Navarre, *ibid.*, **8**, 371 (1957).
(36) A. H. Beckett, S. J. Patki, and A. E. Robinson, *Nature*, **181**, 712 (1958).
(37) A. H. Beckett and A. E. Robinson, *Soap Perfum. Cosmet.*, **31**, 454 (1958).
(38) A. H. Beckett, S. J. Patki, and A. E. Robinson, *J. Pharm. Pharmacol.*, **11**, 421 (1959).
(39) D. L. Wedderburn, *J. Soc. Cosmet. Chem.*, **9**, 210 (1958).
(40) J. Judis, *J. Pharm. Sci.*, **51**, 261 (1962).
(41) S. J. A. Kazmi and A. G. Mitchell, *Soap Perfum. Cosmet.*, **45**, 549 (1972).

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Preservation of Solubilized and Emulsified Systems II: Theoretical Development of Capacity and Its Role in Antimicrobial Activity of Chlorocresol in Cetomacrogol-Stabilized Systems

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Abstract □ The preservation of solublized and emulsified disperse systems against microbial spoilage depends on the free (unbound) preservative concentration in the aqueous phase and the capacity of the system. The capacity may be defined as the system's ability to resist losses in free preservative concentration. The theory of capacity is developed quantitatively for solubilized and emulsified systems containing the preservative chlorocresol stabilized by the nonionic surfactant cetomacrogol. Equations are derived for solubilized systems that relate capacity to surfactant concentration and the interaction between the surfactant and the preservative. Additional terms are included in the equations to account for the effects of the oil phase on the capacity of oil-in-water emulsions.

Keyphrases □ Chlorocresol—in solubilized and emulsified systems, role of capacity in antimicrobial activity □ Capacity—theoretical development, role in antimicrobial activity of chlorocresol in solubilized and emulsified systems □ Antimicrobial activity—chlorocresol, role of capacity in solubilized and emulsified systems

For solubilized systems and oil-in-water emulsions stabilized with the nonionic surfactant cetomacrogol, the kill rate of *Escherichia coli* over 6–8 hr depends on the free chlorocresol concentration (1). However, for long-term protection against microbial contamination, the preservative's effectiveness should depend on the capacity of the system in addition to the free preservative concentration.

The term capacity was first used (2) in discussing the antibacterial activity of iodine solubilized by a nonionic surfactant[1]. The saturation solubility of the iodine was used as a measure of capacity. This definition implies that depletion of preservative due to interaction with microorganisms or foreign materials[2], volatilization, chemical decomposition (4), or metabolism by microorganisms (5) will be relatively greater from a solution in water than from a surfactant solution where the micelles act as a reservoir of preservative.

In this paper, the theoretical basis of capacity is developed more fully for solubilized systems and the theory is extended to oil–water dispersions and oil-in-water emulsions. The necessary physicochemical parameters were obtained previously (1, 6).

THEORETICAL

Capacity of Solubilized Systems—For a given loss of preservative, the decrease in concentration of free (unbound or nonmicellar) preservative in a solubilized system is less than from a solution in water and decreases with surfactant concentration. The percent decrease in con-

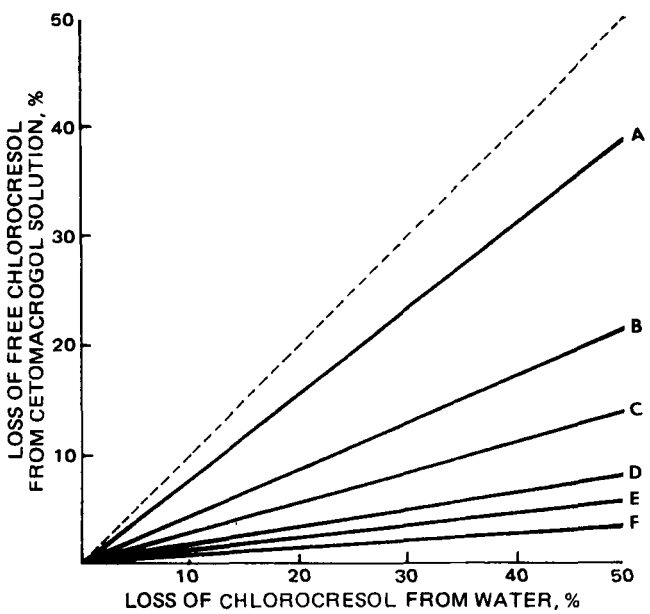

Figure 1—*Correlation between percent loss of chlorocresol from water and free chlorocresol from cetomacrogol solution when equal amounts of chlorocresol are removed from water and the surfactant solution. Key [cetomacrogol concentration (percent)]: A, 0.1; B, 0.5; C, 1.0; D, 2.0; E, 3.0; and F, 4.0 Curves were calculated using Eq. 1.*

[1] Antarox A-400.
[2] See Tables III and IV of Ref. 3.

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