DISTRIBUTION AND ANTIMICROBIAL ACTIVITY OF
PRESERVATIVES IN SOLUBILIZED AND
EMULSIFIED SYSTEMS

by

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A THESIS SUBMITTED IN PARTIAL FULFILMENT OF
THE REQUIREMENTS FOR THE DEGREE OF
DOCTOR OF PHILOSOPHY

in the Division of
Pharmaceutics
of the
Faculty of Pharmaceutical Sciences

We accept this thesis as conforming to
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Date May 28th, 1974
ABSTRACT

Until recently, evaluation of the effectiveness of preservatives in solubilized and emulsified systems has depended largely on time-consuming microbiological techniques. Mathematical models have now been developed which enable the amount of preservative necessary for adequate preservation to be calculated. These mathematical models have been derived on the assumption that the antimicrobial activity of preservative in solubilized and emulsified systems is largely a function of the free preservative concentration in the aqueous phase. In this work, a physicochemical and microbiological evaluation was made to test the validity of the above assumption and to study the possible effects of changes in surfactant concentration and oil-water ratio on the antimicrobial activity of preservative in solubilized and emulsified systems. Some weaknesses of the earlier models were pointed out. In particular, the concept of the capacity of the system to resist the changes in effective free preservative concentration was developed.

The permeability of cellophane membranes to the nonionic surfactant cetomacrogol was investigated using equilibrium dialysis, dynamic dialysis, and an ultrafiltration technique.

Cellophane and silicone rubber membranes were compared in an equilibrium dialysis study of the interaction of chlorocresol with nonionic surfactants. The magnitude of errors introduced into the binding parameters using cellophane membrane were related to the permeability of the membranes to the nonionic surfactants and to changes in volume and in surfactant concentration which occurred as a result of osmotic differential across
The diafiltration technique was evaluated for the interaction of benzoic acid with cetomacrogol. The results of the diafiltration technique were compared with those obtained using the equilibrium dialysis technique. Various technical artifacts of the diafiltration technique were pointed out.

Interaction of preservative mixtures with the nonionic surfactant cetomacrogol was studied using the equilibrium dialysis technique. Attempts to correlate the data with theory of competitive protein binding were unsuccessful.

The interaction of chlorocresol with certain nonionic surfactants and their mixtures was studied using the equilibrium dialysis technique. Binding parameters which characterized the interaction of the preservative with each individual surfactant were used to predict the binding behaviour of surfactant mixtures.

Various factors affecting the distribution of preservatives between oil and water and the interaction between preservatives and surfactants are discussed. These factors were related to the problem of the distribution of a preservative in oil and water emulsion systems. Methodology used to evaluate the various physicochemical parameters and the antimicrobial activity is reviewed, and equations for representing the results are discussed.

The Millipore filtration method was compared with a pour-plate technique for the viable counting of *E. coli*.
The bactericidal activity of chlorocresol in aqueous cetomacrogol solutions and liquid paraffin emulsions of varying oil-water ratios against E. coli was studied using a viable count method. The microbiological results were related with the physicochemical models of preservative distribution in solubilized and emulsified systems.

This abstract represents the true contents of the thesis submitted.

Signatures of Examiners:
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I wish to express my sincere gratitude and appreciation to:

Dr. A.G. Mitchell for his encouragement and guidance during the course of this investigation.

Dr. J.E. Axelson, Dr. M. Pernarowski and Dr. B.D. Roufogalis for many valuable, informative and helpful discussions.

Dr. B.C. McBride (Department of Microbiology) for counselling in the microbiological aspects of this study.

Dr. F.P. Glick (Department of Mathematics) for advice in the statistical analysis of microbiological data.

Dr. M.H. Chaudhry (IPEC Ltd.), Dr. A.W. Khanzada (Department of Chemistry), Mr. A.K. Khatry (Department of Mechanical Engineering) and Mrs. P. Haugen for their valuable assistance in preparing computer programs.

Financial support from the University of British Columbia is gratefully acknowledged.
DEDICATION

To my parents.
INTRODUCTION

Modern usage of the word solubilization appears to have given it a more general meaning and, therefore, it would perhaps require a specific definition for the present work. Solubilization can be defined as the preparation of a thermodynamically stable isotropic solution of a substance normally insoluble or very slightly soluble in water by the introduction of a surfactant or a mixture of surfactants. Thus, a solubilized system means a system containing a preservative or a mixture of preservatives and one or more types of surfactants.

For the present purposes an emulsified system is defined as a heterogeneous oil-in-water dispersion stabilized by a surfactant. The surfactants used in this work were of the nonionic type.

The microbiological activity of a preservative in solubilized and emulsified systems is much more complex than in simple aqueous systems but is generally less than the same amount of preservative in an aqueous system. An understanding of the factors controlling the effectiveness of a preservative in these systems can only be achieved by a thorough study of the various physical, chemical, and microbiological parameters governing the distribution and the antimicrobial activity.

At the present time, assessment of the ability of preservatives to prevent microbial spoilage of solubilized and emulsified products depends largely on empirical tests involving inoculation of the finished product, and examination during a prolonged period of storage. These methods are laborious, time consuming and are mainly qualitative in nature. No
information is obtained with regard to the mechanism of inactivation of the preservative or the concentration of preservative in various phases of the surfactant solution or emulsion.

Mathematical models have now been developed which permit a prediction to be made of the preservative concentration required in a solubilized system or an emulsion in order to achieve adequate preservation. The development of these models has been based on the basic assumptions that the antimicrobial activity is largely a function of the concentration of preservative in the aqueous phase, and that the preservative partitioned into the oil phase or bound with the surfactant micelles is biologically inactive. In this work a physico-chemical and microbiological evaluation of two mathematical models, one for solubilized systems and the other for emulsions, has been made to test the validity of the above assumptions, and to study the influence of factors such as surfactant concentration and oil-water ratio on the antimicrobial activity.

The antimicrobial activity of a preservative in a system is governed by the availability of the effective free preservative concentration to the micro-organisms. Depletion of the preservative due to factors such as interaction with micro-organisms or foreign materials, chemical decomposition, or even metabolism by the micro-organisms, will reduce the free preservative concentration and hence the antimicrobial activity. The duration of activity will depend on the capacity of the system. Capacity is defined as the ability of the system to resist changes in the effective free preservative concentration. In aqueous solutions the activity decreases rapidly because these systems lack capacity. In solubilized and emulsified
systems the surfactant micelles and the oil phase act as reservoirs of preservative and hence, capacity is controlled by the surfactant concentration, the oil-water partition coefficient and the oil-water ratio. Hence, the overall effectiveness of the preservative is determined not only by the effective free preservative concentration but also by the capacity of the system. In the present work, an attempt has been made to develop a numerical expression for the capacity and to examine the relationship between capacity and the parameters such as surfactant concentration, oil-water partition coefficient and oil-water ratio for various solubilized and emulsified systems.

The use of mathematical models to calculate the total concentration of preservative necessary to achieve the desired concentration in the aqueous phase of a surfactant solution or an emulsion requires the determination of various physico-chemical parameters. Methods for determining the distribution of preservative between oil-water phases are well established. The interaction between preservatives and surfactants has been studied using a variety of methods, e.g., equilibrium dialysis, dynamic dialysis, gel filtration, etc. Dialysis methods are slow and require numerous experiments to fully characterize the interaction. The selection of an adequate semipermeable membrane is a very crucial aspect of dialysis methodology. Ideally dialysis investigations require that the membrane be impermeable to the nonionic surfactant while allowing diffusion of the solute and that the osmotic differential across the membrane is negligible. Cellophane membranes are widely used in equilibrium and dynamic dialysis studies involving interaction of preservatives and drugs with nonionic surfactants. Cellophane has also been used to study the effects of surfactants on the diffusion of
drugs across the membranes. There is some controversy concerning the permeability of cellophane dialysis membranes to nonionic surfactants. Hence, in view of the continued use of cellophane, quantitative measurements of the permeability of cellophane membranes to nonionic surfactants, and an assessment of the effects of surfactant permeation and osmosis on the binding constants for the interaction between a preservative and nonionic surfactants has been made in this study. Because of the difficulties in dialysis methods it is realized that there is still need for a simple, rapid and reliable means of evaluating the binding parameters. The diafiltration technique appeared to answer these needs. Therefore, the diafiltration technique was used in an attempt to determine binding parameters for the preservative-surfactant interaction.

While the interaction of preservatives with nonionic surfactants has been studied extensively, little information is available concerning the solubilization of preservative mixtures by nonionic surfactants. Since preservative mixtures are often employed in cosmetic and pharmaceutical preparations containing nonionic surfactants, an attempt has been made to examine the binding behavior of binary preservative mixtures with a nonionic surfactant, and to correlate the binding results with the theory of competitive protein binding.

Surfactant mixtures offer many advantages over individual surfactants and are extensively used therefore in the formulation of solubilized and emulsified systems. Hence, an attempt has been made to use binding constants characterizing the interaction of a preservative with individual surfactants to predict the binding behavior of surfactant mixtures.
Pour-plate technique or roll tube method are generally used for the viable counting of micro-organisms. These techniques involve dilution of the sample in some non-nutrient medium, such as normal saline or quarter strength Ringer's solution, and subsequent plating on the agar. A few difficulties arise with the use of these techniques for studying the death rate of micro-organisms in systems containing preservatives. Large dilutions are required to reduce the concentration of preservative which would otherwise inhibit the growth of micro-organisms upon plating. This is a serious limitation, especially when the death rate is followed up to 100% mortality. Since at high mortality levels large dilutions are not possible, the chances of carry-over of preservative to the growth medium are great. Recently membrane filtration methods have been used extensively for the viable counting of micro-organisms. These methods are said to obviate the aforementioned problems of the pour-plate or roll tube methods by rinsing the test filter with sterile fluid after sample filtration. Hence, in the present work the Millipore filtration method has been evaluated for the viable counting of E. coli.
A. Distribution of Preservatives in Oil-Water Systems

(a) Factors affecting the antimicrobial activity of preservatives in oil-water systems

A preservative added to an oil-water mixture partitions between the two phases (Hibbott and Monks, 1961; Bean, Richard and Thomas, 1962). The antimicrobial activity is mainly a function of the availability, or thermodynamic activity, of a biologically effective concentration of preservative in the aqueous phase, and not the total amount added, i.e., preservative in the oil phase is biologically inactive (Wolffhugel and Von Knorre, 1881; Clark, 1939; Gershenfeld and Brillhart, 1939; Atkins, 1950; Bean, Richard and Thomas, 1962; Bean and Heman-Ackah, 1964; Bean, Heman-Ackah and Thomas, 1965). The availability of preservative in the aqueous phase is controlled by various factors:

1. Dissociation constant \((K_a)\) of the preservative and the pH of the aqueous phase.
2. Oil-water partition coefficient \((K_w^o)\).
3. Oil-water ratio \((q)\).
4. Interfacial factor.
5. Temperature.

(1) Dissociation constant of preservative and pH of aqueous phase:
Weak acid preservatives are generally most effective in their undissociated form (Rahn and Conn, 1940; Bandelin, 1958), the equilibrium between
undissociated acid and anion being a function of pH. It can be observed from Table 1 that 60 times as much benzoic acid is required at pH 6 as at pH 3 to achieve equivalent antimicrobial activity.

Table 1

Proportions of Benzoic Acid Undissociated at Various pH Values (Kostenbauder, 1962)

<table>
<thead>
<tr>
<th>pH</th>
<th>Undissociated Benzoic Acid %</th>
</tr>
</thead>
<tbody>
<tr>
<td>2</td>
<td>99.4</td>
</tr>
<tr>
<td>3</td>
<td>94.3</td>
</tr>
<tr>
<td>4</td>
<td>62.5</td>
</tr>
<tr>
<td>5</td>
<td>13.7</td>
</tr>
<tr>
<td>6</td>
<td>1.6</td>
</tr>
</tbody>
</table>

\[ K_a = 6.3 \times 10^{-5} \quad \text{pK}_a = 4.2 \]

If the minimum inhibitory concentration of undissociated acid is known, the total concentration of acid required in the aqueous phase can be calculated from the following equation (Kostenbauder, 1962):

\[
\text{Total required preservative} = \frac{[\text{Inhibitory Concentration}]_{\text{of undissociated acid}} [H^+] + K_a}{[H^+]}
\]

A close relation is generally found between pH and the antimicrobial activity of weak acid preservatives (Rahn and Conn, 1944; Wolf and Westveer, 1950; Simon, 1952; Von Schelhorn, 1952; Albert, 1957; Bandelin, 1958; Bell et al., 1959; de Navaree, 1959; Enterikin, 1961).
Since the dissociation constant of different preservatives varies, their behavior in different pH conditions also varies. Some are inactivated by small increases in pH while others are not influenced at all. When the pH of the environment is below the pKₐ, changes of pH are of little consequence but as the pH is increased above the pKₐ, higher concentrations are required to produce a standard response (Simon, 1952). In the case of benzoic acid a constant concentration of undissociated molecules does not produce the same response at different pH levels. This indicates that anions are also slightly toxic (Evans and Dunbar, 1965; Anderson and Cho, 1967).

(2) **Oil-water partition coefficient:** Partitioning has significant effect on the availability of preservative in the aqueous phase (Husa and Radin, 1932; Atkins, 1950; Allawala and Riegelmann, 1953; Garrett and Woods, 1953; Hibbott and Monks, 1961; Bean, Heman-Ackah and Thomas, 1965; and others). Assuming that the amount in the aqueous phase is active and if some transfers to the oil phase, sufficient additional preservative should be provided to maintain the required concentration in water. Thus, the total preservative to be added to a two-phase system can be calculated by knowing the concentration required in the aqueous phase (C₂H₅O) and knowing the volume of each phase (V_{oil} and V_{H₂O}) and the distribution coefficient (Eq. 1):

\[
\text{total preservative} = C_{H_2O} \cdot V_{H_2O} + K^O \cdot C_{H_2O} \cdot V_{oil}
\]

(Kostenbauder, 1962).

(3) **Oil-water ratio:** The concentration of preservative in the aqueous phase is not controlled by the partition coefficient alone, but by the interaction between the partition coefficient and the phase volume ratio (Bennett, 1962; Bean and Heman-Ackah, 1964; Bean, Heman-Ackah and Thomas,
1965; Bean, Konning and Malcolm, 1969). Table 2 shows the influence of partition coefficient and phase volume ratio on the concentration of preservative in the aqueous and oil phases of a two phase system. When the partition coefficient is less than one, the majority of the preservative is in the aqueous phase and an increase in the oil-water ratio increases the aqueous phase concentration. When the partition coefficient is greater than one, most of the preservative is in the oil phase and an increase in the oil-water ratio reduces the concentration of preservative in the aqueous phase. When partition coefficient is equal to one, changing the oil-water ratio has no effect on the concentration of the preservative in either phase. Thus, when selecting a compound for study as a possible preservative for a product, both the partition coefficient and the oil-water ratio must be considered.

(4) **Interfacial factor**: Bean et al. (1962, 1965) have shown that the bactericidal activity of a given concentration of preservative in simple aqueous solutions is less than the bactericidal activity of same concentration of preservative in the aqueous phase of an oil-water mixture. As the ratio of oil-water increases, the bactericidal activity of the preservative increases considerably. It was suggested that preservative molecules are adsorbed at the oil-water interface, with the polar portion of the molecule projecting into the aqueous phase, and the nonpolar portion projecting into the oil phase. In this way the concentration of preservative at the interface is higher than the bulk aqueous phase. When bacteria are added to such a system they are also adsorbed at the oil-water interface, being heavier (diameter in microns) than preservative molecules (diameter in Å) they penetrate the aqueous phase more deeply than the preservative molecules. It
Table 2
Influence of Partition Coefficient and Phase-Volume Ratio on Concentration of Preservative in Aqueous and Oil Phase of a Two-Phase System
(Bean, Heman-Ackah and Thomas, 1965)

<table>
<thead>
<tr>
<th>K°&lt;sub&gt;W&lt;/sub&gt; Oil/Water ratio</th>
<th>0.2</th>
<th>1.0</th>
<th>2.5</th>
<th>5.0</th>
<th>10.0</th>
</tr>
</thead>
<tbody>
<tr>
<td>at 25°</td>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>0.4% w/v phenol in liquid</td>
<td>0.067</td>
<td>Preservative in oil %</td>
<td>0.031</td>
<td>0.050</td>
<td>0.080</td>
</tr>
<tr>
<td>paraffin/water dispersions</td>
<td></td>
<td>Preservative in water %</td>
<td>0.474</td>
<td>0.750</td>
<td>1.199</td>
</tr>
<tr>
<td>1% hypothetical preservative</td>
<td>1.000</td>
<td>Preservative in oil %</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td></td>
<td></td>
<td>Preservative in water %</td>
<td>1.000</td>
<td>1.000</td>
<td>1.000</td>
</tr>
<tr>
<td>4.0% w/v chlorocresol</td>
<td>116.7</td>
<td>Preservative in oil %</td>
<td>22.96</td>
<td>7.93</td>
<td>5.60</td>
</tr>
<tr>
<td>in peanut oil/water dispersions</td>
<td></td>
<td>Preservative in water %</td>
<td>0.197</td>
<td>0.068</td>
<td>0.048</td>
</tr>
</tbody>
</table>
is therefore probable that part of the bacterial surface at the interface is in contact with a higher concentration of preservative, but this is certainly not true for the whole cell. If the bacteria were strongly adsorbed at the interface, as shown by Kamakaka (1956), then the preservative adsorbed at the interface would have been much more effective than was observed in these studies.

(5) **Effect of temperature:** Temperature influences the activity of preservatives in oil-water dispersions in a complex manner (Bean and Heman-Ackah, 1965; Bean, Heman-Ackah and Thomas, 1965). Temperatures above $50^\circ$ cause the death of vegetative cells by protein coagulation, enzyme inactivation, or both. In addition there are indirect effects of temperature on: (i) the oil-water partition coefficient (Bean and Heman-Ackah, 1963); (ii) the oil-water interfacial activity which diminishes with rise in temperature (Heman-Ackah, 1965); (iii) the velocity of bactericidal action which increases with temperature (Madsen and Nyman, 1907; Chick, 1908; Phelps, 1911).

(6) **Effect of additives:** Additives such as propylene glycol, glycerin, etc., are often included in pharmaceutical and cosmetic emulsions as humectants. These additives often bring about an increased solubility of preservative in the aqueous phase that reduces the oil-water partition coefficient (Hibbott and Monks, 1961; Anderson and Cho, 1967). It has been suggested (Hibbott and Monks, 1961) that this makes more preservative available in the aqueous phase with a consequent increase in antimicrobial activity. On the other hand, Anderson and Cho (1967) showed a reduction in preservative activity with the addition of glycerin. They suggested that
although the inclusion of glycerin reduces the oil-water partition coefficient, it also reduces the availability of the preservative to the microbial biophase. Consequently a higher concentration is required. In addition to reducing the oil-water partition coefficient, Barr and Tice (1957a,b) found that glycerin and sorbitol supplement each other in their inhibition of both bacteria and moulds. This effect was attributed to an osmotic effect of the high concentrations of the humectants. Propylene glycol, however, appeared to have a specific inhibitory effect in addition to its effect on the osmotic pressure of aqueous solutions. It was concluded that propylene glycol would have a significant and useful preservative effect. de Navarre (1962) reported that propylene glycol was a reliable preservative at 16% v/v in many cosmetic products because its antimicrobial properties were three or four times that of the equivalent amount of glycerin.

(b) Representation of distribution data

The distribution of preservatives between oil-water systems can be represented by the Nernst Equation (Nernst, 1891):

\[ K_w^0 = \frac{[D_o]}{[D_f]} \]  

(Eq.1)

where \( K_w^0 \) is the distribution or partition coefficient; \([D_o]\), the concentration of preservative in the oil phase; \([D_f]\), the concentration of preservative in the aqueous phase.

The ratio, \( K_w^0 \), is constant only for ideal solutions and is most closely approximated when (a) the preservative neither dissociates (ionizes) nor associates in either phase, (b) the preservative concentrations are approximately equal to activities, and (c) the two phases are completely immiscible (Reese et al., 1964).
When the preservative is monomeric in the oil phase, but ionizes in the aqueous phase, then the ratio of concentration of preservative in the oil phase to the concentration of unionized preservative in the aqueous phase will be constant.

\[
\frac{[\text{HA}]_o}{[\text{HA}]_w} = \frac{K^O_{dW}}{K_{dW}}
\]  

(Eq.2)

where \( K^O_{dW} \) is the distribution coefficient for monomer; \([\text{HA}]_o\), is the concentration of monomer in the oil phase; \([\text{HA}]_w\), the concentration of monomer in the aqueous phase.

In case of acid preservatives, such as benzoic acid and sorbic acid, the degree of ionization in the aqueous phase is a function of the pH of the aqueous phase and the ionization constant, \( K_a \), of the preservative. This can be expressed

\[
K_a = \frac{[\text{H}^+] [\text{A}^-]_w}{[\text{HA}]_w}
\]  

(Eq.3)

\[
[\text{A}^-]_w = \frac{K_a [\text{HA}]_w}{[\text{H}^+]}  
\]  

(Eq.4)

where \([\text{H}^+]\) is the hydrogen ion concentration in the aqueous phase.
The observed pH dependent distribution coefficient, \( K_w^0 \), is given by:

\[
K_w^0 = \frac{[Do]}{[Df]} = \frac{[HA]_o}{[HA]_w + [A^-]_w}
\]  
(Eq. 5)

where \([A^-]_w\) is the concentration of anion in the aqueous phase.

Substitute \([A^-]_w\) from Eq. 4 in Eq. 5 and rearrange

\[
K_w^0 = \frac{[HA]_o}{\frac{\beta A}{[HA]_w} + Ka \frac{[HA]_w}{[H^+]}}
\]  
(Eq. 6a)

or

\[
K_w^0 = \frac{[HA]_o}{[HA]_w} \cdot \frac{1}{1 + \frac{Ka}{[H^+]}}
\]  
(Eq. 6b)

or

\[
K_w^0 = K_{dw}^0 \cdot \frac{1}{1 + \frac{Ka}{[H^+]}}
\]  
(Eq. 6c)

A plot of \( K_w^0 \) versus \( 1/(1 + Ka/[H^+] \) will give a straight line with a slope equal to \( K_{dw}^0 \), the monomer distribution coefficient.

Rearranging Eq. 6c in double reciprocal form gives

\[
\frac{1}{K_w^0} = \frac{1}{K_{dw}^0} \cdot 1 + \frac{Ka}{[H^+]}
\]  
(Eq. 7a)

or

\[
\frac{1}{K_w^0} = \frac{1}{K_{dw}^0} + \frac{Ka}{K_{dw}^0} \cdot \frac{1}{[H^+]}
\]  
(Eq. 7b)
A plot of $1/K_w^O$ versus $1/[H^+]$ gives a straight line with a slope of $Ka/K_{dw}^O$ and intercept equal to $1/K_{dw}^O$. Thus from Equations 6 and 7, the monomer distribution coefficient can be calculated from the values of the observed pH dependent, concentration dependent distribution coefficient, $K_{dw}^O$, obtained over a range of hydrogen ion concentrations.

An equation similar to Equations 6 and 7 has been derived by Garrett and Woods (1953) to determine the monomer distribution coefficient when the preservative ionizes in the aqueous phase.

$$\frac{Ka + [H^+]}{[Df]} = \frac{q K_{dw}^O + 1}{[D]} \cdot [H^+] + \frac{Ka}{[D]}$$  \hspace{1cm} (Eq. 8)

where $q$, is the oil-water ratio; $[D]$, the total concentration of preservative in the oil-water system.

A plot of $\frac{Ka + [H^+]}{[Df]}$ against $[H^+]$ yields a straight line with a slope, $(q K_{dw}^O + 1)/[D]$ and intercept, $Ka/[D]$. The monomer distribution coefficient can thus be calculated from slope and intercept over the range of hydrogen ion concentration considered, as illustrated below:

$$m = \frac{(q K_{dw}^O + 1)}{[D]} = \text{slope} \hspace{1cm} (Eq. 9a)$$

or

$$q K_{dw}^O = m[D] - 1 \hspace{1cm} (Eq. 9b)$$

or

$$K_{dw}^O = \frac{m[D] - 1}{q} \hspace{1cm} (Eq. 9c)$$
\[
c = \frac{K_a}{[D]} = \text{Intercept} \quad \text{(Eq. 10a)}
\]

or

\[
[D] = \frac{K_a}{c} \quad \text{(Eq. 10b)}
\]

Substituting the value of \([D]\) in Eq. 9c

\[
K_{dw}^O = \frac{mK_a}{qc} - 1 \quad \text{(Eq. 11a)}
\]

or

\[
K_{dw}^O = \frac{mK_a - qc}{qc} \quad \text{(Eq. 11b)}
\]

Thus, from Eq. 8, if pH of the aqueous solution and the concentration of preservative in the aqueous phase, \([D_f]\), are known, the monomer distribution coefficient can be calculated. Unlike Equations 6 and 7 there is no need to calculate the observed pH dependent, concentration dependent distribution coefficient.

Alternatively, the monomer distribution coefficient, \(K_{dw}^O\), can be obtained according to Eq. 2 by analysis of the oil phase and aqueous phase at a pH sufficiently low to ensure that the preservative exists completely in the unionized form.

When the preservative is monomeric in the aqueous phase, but associates to one species of \(m\) - mer (that is, the association of more than one preservative molecule) in the oil phase, three equilibrium constants are considered:
where $K'_{Ow}$, the pH independent, concentration dependent distribution coefficient; $K'$, the association equilibrium constant for monomer and $m$ - mer.

Rearranging Eq.13 gives

$$K'_{Ow} = \frac{[HA]_{Ow}}{[HA]_{w}} + \frac{m \cdot [HA]_{om}}{[HA]_{w}} \quad \text{(Eq.14)}$$

or

$$K'_{Ow} = K_{dw}^o + \frac{m \cdot [HA]_{om}}{[HA]_{w}} \quad \text{(Eq.15)}$$

Substituting the value of $[HA]_{om}$ from Eq.12 into Eq.15 gives

$$K'_{Ow} = K_{dw}^o + \frac{m \cdot K' \cdot [HA]_{Oo}^m}{[HA]_{w}} \quad \text{(Eq.16a)}$$

or
\[ K'_{w}^0 = K_{d}^0 + m K' (K_{d}^0)^m [HA]^m_{w} \]  
(Eq.16b)

If \([HA]_{w} = [D']_{f} = \) concentration of unionized preservative in aqueous phase.

\[ K'_{w}^0 = K_{d}^0 + m K' (K_{d}^0)^m [D']^m_{f} \]  
(Eq.17)

The simplest case, i.e., dimerization of preservative in oil phase, corresponds to \(m=2\) and then Eq.17 becomes

\[ K'_{w}^0 = K_{d}^0 + 2K' (K_{d}^0)^2 [D']^2_{f} \]  
(Eq.18)

This linear relationship between \(K'_{w}^0\) and \([D']_{f}\) is the one used by Gross and Schwarz (1930). Its form has since been deduced by other authors (Shikata, 1931; Philbrick, 1934; Moelwyn-Hughes, 1940; Davies and Hallam, 1956). In the case of dimerization, a plot of \(K'_{w}^0\) against \([D']_{f}\) gives a straight line extrapolating to \(K_{d}^0\) at \([D']_{f} = 0\), and of slope \(2K' (K_{d}^0)^2\), from which the association constant, \(K'\), is calculated. In cases other than \(m = 2\) Eq.17 leads to simple curves if \(K'_{w}^0\) is plotted against \([D']_{f}\), showing \(m\) - merization of preservative in the oil phase.
B. Interaction of Preservatives with Nonionic Surfactants

This aspect has been the subject of numerous investigations and has been reviewed elsewhere (Allawala and Riegelmann, 1953; Wedderburn, 1964; Evans and Dunbar, 1965; Elworthy, Florence and Macfarlane, 1968). The following is a brief account of the factors pertinent to an understanding of this problem.

(a) Potentiation of preservative activity

Early observations of the inactivation and inhibition of germicides and preservatives were made in presence of ionic surfactants (Frobisher, 1927; Hampil, 1928; Ordal et al., 1941; Alexander and Tomlison, 1949; Bean and Berry, 1950, 1951). Most of these workers found that low concentrations of surfactants (i.e., below critical micelle concentration) enhanced the effects of germicides, while higher concentrations led to varying degrees of inactivation. Alexander and Trim (1946) studied the effects of ionic surfactants on the penetration of hexylresorcinol into the Ascaris worm. Maximum penetration was found to occur at the critical micelle concentration (CMC) which corresponds to the maximum concentration of monomolecularly dispersed surfactant. Schoog (1957) reported that the activity of hexachlorophene was increased by concentrations of a polyoxyethylene lauryl ether up to $10^{-4}$M, but reduced by higher concentrations of the nonionic surfactant. Brown and Richards (1964) showed that the antibacterial activity of chlorhexidine was enhanced by the presence of 0.02% polysorbate 80 but was reduced by 0.05% solution of the same surfactant. Bradshaw et al. (1972) showed that low concentrations of Tween 80 potentiated the effect of cetylpyridinium chloride against E. coli.
The exact mechanism of potentiation of preservative activity by surfactants, below their CMC, is not well understood. It has been ascribed to the surface-active properties of the surfactant monomers. The surfactant monomers possibly help in the adsorption of preservative molecules at the bacteria/water interface by reducing the interfacial tension.

(b) Inactivation of preservative activity

Since their introduction about 25 years ago, nonionic surfactants have found ever increasing use in the preparation of solubilized and emulsified systems. Despite their many advantages in formulation, they have the serious disadvantage of suppressing or even inactivating the efficacy of added preservatives. Bolle and Mirimanoff (1950) were the first to point out the importance of this phenomenon and showed that the antimicrobial activity of methyl p-hydroxybenzoate was suppressed in the presence of several structurally different nonionic surfactants. They found that the surface-active sorbitan esters and polyoxyethylene sorbitan esters reduced the effect of methyl p-hydroxybenzoate, oxyquinoline sulfate and dioxydichlorodiphenylmethane, whereas the non surface-active Carbowax 1500, a polyethylene glycol polymer, had no inactivating effect. Lawrence and Erlandson (1953) and Erlandson and Lawrence (1953) found that Tweens and certain other nonionics reduced the germicidal effect of a number of phenolic compounds by a factor of 2000-5000 times. Barr and Tice (1957b) found that 5% Tween 20 rendered ineffective a large number of phenolic substances when tested at the concentrations at which they are normally used. Similar inactivations of commonly used preservatives in the presence of a number of nonionic surfactants have been observed by de Navarre and Bailey (1956) and de Navarre (1957). The preservatives studied included benzoic acid, sorbic acid and methyl p-hydroxy-
benzoate. Wedderburn (1958) assessed the effect of thirty-six different nonionics on twenty-six antimicrobial agents and found that although all the surface-active nonionics exerted some depressant effect, those which were not surface-active had negligible effects. The extent of the adverse effect was different for different combinations of nonionic surfactant and preservative, and appeared to be related not only to the hydrophilic-lipophilic balance of the surfactant but also to the chemical structure of the preservative. Blaug and Ahsan (1961b) examined the interaction of the methyl, ethyl, propyl and butyl p-hydroxybenzoates with several nonionic macromolecules and reported that p-hydroxybenzoates of higher molecular weights were influenced to a greater extent than those of lower molecular weight. In determining the binding tendencies of the p-hydroxybenzoates with Tween 80 (polyoxyethylene sorbitan mono-oleate), Myrj 52 (polyoxyethylene monostearate), polyethylene glycol 4000, 6000 and Pluronic F-68 (polyethylene polypropylene glycol), they confirmed that the hydrophilic-lipophilic balance strongly influenced the effect. Those compounds with greater lipophilic tendencies reduced the effectiveness more than the more hydrophilic macromolecules. Richards and Hardie (1972) showed that polysorbate 80 antagonized the activity of bis (2-hydroxy-5 chloro-phenyl) sulfide (fentichlor) against exponential phase cultures of *S. aureus*, *Pr. vulgaris*, and *E. coli*.

The above studies have demonstrated qualitatively the failure of preservatives in the presence of nonionic surfactants. It is evident that the inactivation results from an association of the preservative molecule with the macromolecule. Many quantitative studies have been carried out to determine the nature and extent of the interactions which occur, from both
a physical and microbiological point of view.

In these systems an equilibrium may be postulated (Kostenbauder, 1962; Garrett, 1966) of the form of Eq.19, which for most of the interactions reported has been shown to be reversible.

\[ \text{Preservative} + \text{Macromolecule} \xrightarrow{\text{Eq.19}} \text{Preservative} - \text{Macromolecule} \]

It is generally agreed that the antimicrobial activity of such systems depends mainly on the concentration of unbound or free preservative (Wedderburn, 1964; Mitchell, 1964) rather than the total concentration present. However, Humphreys, Richardson and Rhodes (1968) showed that the antimicrobial activity of a concentration of preservative in aqueous solution was less than the same concentration of preservative in an aqueous phase of a surfactant solution. They concluded that antimicrobial activity is not only a function of the concentration of preservative in the aqueous phase but some additional factors also control the extent of antimicrobial activity.

(c) Mechanism of inactivation of preservatives by nonionic surfactants

An examination of the literature reveals that the mechanism of interaction has been a subject of much debate, and the controversy has centred between two schools of thought. One maintains that the interaction between preservative and surfactant is due to complex formation, while the other believes that interaction is due to partitioning of preservative into surfactant micelles.

Higuchi and Lach (1954) reported the formation of hydrogen bonded complexes between polyethylene glycols and phenols and between
polyethylene glycols and organic acids. Since most nonionic surfactants have polyethylene glycol chains, many authors (Guttman and Higuchi, 1956; Mulley and Metcalf, 1956; de Navarre, 1956, 1957; Barr and Tice, 1957b) have attributed both the solvent properties and the inactivation of preservative to complex formation. Evans (1964) showed that complex formation between surfactant monomer and preservative is unlikely and suggested that inactivation arises from solubilization of preservative within the surfactant micelles. Mulley (1964) collected evidence from a number of sources which indicates that the solubilization of a wide range of solutes in nonionic surfactants can be treated as a solution process within the hydrocarbon-like interior of the micelle. He considered that the data does not support suggestions that solubilization is controlled by more specific factors such as complex formation. Some workers (Evans and Dunbar, 1965; Wedderburn, 1964) suggested that since inactivation occurs with preservatives and surfactants of such diverse chemical structures, solubilization rather than specific complexing is a more probable explanation. Support for these authors is found in the fact that non-surface active macromolecules interact with preservatives to a much lesser extent than surfactant molecules. Kostenbauder (1962) maintains that it is unnecessary to distinguish between the mechanism of complex formation and micellar solubilization and considers that solubilization and micelle formation itself fall within the broad scope of complex formation described by Higuchi and Lach (1954), because both processes obey the law of mass action. Micelles of nonionic surfactants appear to provide ideal conditions for association with preservatives. They afford the possibility for hydrogen bonding and solubilization within the hydrocarbon-like interior of the micelle. It seems likely that under suitable conditions both these mechanisms may operate simultaneously.
(d) Possible sites for the interaction of preservatives in a surfactant micelle

The site of incorporation of solubilizate in a surfactant micelle is believed to be closely related to its chemical nature, as well as the chemical nature of the surfactant. In aqueous solution it is generally accepted that nonpolar solubilizates, e.g., aliphatic hydrocarbons, are dissolved in the hydrocarbon core of the micelle (Fig. 1(a)). Semipolar or polar solubilizates, e.g., fatty acids and alkanols, are taken up in what is often termed the palisade (or oxyethylene) layer of the micelle, oriented with their hydrophobic moieties towards the centre of the micelle and their polar groups in its surface.

Riegelman et al. (1958) studied various aromatic compounds solubilized in aqueous solutions of potassium laurate, dodecyl amine hydrochloride and a polyoxyethylene ether of lauryl alcohol using UV spectroscopy and concluded that ethyl-benzene was incorporated in the hydrocarbon portion of the micelle (a), o-nitroaniline was located at a position of short penetration into the palisade layer (b), azobenzene, naphthalene and anthracene were in a position of deep penetration of palisade layer (c), and dimethyl phthalate was adsorbed on the surface of the micelle (d), (Fig. 1).

Mulley and Metcalf (1956) suggested from their UV spectroscopic studies that chloroxylenol solubilized in the micelles of cetomacrogol formed hydrogen bonds between phenolic hydroxyl groups and oxygen atoms of the polyoxyethylene chains (Fig. 1(e)).
Fig. 1. Possible sites of incorporation of solubilizate in a micelle (Elworthy et al., 1958): (a) in the hydrocarbon core; (b) short penetration of the palisade layer; (c) deep penetration of the palisade layer; (d) adsorption on the surface of the micelle; (e) in the polyoxyethylene shell of the micelle of a nonionic surfactant; (f) at the junction of hydrocarbon core and polyoxyethylene chain of nonionic surfactant. A, micelle of ionic surfactant. B, micelle of nonionic surfactant.
Anderson and Slade (1965) compared the UV spectra of benzoic acid in hexane, water and Myrj 59 solutions. In hexane a typical benzenoid pattern was obtained, whereas the spectra in all surfactant solutions were similar to that in water and showed no fine structure. From these observations the authors concluded that solubilized benzoic acid was located largely in a polar environment, i.e., the oxyethylene layer of the micelle (Fig. 1(e)).

Donbrow and Rhodes (1966) used UV and NMR spectroscopy to examine the location of benzoic acid in cetomacrogol micelle. They favoured the view that the position of the benzoic acid molecules in the micelle was at the junction of the hydrocarbon core and the polyoxyethylene chains (Fig. 1(f)), with the benzene ring enclosed in the former and the carboxylic acid group protruding outwards. Such a position would still allow for hydrogen bonding between carboxyl group and the innermost ether oxygen. Their previous potentiometric studies also supported the same view (Donbrow and Rhodes, 1964). In another study, Donbrow et al. (1967) employed potentiometric and NMR techniques which suggested that m- and p-hydroxybenzoic acids were located at the polyoxyethylene chains of cetomacrogol micelle (Fig. 1(e)).

Jacobs et al. (1971), examining the interaction of phenol with cetomacrogol using NMR spectroscopy, suggested that phenol was located in the polyoxyethylene region of the cetomacrogol micelle.

Corby and Elworthy (1971a,b) identified the sites of solubilization of esters of p-hydroxybenzoic acid in a cetomacrogol micelle using UV, NMR, solubility and viscometric techniques. The sites of solubilization were suggested to be as follows: p-hydroxybenzoic acid was wholly
solubilized deep in the oxyethylene layer of the micelle, ethyl p-hydroxybenzoate was solubilized mostly in the oxyethylene layer situated adjacent to the core, with some solubilization occurring within the core, butyl p-hydroxybenzoate was solubilized mainly at the oxyethylene layer with the butyl chain in the core while some solubilizate was wholly present in the core, methyl p-methoxybenzoate was mostly dissolved in the micellar core, but some was also present in the oxyethylene layer, benzene was solubilized in a similar manner to methyl p-methoxybenzoate.

(e) Effect of preservatives on the micellar molecular weight of surfactants

The effect of solubilize on micellar size has been examined in relatively few systems. In a light-scattering study of solubilization by hexadecytrimethylammonium bromide (Hyde and Robb, 1964) it was shown that the incorporation of increasing amounts of the non-polar molecules, decane, octane and cyclohexane, caused a pronounced increase in the micellar molecular weight (Mµ). This was due to an increase in the number of solubilizate and surfactant molecules in each micelle. However, the solubilization of the polar molecule, octanol, although increasing the Mµ, caused a decrease in the number of surfactant molecules in each micelle.

Nakagawa et al. (1959, 1960) found that the solubilization of decane and decanol by three methoxypolyoxyethylene decyl ethers resulted in increases in the micellar weight of the micelles of these nonionic surfactants. Each weight increase was a consequence of increases in the amount of solubilizate and surfactant per micelle. Viscosity and sedimentation studies of the solubilization of 1,2,4-trichlorobenzene and toluene by cetylpypyridinium chloride (Smith and Alexander, 1957) have indicated an increase in Mµ and in
micellar asymmetry with increase in solubilizate concentration up to a maximum, after which further solubilizate promoted the formation of a more spherical micelle which existed in equilibrium with the rod-like micelles produced initially. In contrast, the solubilization of methylcyclohexane by the same surfactant resulted in only a small regular increase in Mu and viscosity.

In most cases, the differences in the effects of the various solubilizates on the micelle size and shape have been attributed to differences in the location of solubilizate within the micelle. Recently, Attwood, Elworthy and Kayne (1971) examined the solubilization of decane, ethyl p-hydroxybenzoate, methyl anisate and p-hydroxybenzoic acid by aqueous micellar solutions of cetomacrogol using membrane osmometry and viscosity techniques. The effect of solubilizates on the number average micellar molecular weight, Mn, was related to their site of incorporation in the micelle (Fig. 2). Decane and methyl anisate were solubilized in the hydrocarbon core of the micelles and both compounds produced an increase in Mn up to a maximum value of $2.0 \times 10^5$, at a solubilizate concentration of approximately 80% of the saturation limit for each compound. This increase was shown to result from an increase in the number of molecules of both solubilize and of surfactant per micelle. Further addition of solubilizate, to produce a saturation level in excess of 80%, results in a decrease in micellar size in both systems. The solubilization of ethyl p-hydroxybenzoate and p-hydroxybenzoic acid was thought to involve the oxyethylene region of the micelle and both the solubilizates cause an increase in Mn owing to the inclusion of solubilizate into the micelle, the number of molecules of surfactant per micelle being unaffected by the solubilization process.
Figure 2. Variation of micellar molecular weight, $M_n$, with the degree of saturation of cetomacrogol micelle with $\bullet$, decane; $\times$, methyl anisate; $\circ$, p-hydroxybenzoic acid and $\triangle$, ethyl p-hydroxybenzoate (Attwood, Elworthy and Kayne, 1971).
(f) Representation of interaction data

The mathematical treatment of results for the interaction between preservatives and surfactants has been presented in a variety of ways. Since the mechanism of interaction proposed governs the mathematical model used in the calculation, literature on the subject has been controversial. A more detailed review and evaluation of these approaches is given elsewhere (Kazmi and Mitchell, 1971a).

The interaction between preservative and surfactant can be represented by the law of mass action. Consider a simple situation where a macromolecule (M) combines with one molecule of preservative (D) to form a complex (DM), then at equilibrium

\[
D + M \underset{K}{\overset{K}{\rightleftharpoons}} DM
\]  
(Eq.19)

the association constant, K, is defined by the following equation:

\[
K = \frac{[Db]}{[Mf][Df]} \quad \text{(Eq.20a)}
\]

or

\[
[Db] = K [Df][Mf] \quad \text{(Eq.20b)}
\]

where \([Df]\), the concentration of free preservative; \([Db]\), the concentration of preservative bound with macromolecule; \([Mf]\), the concentration of free macomolecule. But

\[
[M] = [Mf] + [Db] \quad \text{(Eq.21a)}
\]

or

Total concentration of macromolecule = Free concentration of macromolecule + Bound concentration of macromolecule
or

\[ [M_f] = [M] - [Db] \]  \hspace{1cm} (Eq.21b)

Substitute the value of \([M_f]\) in Eq.20b and rearrange

\[ [Db] = K [Df] \{ [M] - [Db] \} \]  \hspace{1cm} (Eq.22)

\[ \frac{[Db]}{[M]} = \frac{K [Df]}{1 + K [Df]} \]  \hspace{1cm} (Eq.23)

If the macromolecule has \(n\) independent binding sites and each site has the same intrinsic affinity for preservative and is not influenced by its neighbours, the Eq.23 is multiplied by \('n'\), and

\[ r = \frac{[Db]}{[M]} = \frac{n K [Df]}{1 + K [Df]} \]  \hspace{1cm} (Eq.24)

where \(r\) represents molar ratio, i.e., the number of moles of preservative bound per mole of macromolecule. This ratio indicates the extent of binding.

If all the monomers in a surfactant micelle behave independently and the interaction of preservative with the micelle does not change the micellar molecular weight, then Eq.24 can be utilized to represent the interaction of preservative with surfactant micelles. In this case, \(r\) will give number of moles of preservative bound \([D_b]\), per mole of micelle \([M]\), \(n\), the number of binding sites per micelle, and \(K\), the association constant for interaction with the micelle.

Assume, as a further extension, that two entirely different classes of sites exist in the surfactant molecule and that as before, all sites are independent and sites within the class are equivalent. A similar treatment can be used to derive an expression for 'r' for this case. Thus,
\[
\frac{r}{1+K_1[D_f]} + \frac{n_2K_2[D_f]}{1+K_2[D_f]}
\]

(Eq. 25)

where:

\(n_1\) and \(n_2\) = number of independent binding sites of class I and class II, respectively, on the surfactant molecule.

\(K_1\) and \(K_2\) = intrinsic association constant for the binding of a molecule of preservative to one of the binding sites of class I and class II, respectively, in the surfactant molecule.

In this work, Eq. 25 will be used to characterize interaction of various preservatives with some nonionic surfactants.

A general expression which describes binding to 'm' different classes of sites is:

\[
r = \sum_{i=1}^{m} \frac{n_1K_1[D_f]}{1 + K_1[D_f]}
\]

(Eq. 26)

The total preservative concentration, \([D_t]\), required in a surfactant solution can be calculated by substituting the values of binding parameters (\(n\)'s and \(K\)'s), \([M]\) and \([D_f]\) into Eq. 27, if only one class of binding sites is involved in the interaction, or Eq. 28, if two classes of binding sites are assumed to be involved in the interaction.

\[
[D_t] = [D_f] \left\{ 1 + \frac{nK[M]}{1 + K[D_f]} \right\}
\]

(Eq. 27)

\[
[D_t] = [D_f] \left\{ 1 + \frac{n_1K_1[M]}{1 + K_1[D_f]} + \frac{n_2K_2[M]}{1 + K_2[D_f]} \right\}
\]

(Eq. 28)
C. Interaction of Preservative Mixtures with Nonionic Surfactants

In recent years much emphasis has been given to the use of a combination of preservatives rather than a single preservative for the preservation of cosmetic and pharmaceutical preparations. (Boehm et al., 1957, 1959, 1968, 1970; Gershenfeld, 1963; Garrett, 1966; Casely et al., 1968; Kostenbauder, 1968; Richards, 1971; Richards and Hardie, 1972; Richards and MacBride, 1971, 1973; Boehm and Maddox, 1971; Parker, 1971, 1972, 1973; Proserpio, 1972; Fontana and Proserpio, 1972). The rationales for combinations are that the spectrum of activity can be increased; that the physiologically harmful effects of a dose of one preservative alone giving an equivalent effect may be averted; that the development or modification of the resistance of an organism to one preservative alone may be prevented; that response may exceed prediction from the separate preservative action or from any concentrations of one preservative alone; that convenience of administration of smaller amounts or economic savings may result (Garrett, 1966).

The interaction of preservatives with nonionic surfactants has been studied extensively (Patel and Kostenbauder, 1958; Mitchell and Brown, 1966; Kazmi and Mitchell, 1971a, 1973, and many more). Little information is available concerning the solubilization of preservative mixtures by nonionic surfactants. Recently, Crook and Brown (1973) reported the interaction of several preservative combinations with cetomacrogol using a solubility technique. They found that the solubility of a given preservative in cetomacrogol solutions was altered by the addition of another preservative.

Since preservative mixtures are often employed in cosmetic and...
pharmaceutical preparations containing nonionic surfactants, the present study was undertaken to examine the binding behavior of preservative mixtures with a nonionic surfactant, cetomacrogol. The binding was studied in less than saturated conditions because the effective concentration of most preservatives in the aqueous phase of a surfactant solution lies below the saturation solubility in water.

D. Theory of Competitive Binding of Drugs with Macromolecules

The competitive binding of drugs with proteins (Meyer and Guttman, 1968) and enzymes (Brand et al., 1967) occurs when two or more drugs compete for the same locus on the macromolecule. Mathematical methods used to express the displacement of one drug by another drug from protein binding sites are well established (Klotz et al., 1948; Karush, 1950; Cogin and Davis, 1951; Meyer and Guttman, 1970). In this investigation, an attempt has been made to test these mathematical methods for the binding of preservative mixtures with the nonionic surfactant cetomacrogol. A fit of the binding data to these mathematical models will enable a prediction to be made of the total concentration of preservative mixture required in a surfactant solution to provide the desired concentration of each preservative free in the aqueous phase.

The interaction of a preservative (D) with surfactant (M) can be characterized by Eq. 25, which can be rewritten in the form of Eq. 29:

\[ r_d = \frac{n_1 K_{d1} [D_f]}{1 + K_{d1} [D_f]} + \frac{n_2 K_{d2} [D_f]}{1 + K_{d2} [D_f]} \]  

(Eq. 29)

In the presence of another preservative (C), the competitor, Eq. 29, can be
written:

\[ r_d = \frac{n_1 K_{d1} [D_f]}{1 + K_{d1} [D_f] + K_{C1} [C_f]} + \frac{n_2 K_{d2} [D_f]}{1 + K_{d2} [D_f] + K_{C2} [C_f]} \]  

(Eq. 30)

where \([C_f]\) is the concentration of free competitor, \(K_{C1}\) and \(K_{C2}\) are the intrinsic association constants for the binding of a molecule of competitor to one of the binding sites of class I and class II, respectively, in the surfactant molecule.

If \(n_1\) and \(n_2\) are equivalent and independent, then:

\[ r_d = \frac{n_1 K'_{d1} [D_f]}{1 + K'_{d1} [D_f]} + \frac{n_2 K'_{d2} [D_f]}{1 + K'_{d2} [D_f]} \]  

(Eq. 31)

where \(K'_{d1}\) and \(K'_{d2}\) are given by Eq.s 32a and 32b respectively:

\[ K'_{d1} = \frac{K_{d1}}{1 + K_{C1} [C_f]} \]  

(Eq. 32a)

\[ K'_{d2} = \frac{K_{d2}}{1 + K_{C2} [C_f]} \]  

(Eq. 32b)

Equation 31 implies that in the presence of a constant concentration of \(C\), the binding of \(D\) to \(M\) follows the same pattern as that indicated in Eq. 29, except that \(K'_{d1}\) and \(K'_{d2}\) values will be lower than that observed for \(K_{d1}\) and \(K_{d2}\), and are a function of \(C\). If \(K_{d1}\) and \(K_{d2}\) are known, and \(K'_{d1}\) and \(K'_{d2}\) are determined for a known value of \(C\), then \(K_{C1}\) and \(K_{C2}\) can be calculated from Eq.s 33a and 33b respectively:

\[ K_{C1} = \frac{1}{[C_f]} \left( \frac{K_{d1}}{K'_{d1}} - 1 \right) \]  

(Eq. 33a)
The above equations are derived in terms of \( r_d \), but analogous equations can be derived in terms of \( r_C \), the moles of species C bound per mole of surfactant.

E. Interaction of a Preservative with Mixtures of Nonionic Surfactants

The use of more than one nonionic surfactant is common in solubilized and emulsified systems. Surfactant mixtures offer many advantages over individual surfactants, e.g., adjustment to a desired HLB value (Griffin, 1949), improvement of emulsion stability (Boyed, Parkinson and Sherman, 1972), and attainment of desired rheological properties (Barry, 1969; Barry and Saunders, 1972; Eccleston, Barry and Davis, 1973).

While some studies have been made of the solubilization behavior of mixtures of anionic and nonionic surfactants (Fukuda and Taniyama, 1958; Saito and Hirata, 1959; Narasaki, 1961; Narasaki and Suzuki, 1962; Hall and Soudah, 1966), little information is available regarding the interaction of solutes with mixtures of nonionic surfactants. Hall (1963) studied the effect of polysorbate 20 on the solubilizing power of polysorbate 80 and demonstrated that polysorbate 20 decreased the "critical miscibility ratio" of salicylic acid in a linear fashion.

In the present investigation, the interaction of chlorocresol with certain nonionic surfactants and their mixtures is reported. Binding parameters which characterize the interaction of a preservative with each individual surfactant have been used to predict the binding behavior of surfactant mixtures.
F. Representation of Interaction Data for the Binding of a Preservative with Mixtures of Nonionic Surfactants

For the interaction of a preservative with a surfactant, \([D_t]\) for a given \([D_f]\) can be calculated using Eq.27.

If there are several types of surfactants of concentrations \(M_1, M_2, M_3 \ldots M_m\) with number of binding sites per molecule \(n_1, n_2, n_3 \ldots n_m\) and with intrinsic association constants \(K_1, K_2, K_3 \ldots K_m\), it follows by reasoning similar to that for Eq.27 that:

\[
[D_t] = [D_f] \left\{ \frac{n_1K_1 [M_1]}{1 + K_1 [D_f]} + \frac{n_2K_2 [M_2]}{1 + K_2 [D_f]} + \cdots + \frac{n_mK_m [M_m]}{1 + K_m [D_f]} \right\}
\]  
(Eq.34)

or

\[
[D_t] = [D_f] \left\{ 1 + \sum_{i=1}^{m} \frac{n_iK_i [M_i]}{1 + K_i [D_f]} \right\}
\]  
(Eq.35)

Similarly for a mixture of surfactants, each with two classes of binding sites:

\[
[D_t] = [D_f] \left\{ 1 + \sum_{i=1}^{m} \frac{n_{i1}K_{i1} [M_i]}{1 + K_{i1} [D_f]} + \frac{n_{i2}K_{i2} [M_i]}{1 + K_{i2} [D_f]} \right\}
\]  
(Eq.36)

For a mixture of two types of surfactants, the Eq.36 can be written:

\[
[D_t] = [D_f] \left\{ 1 + \frac{n_1K_1 [M_1]}{1 + K_2 [D_f]} + \frac{n_2K_2 [M_1]}{1 + K_2' [D_f]} + \frac{n_1K_1' [M_{II}]}{1 + K_1' [D_f]} + \frac{n_2K_2' [M_{II}]}{1 + K_2' [D_f]} \right\}
\]  
(Eq.37)
where:

\[ M_1 = \text{concentration of surfactant of type I.} \]
\[ M_{II} = \text{concentration of surfactant of type II.} \]
\[ n_1^1 = \text{number of independent binding sites of class I on the surfactant molecule of type I and II respectively.} \]
\[ n_2^1 = \text{number of independent binding sites of class II in the surfactant molecule of type I and II respectively.} \]
\[ K_1 = \text{intrinsic association constants for the binding of a molecule of preservative to one of the binding sites of class I in surfactant molecules of type I and II respectively.} \]
\[ K_2 = \text{intrinsic association constants for the binding of a molecule of preservative to one of the binding sites of class II in surfactant molecules of type I and II respectively.} \]

In the present work, Eq.37 has been used to characterize the interaction of a preservative with a mixture of two types of surfactants. The values of binding parameters \((n's \text{ and } K's)\) for the substitution of Eq.37 were estimated using Eq.25, which describes the binding of a preservative with a surfactant, assuming that two classes of binding sites are involved in the interaction.

G. Distribution and Antimicrobial Activity of Preservatives in Emulsified Systems

(a) Introduction

Factors affecting the antimicrobial activity of preservatives in emulsified systems are much more complex than simple aqueous systems. Some notable reviews on the subject have appeared in the literature (Tice and
Barr, 1958; Kostenbauder, 1962; Bennett, 1962; de Navarre, 1962; Wedderburn, 1964). Aoki et al. (1957) studied various factors important in the preservation of hydrophilic ointment (U.S.P. XV; J.P. VI) and similar emulsions by p-hydroxybenzoates. They found that the amount of ester required was dependent not only on the nature of the ester but also on the nature of components in the oil and water phases. Matsumoto and Aoki (1962) later found that the degree of inactivation of p-hydroxybenzoates was dependent upon the relationship between the properties of the ester, the surfactant and the oily substance to be incorporated in the emulsion. Oily substances, such as isopropyl myristate, olive oil, lauryl alcohol, xylene and dibutyl phthalate, considerably altered the activity of the esters. Propyl p-hydroxybenzoate was found to be more subject to inactivation than the methyl ester. It was suggested that the propyl p-hydroxybenzoate was inactivated by ready solubilization into the lipophilic part of the micelle. Kostenbauder (1962) derived an equation which involved the use of the oil-water partition coefficient and preservative-macromolecule binding data (see Eq. 44). The equation permitted a calculation to be made of the amount of preservative required in an emulsion to provide a concentration of preservative in the aqueous phase sufficient to inhibit microbial growth. Garrett (1966) subsequently developed a more comprehensive mathematical model, which quantified all the pertinent factors responsible for the inactivation of preservatives in heterogeneous systems. Anderson and Cho (1967) reported on the distribution and activity of benzoic acid in oil-water systems emulsified with 0.1% polyoxyethylene lauryl ether (Brij 35). The antifungal activity of benzoic acid was related to its concentration in the aqueous phase. Bean, Konning and Malcolm (1969) found close agreement between
predictions made using an equation similar to that of Kostenbauder (1962) and the results obtained from microbiological studies. They concluded that the activity of preservatives in emulsified systems was related to the concentration free in the aqueous phase. Patel and Romanowski (1970) also verified Kostenbauder's equation (1962) by an in-vitro microbiological procedure. They showed that the fungistatic activity of methyl and propyl p-hydroxybenzoates in emulsified systems was primarily a function of the free paraben concentration in the aqueous phase. Marszall (1972) reviewed various mathematical models used for predicting the required preservative concentration in emulsified systems.

(b) Calculation of total preservative concentration required in an emulsion

When a preservative is added to a simple emulsion consisting of oil, water and surfactant phases, part of it is partitioned into the oil phase and part is complexed or solubilized within the surfactant micelles. The rest of the preservative remains in the aqueous phase.

Preservative partitioned into the oil phase, or bound to the surfactant micelles, has little antimicrobial activity and the antimicrobial activity is said to depend mainly on the concentration of preservative in the aqueous phase (Anderson and Cho, 1967; Bean, Konning and Malcolm, 1969; Patel and Romanowski, 1970).

Thus from the physico-chemical parameters governing the distribution of preservatives in oil-water systems and the binding of preservative with surfactant micelles it should be possible to calculate the total concentration of preservative required in an emulsion to provide a concentration of preservative in the aqueous phase sufficient to inhibit
microbial growth.

The total amount of preservative in an emulsion, \( W \), is given by

\[
W = [Dt] Vw + [Do] Vo \quad \text{(Eq.38)}
\]

where \([Dt]\) is the total concentration of preservative in aqueous phase, \([Do]\) is the concentration of preservative in oil phase, \(Vw\) is the volume of the aqueous phase and \(Vo\) is the volume of the oil phase. But

\[
W = [D] (Vo + Vw)
\]

and

\[
[ Dt ] = [Df] + [Db]
\]

where \([D]\) is the total concentration of preservative in the emulsion, \([Df]\) is the concentration of free preservative in the aqueous phase and \([Db]\) is the concentration of bound preservative in the aqueous phase.

Substitute the values of \(W\) and \([Dt]\) in Eq.38 and rearrange

\[
[D] (Vo + Vw) = \left[ [Df] + [Db] \right] Vw + [Do] Vo \quad \text{(Eq.39)}
\]

\[
[D] = \left\{ \left[ [Df] + [Db] \right] Vw + [Do] Vo \right\} / (Vo + Vw) \quad \text{(Eq.40)}
\]

But

\[
[Db] = n K [M] [Df] / (1 + K [Df]) \quad \text{(Eq.24)}
\]

and

\[
[Do] = K^o_w [Df] \quad \text{(Eq.5)}
\]

\[
q = \frac{Vo}{Vw} = \text{oil:water ratio}
\]

\[
Vo = q Vw
\]

Substitute the values of \([Db]\), \([Do]\) and \(Vo\) in Eq.40 and rearrange

\[
[D] = \left\{ \left[ [Df] + n K [M] [Df] / (1 + K [Df]) \right] Vw + K^o_w [Df] q Vw \right\} / (q Vw + Vw) \quad \text{(Eq.41)}
\]
or
\[
[D] = \left\{ [D_f] \left[ 1 + n K \frac{[M]}{(1 + K [D_f]) + K_w^0 q} \right] \right\} / (q + 1)
\]
(Eq.42)

For an acid preservative:
\[
[D_f] = [D_f'] (1 + Ka/[H^+])
\]

where \([D_f']\) is the concentration of unionized preservative in the aqueous phase, \(Ka\) is the ionization constant, \([H^+]\) is the hydrogen ion concentration.

and
\[
K_w^0 = \frac{[D_o]}{[D_f]} = \frac{[D_o]/[D_f'] (1 + Ka/[H^+])]}
\]
\[
K_w^0 (1 + Ka/[H^+]) = \frac{[D_o]/[D_f']} = K_w^{0'}
\]

where \(K_w^{0'}\) is the pH independent, concentration dependent distribution coefficient.

Substituting the values of \(K_w^0\) and \([D_f]\) in Eq.42 gives:
\[
[D] = \left\{ [D_f'] (1 + Ka/[H^+]) \left[ 1 + n K \frac{[M]}{(1 + K [D_f])}
\]
\]
\[
(1 + Ka/[H^+])) + K_w^{0'} q/(1 + Ka/[H^+]) \right\} / (q + 1) \) (Eq.43)

Equations similar to 42 and 43 have been derived by other authors:

(i) Kostenbauder (1962); Patel and Romanowski (1970).
\[
W = [D_f] \left\{ R V_w + K_w^0 V_o \right\}
\]
(Eq.44)

and for acid preservative,
\[
W = [D_f] \left\{ \left[ R + Ka/[H^+] \right] V_w + K_w^0 V_o \right\}
\]
(Eq.45)

\[
[D] = [D_f'] . f1 . f2 . f3 = [D_f'] \left\{ 1 + \sum_{i=1}^{m} n_i [M_i] /
\]
\[
\left[ K_i + [D_f'] (1 + Ka/[H^+]) + K_w^0 q \right] \right\} \left\{ 1 + Ka/[H^+] + K_w^0 q \right\}
\]
\[
\left\{ e^{k't} \right\}
\]
(Eq.46)
where \( f_1 \) is the binding enhancement factor, \( f_2 \) is the oil-water distribution and ionization enhancement factor, \( f_3 \) is the instability enhancement factor, \( K_r \) is the intrinsic dissociation constant for preservative-surfactant complex, \( k' \) is the 1st order rate constant for the decomposition of preservative and \( t \) is the time required for decomposition.


\[
[D] = [D_f] \left\{ R + K_w^o q \right\} / (q + 1) \quad (Eq.47)
\]

Basically, all these equations are similar except for the binding parameters used. In the equation of Kostenbauder (1962), Bean, Konning and Malcolm (1969) and Patel and Romanowski (1970), the term \( 1 + n K [M] / (1 + K[D_f]) \) of Eq.42 is replaced by \( R \), where \( R = 1 + n K [M] \), i.e., for a given macromolecule concentration the \( R \) value, or binding or solubilization constant, was assumed to be independent of \([D_f]\). As has been described elsewhere (Kazmi and Mitchell, 1971a), this is true only in special cases (see page 167). In Garrett's equation (1966) the term \( 1 + n' K [M] / (1 + K [D_f]) \) is replaced by \( 1 + n [M] / (K' + [D_f]) \) where \( K' \) is the intrinsic dissociation constant and is equal to \( 1/K \). Garrett (1966) also takes into account an instability factor where the preservative degrades by 1st order kinetics. This correction is seldom necessary because most of the commonly used preservatives are stable under the conditions of use.

For an emulsion containing a surfactant with two classes of binding sites, Eq.42 and 43 can be written:

\[
[D] = \left\{ [D_f] \left[ 1 + n_1 K_1 [M] / (1 + K_1 [D_f]) + n_2 K_2 [M] / (1 + K_2 [D_f]) \right. \right. \\
\left. \left. + K_w^o q \right\} \right\} / (q + 1) , \quad (Eq.48)
\]
\[
[D] = \left\{\frac{[D_f^1]}{(1 + \frac{K_a}{[H^+]})} \left[1 + n_1 K_1 [M]/(1 + K_1 [D_f^1] (1 + \frac{K_a}{[H^+]})
+ n_2 K_2 [M]/(1 + K_2 [D_f^1] (1 + \frac{K_a}{[H^+]}) + K_w^o q/(1 + \frac{K_a}{[H^+]})\right]}{(q + 1)} \right\}
\]

(Eq. 49)

H. Methodology

(a) Distribution of preservatives in oil-water systems

Methods for the determination of the oil-water partition coefficient of preservatives and drugs can be divided into two categories, (I) the shake-out method, resulting in the formation of oil-water emulsions and (II) methods where emulsion formation is avoided.

(I) - The shake-out methods: In these methods (Garrett and Woods, 1953; Hibbott and Monks, 1961; Bean and Heman-Ackah, 1964; Anderson and Cho, 1967; Bean, Konning and Malcolm, 1969) the oil-water mixture with preservative or drug is shaken at constant temperature until equilibrium is attained. The mixture is allowed to stand and the oil-water phases are separated and analyzed for preservative. An inherent problem associated with this method is that of emulsification or sometimes dispersion of fine droplets of one phase into the other phase. As a result complete separation of the two phases becomes very difficult. Allen and McDowell (1960) reported that shake-out methods can also result in anomalous equilibria. In spite of these drawbacks, these methods are popular because of convenience and compared with non-shake-out methods less time is required for equilibrium to be attained. Where emulsification is a problem, separation of the oil-water phases can be achieved by ultracentrifugation (Garrett, 1962).
(II) - Other methods: To avoid emulsification, other methods have been devised for the determination of the oil-water partition coefficient of preservatives and drugs. Patel and Romanowski (1969) determined partition coefficients using a two-chambered dialysis technique, with oil in one compartment and water in the other. The two compartments were separated by a semipermeable membrane, permeable only to preservative molecules. They found close agreement between partition coefficients determined by the dialysis technique and those determined by shake-out method. Reese et al. (1964) and Doluisio and Swintosky (1964) developed a simple rocking apparatus for routine determination of distribution coefficients. With this apparatus, up to 36 two-phase samples in cylindrical tubes were equilibrated by rocking the horizontal tubes at one cycle per minute through an arc of 45°. This rocking causes the interface between the two immiscible phases to expand and contract slowly. It also causes the shape of each phase to vary constantly. These two actions facilitate uniform distribution of solute within each phase and facilitate drug transfer from one phase to the other. Emulsion formation is negligible since little turbulence is created. The authors found good agreement between results obtained by this method and results from shake-out methods. Distribution coefficients of some drugs sparingly soluble in the aqueous phase has also been determined by paper chromatography (Bowen, James and Roberts, 1970).

(b) Interaction of preservatives with nonionic surfactants

Various methods used for assessing preservative-surfactant interaction have been reviewed elsewhere (Wedderburn, 1964; Parker and Barnes, 1967; Elworthy, Florence and Macfarlane, 1968). These methods
fall into two groups: (I) physico-chemical methods and (II) biological methods.

(I) **Physico-chemical methods:**

These methods can be divided into two categories. The first group depends upon the properties of the interacting molecule; the second, on the behavior of the macromolecule. Methods depending on the properties of the interacting molecule include solubility analysis (Patel and Kostenbauder, 1958; Blaug and Ahsan, 1961; Goodhart and Martin, 1962; Lundi and Held, 1966; Mitchell and Brown, 1966; Humphreys and Rhodes, 1968; Kazmi and Mitchell, 1971, and others), equilibrium dialysis (Patel and Kostenbauder, 1958; Patel and Foss, 1964, 1965; Breuninger and Goettsch, 1965; Mitchell and Brown, 1966; Anderson and Morgan, 1966; Kazmi and Mitchell, 1971, 1973, and many others), dynamic dialysis (Ikeda et al., 1971; Crook and Brown, 1973), turbidimetric titration (a. visual: Higuchi and Lach, 1954; Guttman and Higuchi, 1956. b. photometric: Kabadi and Hammarlund, 1966), potentiometric titration (Donbrow and Rhodes, 1963a, 1964, 1965; Evans, 1964, 1966; Donbrow and Jacobs, 1966), pH measurement (Mitchell and Brown, 1966), molecular sieve technique (Ashworth and Heard, 1966; Donbrow, Azaz and Hamburger, 1970), etc. Methods depending on the behavior of the macromolecule include differential interference refractometry (Choulis, 1970; Choulis and Rhodes, 1970), surface tension (Horin and Arai, 1970), viscometry (Horin and Arai, 1970), density measurements (Harkins et al., 1946), X-ray diffraction (Harkins et al., 1946). Ultraviolet spectroscopy (Riegelman et al., 1958; Anderson and Slade, 1965; Donbrow and Rhodes, 1966; Kabadi and Hammarlund, 1966; Corby and Elworthy, 1971a,b; Choulis,
1973) and NMR spectroscopy (Donbrow and Rhodes, 1966, 1967; Jacobs et al., 1971; Corby and Elworthy, 1971a,b) have also been used to study preservative-surfactant interaction by comparing the spectrum of preservative in the presence of surfactant. Equilibrium dialysis and equilibrium ultrafiltration techniques were used for studying preservative-surfactant interaction in the present work. Therefore, these two techniques will be discussed in more detail.

(i) **Equilibrium dialysis**: Interactions between preservatives and surfactants may be studied quantitatively using the equilibrium dialysis technique. A container is divided into two compartments by a semipermeable membrane. A surfactant solution with preservative is placed in one compartment and aqueous solution with preservative is placed in the other compartment. The semipermeable membrane should be permeable only to preservative molecules but not to surfactant monomer. At equilibrium, the total number of preservative molecules in the surfactant compartment will exceed that in the aqueous compartment. The difference between the concentrations in the two compartments is a measure of [Db]. Two possible sources of error, namely the Donnan effect and membrane binding of preservatives, must be considered before applying this technique.

When a charged macromolecule, [M], is retained in one of the two compartments, at equilibrium, the concentration of diffusible ions is no longer identical across the membrane. This phenomenon is described as the Donnan equilibrium (Overbeek, 1956). In dilute solution of macromolecule, the Donnan effect can be neglected only if the concentration of the diffusible ions is reasonably high and the valency of the macromolecule is fairly low. In a solvent system of high ionic strength and pH at which the macromolecule
has a small valency charge, the abnormal distribution of small molecules across the membrane due to Donnan equilibrium can be neglected.

The dialysis membrane may act as a binding site for the preservative molecule (Patel and Kostenbauder, 1958; Kazmi, 1971) and a correction must be made for this interaction. Corrections are generally made by using a control in which no macromolecule is present. It is then possible to measure the "loss" of small molecule from one compartment to the other across the semipermeable membrane. It has been observed that the extent of membrane binding is proportional to the amount of preservative added to the system (Patel and Kostenbauder, 1958; Schoenwald and Belcastro, 1969; Patel and Nagabhushan, 1970; Kazmi, 1971).

The main advantage of this method is that an interaction can be studied through a range of free preservative concentration \([D_f]\) and in this way it is possible to cover a wide range of "r" values (see Eq.24). Thus, more information about the interaction can be obtained than with the commonly used solubility method. The solubility method is a "one point method" in which \([D_f]\) is constant for all surfactant concentrations, and therefore only one value of "r" is obtained (see Eq.24).

Techniques employed to carry out equilibrium dialysis have varied from a dialysis bag placed in a bottle (Deluca and Kostenbauder, 1960) to two-chambered dialysis cells with a semipermeable membrane separating the two compartments (Patel and Foss, 1964). The main advantage of cells over a bag is that a better control of membrane binding is attained because the surface area of the membrane remains more or less constant throughout the study.
Various types of dialysis membranes are described by Craig (1965). Kostenbauder et al. (1969) discussed the use of nylon membrane in dialysis studies. Nylon membrane reacts with phenolic compounds (Patel and Kostenbauder, 1958; Patel and Foss, 1964). Hence, rubber membranes have been used in the study of the interaction of phenolic preservatives with surfactants (Patel and Foss, 1964; Mitchell and Brown, 1966; Patel, 1967).

Cellophane membranes are widely used in equilibrium (Chakravarty and Lach, 1959; Breuninger and Goettsch, 1965; Kabadi and Hammarlund, 1966; Matsumoto et al., 1966; Patel, 1967; Patel and Romanowski, 1970; Ikeda et al., 1971) and dynamic (Matsumoto et al., 1966; Matsumoto, 1966; Ikeda et al., 1971) dialysis studies involving the interaction of preservatives and drugs with nonionic surfactants. Cellophane has also been used to study the effects of surfactants on the diffusion of drugs across membranes (Matsumoto et al., 1966; Matsumoto, 1966; Short et al., 1970; Short and Rhodes, 1972; Withington and Collet, 1972; Collet and Withington, 1973). Ideally, such investigations require that the membrane be impermeable to the nonionic surfactant while allowing diffusion of the drug and that the osmotic differential across the membrane is negligible.

There is some controversy concerning the permeability of cellophane dialysis membranes to nonionic surfactants. Patel and Kostenbauder (1958) reported that Visking cellophane membrane was permeable to polysorbate 80, and they considered this membrane unsatisfactory for equilibrium dialysis work. This observation was supported by Nishida et al. (1964). Breuninger and Goettsch (1965) found that, although Visking cellophane membrane was permeable to polysorbate 80, Fisher cellophane membrane was
impermeable to the same surfactant. Matsumoto et al. (1966) studied the permeability of polysorbate 80 and a polyoxyethylene lauryl ether through Visking cellophane membrane, using dynamic dialysis (without stirring the solutions) under sink and nonsink conditions. They found that Visking cellophane membrane was practically impermeable to the nonionic surfactants and that only impurities such as low molecular weight polyethylene glycols, passed through it.

Patel (1967) compared Fisher cellophane membrane with nylon and rubber membranes in equilibrium dialysis studies, involving the interaction of several preservatives with cetomacrogol and polysorbate 80. Since close agreement was found between data obtained using Fisher cellophane membrane and those obtained using the rubber or nylon membranes, it was assumed that the cellophane membrane was impermeable to cetomacrogol and polysorbate 80. Ikeda et al. (1971) used Visking cellophane membrane in equilibrium and dynamic dialysis techniques to study the interaction of barbiturates with a polyoxyethylene ether surfactant. Although this surfactant permeated through the cellophane membrane, the amount passed during 48 hours was below the CMC of the surfactant.

Short et al. (1970) reported that Visking cellophane membrane was impermeable to Texofor A30, 45 and 60 and that only small traces of nonsurface-active impurities passed through it.

Although the permeability of cellophane to polyethylene glycols has been studied less extensively than the nonionic surfactants, it has been shown that cellophane is permeable to polyethylene glycols with a molecular weight less than 20,000 (Shaffer et al., 1950; Kabadi and Hammarlund, 1966).
Polyethylene glycols permeate cellophane membranes more readily than nonionic surfactants of similar molecular weight.

Rubber (Mitchell and Brown, 1966) and nylon (Kazmi and Mitchell, 1970) membranes were used in equilibrium dialysis studies of the interaction of various preservatives with the nonionic surfactant cetomacrogol, since qualitative tests showed that cellophane is permeable to the nonionic surfactant and that volume changes occurred as a result of osmosis. Apart from Matsumoto et al. (1966), few workers using cellophane appear to have corrected for the volume changes. In view of the continued use of cellophane, it seemed desirable as part of this work to make quantitative measurements of the permeability of cellophane membranes to cetomacrogol and to assess the effects of surfactant permeation and osmosis on the binding constants for the interaction between the surfactant and a preservative determined using the equilibrium dialysis technique.

(ii) Diafiltration (or Equilibrium Ultrafiltration): Various methods have been used to study interaction between preservatives and nonionic surfactants. Most of these methods are slow and require numerous separate experiments to fully characterize the interaction. Thus, there was still need of a simple, rapid and reliable means of evaluating binding parameters. The diafiltration technique appeared to answer these needs. Therefore, an attempt was made to evaluate diafiltration technique for studying preservative – surfactant interaction. In the present investigation, a comparison has been made between the binding parameters obtained using diafiltration and equilibrium dialysis techniques.

Diafiltration is a relatively new technique which has been used to study drug-protein interactions (Blatt, Robinson and Bixler, 1968; Farese,

The experimental procedure can be best described with reference to Fig. 3. Solution of drug in water or buffer in the reservoir is forced under pressure into the ultrafiltration cell containing the solution of macromolecule. The macromolecule-free ultrafiltrate passes through the membrane and is directed to a fraction collector. Ultrafiltrate is collected until the effluent drug is in equilibrium with the free drug in the cell which is equal to the feed concentration. Drug bound to macromolecule is in equilibrium with the free drug in the cell which is equal to the concentration in the ultrafiltrate. Hence, analysis of the drug concentration in the ultrafiltrate permits calculation of the fraction of drug bound to macromolecule from zero free drug concentration to that of the feed solutions. The results for bound and free drug can be treated in a number of ways to obtain values for the association constant, K, and number of binding sites, n (Blatt, Robinson and Bixler, 1968; Ryan and Hanna, 1971).

The diafiltration technique appears to offer several advantages over commonly used techniques such as equilibrium dialysis and gel filtration etc:

1. This technique enables a complete binding curve to be determined from a single experiment lasting a few hours.

2. This technique lends itself to complete automation and computer analysis of data.
Figure 3. Equilibrium Ultrafiltration Apparatus
A. C/D Selector
B. Reservoir containing feed solution
C. Ultrafiltration cell
D. Stirring bar
E. Magnetic stirrer
F. Membrane
G. Fraction collector
H. Temperature controlled water-baths
3. The binding parameters describing the interaction are readily determined from the binding curve.

II Biological methods

As discussed earlier, physico-chemical methods have been used extensively for studying preservative-surfactant interactions because these methods are less time consuming, quantitative in nature, and give information regarding the mechanism of interaction. However, these methods do not take into account the various biological variables affecting microbial growth. Hence, biological methods are often employed for evaluating preservative efficacy in a system. However, compared with physico-chemical methods, these methods are laborious, time consuming, often qualitative in nature, and give no information about the mechanism of inactivation. A number of authors (Allawala and Riegelman, 1953; Pisano and Kostenbauder, 1959; Blaug and Ahsan, 1961a; Mitchell, 1964; Evans and Dunbar, 1965; Anderson and Morgan, 1966; Patel, 1967; Humphrey, Richardson and Rhodes, 1968; Henderson and Newton, 1969; Bradshaw, Rhodes and Richardson, 1972) have attempted to correlate the data obtained from physico-chemical studies with the biological activity of such systems. Investigations of this type are important because they give information about both physico-chemical as well as biological factors and should save a tremendous amount of time by avoiding much 'trial and error' formulation which is common where purely biological methods are employed for evaluating preservative efficacy.

Biological activities can be evaluated either by measuring the absolute antimicrobial activity of each system, or some biological response which parallels the preservative action on microbial cells. A brief
discussion of the various methods used for evaluating the antimicrobial activity of preservatives in surfactant systems follows.

(i) **Basic microbiological method:** The simplest microbiological technique for demonstrating the antagonism between a preservative and a surfactant is to compare the growth of test organisms in a suitable growth promoting media containing preservative in the presence and absence of surfactant. The criterion chosen to assess microbial proliferation varies from various qualitative tests, such as visual growth (Wedderburn, 1958), to quantitative methods, such as weighing of mycelium (Bolle and Mirimanoff, 1950; de Navarre and Bailey, 1956; de Navarre, 1957).

Bolle and Mirimanoff (1950) investigated interactions between seven nonionic surfactants and three preservatives using *Penicillium spp.* and *Aspergillus niger* as test organisms. Into 50 ml flasks containing 10 ml of Jaag medium was added either (a) a standard suspension of spores, (b) spores and preservative in progressive dilution, (c) spores and surfactant, or (d) a mixture of spores, preservative and surfactant. The clumps of mycelium were weighed after 10 days of incubation at 28°. Similar methods have been used by other workers (de Navarre and Bailey, 1956; de Navarre, 1957; Barr and Tice, 1957; Wedderburn, 1958).

(ii) **Minimum inhibitory concentration tests:** The effect of a surfactant on the bacteriostatic activity of a preservative can be evaluated by determining the minimum inhibitory concentration (MIC) values against a range of organisms in the presence and absence of a surfactant. Essentially a MIC experiment involves setting up a series of plates or tubes containing culture medium, the test organism and increasing concentrations of the preservative.
The MIC is the least concentration of the preservative found to inhibit growth.

Beckett et al. (1959) demonstrated inhibition of bacteriostatic activity of hexylresorcinol against *E. coli* by cetomacrogol, using a MIC test. Pisano and Kostenbauder (1959) obtained MIC values of methyl paraben against *Aerobacter aerogenes* and *Aspergillus niger* in the presence of polysorbate 80. The increased amount of paraben required was compared with the concentration predicted by equilibrium dialysis studies. Similar studies have been carried out by other authors using fungi (Blaug and Ahsan, 1961a; Matsumoto and Aoki, 1962; Patel, 1967), and bacteria (Blaug and Ahsan, 1961a; Evans and Dunbar, 1965) as test organisms.

Since this technique involves a microbiostatic end point it is not suitable for determining concentration required to produce sterility. Further limitations of the use of a MIC are discussed on page 174.

(iii) *Inhibition zone techniques*: Inhibition zone techniques such as the cup-plate, cylinder-plate, paper disc-plate, ditch-plate and wheel-plate methods rely on the diffusion of preservative from a central reservoir into the surrounding medium. Test organisms surrounding the reservoir will be inhibited, resulting in a zone of no growth around the reservoir. By comparing zones produced in the presence and absence of macromolecule in the reservoir, information may be obtained concerning the effect of the macromolecule on diffusion properties of preservative.

Agar diffusion tests are quite unsatisfactory for evaluating the degree of interaction between preservatives and surfactants. Diffusion characteristic of the preservative in agar is not only affected by
preservative-surfactant interaction, but other factors may also be involved. Several authors (Sherwood, 1942; Quisno et al., 1946; Poter and Nisonger, 1950) have demonstrated that agar reduces the germicidal potency of quaternary ammonium compounds (quats) due to physical adsorption. Thus interaction of the preservative with agar will result in a reduced zone of inhibition, and hence results derived from the measurements of the zones will provide a false information regarding preservative-surfactant binding. It is interesting to note that the few authors whose conclusions on the effects of nonionic surfactants on preservatives differ from the generally accepted views on this topic used these kinds of techniques. Schwarz and Levy (1957) used the agar cup-plate technique in a study of the compatibility of certain preservatives, including quats with carbopol 934. They noted no marked decrease in the activity of any of the preservatives, since inactivation of cationic preservatives by the anionic carbopol had been expected. Agar plate diffusion techniques have given results suggesting that surface-active agents enhance the antibacterial activity of hexachlorophene (Gregg and Zopf, 1951). Anderson and Morgan (1966) used the paper disc-plate method to study interaction of hexachlorophene with a number of nonionic surfactants. No correlation was obtained between their physico-chemical and microbiological results.

(iv) Turbidity methods: The growth rate of micro-organisms in broth may be monitored by measuring the change in turbidity with time. The growth rate is characterized by an initial lag phase followed by a logarithmic growth phase where on a statistical basis each viable cell is reproducing at the same rate as all the others (Brown and Garrett, 1964). Brown and Richards (1964a,b) measured the effect of several compounds on the growth rate of exponentially dividing cells of E. coli. They found (1964b) that
the antibacterial activity of polymixin-B sulfate, benzalkonium chloride
and chlorhexidine against \textit{P. aeruginosa} was substantially increased in the
presence of a low concentration of polysorbate 80. The cultures of
\textit{P. aeruginosa} in nutrient broth were measured in the logarithmic phase of
growth spectrophotometrically at 420 nm. Richards and Hardie (1972) used
a similar technique to study the effect of polysorbate 80 and phenylethanol
on the antimicrobial activity of fentichlor. Polysorbate 80 antagonised
the activity of fentichlor against exponential phase cultures of \textit{S. aureus, Pr. vulgaris, and E. coli}, but phenylethanol enhanced the activity of
fentichlor against similar cultures of \textit{E. coli} and \textit{Pr. vulgaris}. Brown
(1966) described a rapid, economical method of evaluating antimicrobial
activity employing turbidimetric measurements, and a similar technique has
been used by other workers (Hugo and Foster, 1964).

In most of the test methods mentioned so far, the biological
activity of the preservative-macromolecule system is determined in the
presence of the culture media. This is a serious limitation because it
has been shown (Pisano and Kostenbauder, 1959; Blaug and Ahsan, 1961a)
that culture media are capable of altering the interaction of preservatives
with macromolecules so that significantly more or less preservative is bound.
Thus, valid comparison of the microbiological data can be made only if the
binding characteristics for the combined macromolecule culture medium system
are known.

(v) \textit{Survivor-concentration-time curve technique:} Microbiocidal activity
may be determined by inoculating a solution of preservative with a known
number of test organisms and subsequently carrying out viable counts.
Either the preservative concentration is kept constant and viable counts
made after increasing time periods (Eisman et al., 1957; Hugo and Newton, 1964; Marx et al., 1968), or increasing concentrations of preservative are tested and viable counts performed after a fixed period of time (Hugo and Newton, 1964; Humphreys et al., 1968; Henderson and Newton, 1969; Bradshaw et al., 1972). The method employed depends on which parameter, sterilizing time or preservative concentration, is the more important. Experiments may be performed in the presence of macromolecules to determine their effect on bactericidal activity.

Marx et al. (1968) studied the behavior of antibacterial agents in solutions of nonionic surfactants using viable count technique. Surfactant solutions containing varying concentrations of a preservative were inoculated with a given number of test organisms and the death rate was followed over a period of several weeks. Surfactant solutions containing preservative showed a steady decline in the microbial count. However, the unpreserved surfactant solutions used as controls showed an increase in the microbial count. Similar techniques have been used by other workers to study the effect of macromolecules on the antibacterial activity of preservatives (Eisman et al., 1957; Taub et al., 1958; Richardson and Woodford, 1964).

(vi) **Extinction time methods:** An extinction time method for evaluating bactericidal activity was described by Cook and Wills (1954) and Berry and Bean (1954). Essentially the method involves: (a) introducing a small volume of preservative solution into each of a replicate series of tubes in a constant temperature water bath; (b) inoculating each tube with a constant number of test organisms; (c) quenching the killing process after a suitable time interval by flooding the tube contents with broth; (d) repeating the whole procedure for several different exposure times; (e) re-
cording the number of negative growth tubes after incubation for each exposure time; and (f) determining the extinction time by statistical analysis. The analysis of extinction time data in bioassays has been described by Mather (1949). The efficacy of a preservative in the presence of a macromolecule can be evaluated by comparing the extinction time in presence and absence of macromolecule (Bean and Berry, 1951, 1953; Allawala and Riegelman, 1953; Beckett et al., 1959; Mitchell, 1964).

(vii) Other methods: Methods of measuring biological responses, which parallel the preservative action on microbial cells, are often used to evaluate preservative efficacy. Ansel (1965) measured the effect of polyethylene glycols in the haemolytic activity of phenol towards rabbit erythrocytes. Judis (1962) examined the protection given by polysorbate 80 to E. coli against chloroxylenol. The release of radio-active material from $^{14}$C labelled cells was measured as an index of cell damage. Measurements of changes in bacterial and fungal spore volume have been used to assess the efficacy of antimicrobial agents in aqueous and surfactant systems (Hitchins et al., 1963; Gould, 1964; Barnes and Parker, 1966, 1967; Parker et al., 1966, 1968; McCafferty and Parker, 1970). Parker et al. (1966) used a coulter counter to measure the germination swelling of Trichoderma and B. subtilis spores in various preservative or preservative-polysorbate 80 mixtures. No spore swelling occurred in phenyl mercuric nitrate or chlorocresol whether the surfactant was present or not, but in solutions of parabens, cetrimide and Nipastat greater swelling occurred in the presence of surfactant. Wailes (1962) and Brown (1968) studied the inhibition of respiration of bakers yeast in preservative and preservative-surfactant mixtures using a Warburg apparatus. Wedderburn (1964) used a similar technique using P. aeruginosa as test organism to evaluate the efficacy
of preservatives in the presence of surface active agents.

(c) Distribution of preservatives in oil-water-surfactant systems

In the previous section (G,b), a mathematical model (Eq. 48) for the preservative distribution in emulsified systems was derived. There are a few limitations to the use of this model.

(i) Determination of necessary parameters in Eq. 48 is a lengthy process, particularly if, as is usual in most pharmaceutical and cosmetic emulsions, preservative mixtures or more than one type of macromolecule is present. Moreover, even a slight change in the formulation of emulsion necessitates a re-evaluation of the various terms.

(ii) Existence of liquid crystalline phases and the presence of reversed micelles in the oil phase (Friberg and Mandell, 1970) in complex emulsions would make the mathematical model difficult or impossible.

(iii) In an emulsion, some surfactant is adsorbed at the oil-water interface and, depending on its oil solubility, some partitions into the oil phase. Both factors reduce the amount of surfactant available for interaction with the preservative and affect the oil-water partition coefficient. Where appreciable amounts of surfactant are adsorbed or partitioned into the oil, determination of the parameters for substitution into Eq. 48 becomes difficult.

In view of these limitations it is necessary that the model should be verified experimentally for the amount of preservative in each 'phase' of the emulsion and hence, the total concentration required to provide desired concentration in the aqueous phase.
Very few techniques are available for a quantitative study of the distribution of a preservative between the different phases of an emulsion system.

Garrett (1966) suggested an ultracentrifuge technique for the separation of the various phases of an emulsion and subsequent analysis of each phase for preservative content. The main drawback associated with this technique is the destruction of emulsion structure which may disturb the equilibrium concentrations of preservative in the various phases of the emulsion.

Patel and Romanowski (1970) used an equilibrium dialysis technique to determine the concentration of free preservative in the aqueous phase, $[D_f]$, of an emulsion. They utilized a two-chambered glass dialysis cell, with emulsion in one compartment and broth in the other (see page 58 for the effect of culture media on preservative–surfactant interaction). The two compartments were separated by a membrane permeable to preservative but impermeable to oil and surfactant. At equilibrium, the concentration of free preservative in the aqueous phase was assumed to be equal on both sides of the membrane. Analysis of the aqueous compartment gave the concentration of free preservative in the aqueous phase of the emulsion. Subtracting the amount of preservative in the aqueous phase from the total amount of preservative added to the dialysis cell gave the total concentration of preservative in the oil and surfactant phases ($[D_o] + [D_b]$). Thus, with this technique, it is not possible to separate the concentrations of preservative in oil, $[D_o]$, and surfactant, $[D_b]$, phases.

Kazmi and Mitchell (1971) developed a three-chambered dialysis
technique. With this it was possible to measure the amount of preservative in each phase of the emulsion, i.e., \([D_f]\), \([D_t]\) and \([D_o]\), and hence the total concentration required to provide the desired concentration in the aqueous phase. The technique also differentiated between free surfactant and surfactant adsorbed at the oil-water interface or partitioned into the oil phase.

Shimamoto et al. (1973) used an ultrafiltration method for measuring free preservative concentration in the aqueous phase of oil-in-water emulsions. The emulsion was centrifuged repeatedly to separate excess of the oil phase. Ultrafiltration of the thin emulsion separated the aqueous phase from the oil and surfactant phases. The ultrafiltrate was analyzed for the free preservative concentration. This technique is not reliable because the procedure involved in the separation of the aqueous phase not only disturbs the emulsion structure but also results in the formation of emulsions of varying oil-water ratios. Both effects may change the equilibrium concentrations of preservative in various phases of the emulsion.

Polderman (1973) reported a dynamic dialysis technique for measuring the free preservative in the aqueous phase of viscous creams. Using this technique it was possible to determine the free preservative concentration in much shorter time than is possible with equilibrium dialysis. Samples of preserved oil-water cream were filled into cellophane tubes and dialyzed against a series of aqueous solutions of its preservative. Based on the overall concentration of the preservative in the cream and its distribution coefficient, a series of aqueous solutions were made,
so that the concentrations were both higher and lower than the estimated concentration in the aqueous phase of the cream. Dialysis in all the tubes was performed under strictly identical conditions such as duration, surface of the membrane, temperature, etc. After a few hours the concentration of the preservative was determined in the dialyzate and the decrease or increase of preservative concentration \((\Delta C)\) was plotted against the starting concentration \([C]\). A straight line was obtained and the point where the line intersected the \(C\)-axis was taken as the concentration of free preservative in the aqueous phase of the cream. The linearity of the line was independent of the concentration of preservative solution under dialysis. The slope of the graph was dependent on the duration of dialysis.

(d) Antimicrobial activity of preservatives in oil-water-surfactant systems

Methods employed for evaluating the efficacy of preservatives in emulsified systems can be divided into two types. Type (I) methods – designed to test predictions of antimicrobial activity based on physico-chemical models. These methods are analogous to methods discussed for studying preservative-surfactant interaction. (II) Methods used to test the efficacy of antimicrobial agents in the final formulations. These methods include normal usage tests, challenge tests and capacity tests.

Type I methods are less time consuming and provide a first step in the formulation of the final emulsion system. However, the reliability of these methods for evaluating the efficacy of preservatives under actual conditions of use has not been sufficiently tested up to the present time. Type II methods, on the other hand, are widely used in industry. They
attempt to simulate conditions in which the final product will be manufactured and utilized.

**Type I Methods**

Anderson and Chow (1967) determined the minimum inhibitory concentration (MIC) of benzoic acid against *Aspergillus niger* spores in emulsified systems. The mycelial growth in the emulsions was examined visually. The concentration of preservative required to prevent fungal growth in half of six or more replicates was chosen as satisfactory means of assessing the fungistatic activity of benzoic acid, and this criterion of assessment was referred to as FC50. Bacteria caused spoilage to an even greater extent than fungi (Wedderburn, 1964), and since their growth in an emulsion cannot be detected visually, the MIC test of Anderson and Chow is not suitable for estimating the efficacy of an antimicrobial agent against bacteria in emulsified systems.

Patel and Romanowski (1970) used a dialysis technique for determining minimum inhibitory concentration of preservatives in emulsified systems. The assumption was made that the biologic activity of preservative would parallel the concentration of free preservative in the aqueous phase. Various limitations and weaknesses of this method will be discussed later (page 173).

Bean *et al.* (1962, 1964, 1965, 1969) employed an extinction time method for studying the effects of oil-water partition coefficient, oil-water ratio, binding with nonionic surfactants and temperature on the antimicrobial activity of preservatives in simple oil-water emulsions and oil-water emulsions stabilized with nonionic surfactants. Various disadvantages
of this method will be discussed later (page 175).

Few attempts (Marx et al., 1968; Shimamoto et al., 1973) have been made to study the death rate of micro-organisms in emulsified systems. The accuracy of these methods has been limited due to difficulties in the isolation of micro-organisms from the emulsion. However, membrane filtration techniques have been found very useful for isolating micro-organisms from emulsions and ointment bases (Sokolski and Chidester, 1964; Buhlmann, 1968; Ko and Vanderwyk, 1968; Hambleton, and Allwood, 1972; Allwood and Hambleton, 1973). Sokolski and Chidester (1964) demonstrated that the membrane filtration technique offered markedly higher recoveries of viable micro-organisms from petrolatum-based ointments than other methods in common use. The advantages of the membrane filtration technique over commonly used isolating procedures will be discussed further under Results and Discussion.

Normal usage tests - These tests (Sykes and Smart, 1968) require that the product is made under normal manufacturing conditions, thus ensuring that it is exposed to the expected contamination hazards, and then stored under various conditions for extended periods, such as months and years. At intervals samples are examined microbiologically for signs of deterioration and spoilage. Another method (Parker, 1969) that has been suggested is daily use of the preparation with daily sampling for viable counts.

There are certain advantages as well as weaknesses associated with the type of normal usage tests described above. The main advantage is that the product is inoculated with 'natural' organisms which have then every opportunity to adapt themselves to grow in the particular medium. Some of the weaknesses of these tests are, (i) it is a long process, involving months and years of storage, (ii) the inoculum can never be standardized...
and is always variable and indeterminate, (iii) more important, these tests do not measure the lethal properties of the preservative in the product but only its ability to prevent microbial growth; such a state is never a stable one and it can easily be upset by small changes in the environmental conditions, or by the occurrence of more resistant organisms.

**Challenge tests** - These are the most commonly used tests (Rdzok *et al.*, 1956; Barr and Tice, 1957; Baker, 1959; Charles and Carter, 1959; Nowak, 1963; Marino, 1966; Bean, 1967; Olson, 1967; Sykes and Smart, 1968; Flawn *et al.*, 1973) for assessing the lethal activities of the product against known micro-organisms.

These tests are carried out by inoculating the product, usually in the final containers, with a given type of micro-organism or a mixture of different types of micro-organisms, and determining their loss of viability over a given period of time. The micro-organisms selected are generally those found to be contaminants in similar types of preparations and important both from clinical and pharmaceutical view points.

Although challenge tests assess lethal activity, are more positive and provide a greater margin of safety, these tests do not take into account the performance of a preservative system under conditions of use in the field where possibly it will be subjected to various cycles of contamination of new unknown micro-organisms during successive use by the patient, or during handling and transfer to other containers by the pharmacist. Thus there is a possibility of reduction of preservative potency in the preparation due to successive contamination and consequent deterioration of the product by multiplication of more resistant organisms.
Capacity tests - Recently these tests have been found very popular and have been used by various authors (Cantor and Shelanski, 1951; Prickett et al., 1961; Bryce and Smart, 1965; MacRae and Johnson, 1965; Lemmex, 1967; Olsen, 1967; Tenenbaum, 1967; Sykes and Smart, 1968; Barnes and Denton, 1969) for evaluating preservative efficacy in various types of formulations, especially cosmetic creams and emulsions. The basis of these tests is to measure the capacity of the preservative system to meet cycles of contamination, as in daily use, and is perhaps the nearest approach available to a true in-use assessment.

In these tests products, containing varying concentrations of preservative, are titrated (repeated inoculation) with micro-organisms and sampled at different time intervals until a positive end point is reached. The time cycle can be varied in accordance with the type of product and its usage.

Criteria for assessing the efficacy of preservative systems using challenge or capacity tests have varied considerably in the literature. Kohn (1963) considers a preservative too slow acting to be used in ophthalmic solutions if it cannot sterilize a given *Pseudomonas* suspension within one hour. The United States Pharmacopoea XVIII (1970) considers that an anti-microbial agent is adequate for use in a product intended for parenteral or ophthalmic administration if there is no significant increase in the number (5 x 10^6/20 ml) of *Candida albicans* or *Aspergillus niger* organisms, and if the number of viable vegetative micro-organisms (*E. coli; P. aeruginosa; S. aureous*) is reduced to not more than 0.1 percent of the initial number (5 x 10^6/20 ml) and remains below that level for a 7 day period within the
28-day test period.

Bean (1967) suggested a time of two hours in which the preservative should be capable of sterilizing vegetative cells introduced into the emulsion. Barnes and Denton (1969) showed from their capacity tests that the standard suggested by Bean was difficult to achieve in formulations such as creams. According to these authors a preservative should reduce viable organisms at approximately $5 \times 10^7$ per g or ml by more than $10^3$ (cream and suspension) or $10^4$ (solution) in 48 hours. Tenenbaum (1967) suggested that the sterilizing time should be measured in days or even weeks, instead of hours. According to Marinaro (1966), "the preservative action is considered excellent, if microbial life is absent or a drastic reduction is observed within 24 hours or less; good, if a satisfactory response is obtained within one week; fair, if in two weeks, and questionable if a response is observed after this period." Baker (1959) considers a preparation ideally preserved if $6 \times 10^6$ organisms per g are sterilized within 2–4 days, and satisfactory if the count is reduced to a few thousand per gram within a period of six days.
EXPERIMENTAL

A. Apparatus.

(a) Amicon Diafiltration Apparatus.
(b) Amsco Medallion Series Autoclave.
(c) Beckman DBGT Double Beam Spectrophotometer, with
    Beckman 10 inch Recorder.
(d) Bioquest Biological Cabinet.
(e) Fisher-Brand Sterilized Disposable Plastic Petri Dishes.
(f) Fisher Colony Counter.
(g) Gallenkamp Oven - Model OV-160.
(h) Hitachi 124 Coleman Double Beam Spectrophotometer with
    Hitachi 165 Recorder.
(i) Haake R\textsubscript{21} Thermoregulator.
(j) Hand Powered Homogenizers (Central Scientific Co.).
(k) International Centrifuge, Size 1, Model SBV.
(l) Isco Fraction Collector.
(m) Labline/Case Imperial II Incubator.
(n) Magniwhirl (Blue M), Constant Temperature Refrigerated
    Shaker Water Bath.
(o) Millipore 6-Place Sterility Test Manifold.
(p) Millipore Sterifil Aseptic Filtration System.
(q) Millipore Vacuum Pump.
(r) Polariter Radiometer PO\textsubscript{4} Polarograph with Radiometer Drop
    Life Timer, Type DLT, and Fisher Calomel Electrode, No. 13-639-51.
(s) Fish Tank, 50 L.
(t) Two Chambered Plexi-Glass Dialysis Cells, as Described by Patel and Foss (1964).

(u) Membrane

(1) Nylon Membrane (0.0005" thick; Capran 77; Allied Chemical Corporation.)

(2) Fisher Cellophane (Dialysis Tubing; 3.6 cm Flat Width; Fisher Scientific Co.)

(3) Visking Cellophane (Dialysis Tubing; 2.4 cm Flat Width; Union Carbide Ltd.)

(4) Millipore Filters (HAEG; 0.45 μm; 47 mm Diameter; Millipore Ltd.)

(5) Diaflo Ultrafiltration Membranes (UM-05; Amicon Corporation.)

(v) Wang 600 Programmable Calculator.

B. Materials

(a) Preservatives

1. Benzoic Acid, reagent grade (Fisher Scientific Co.).
2. Sorbic Acid, reagent grade (Eastman Organic Chemicals).
3. Methyl p-hydroxybénzoate, Propyl p-hydroxy benzoate, reagent grade (British Drug Houses).
4. Chlorocresol, reagent grade (British Drug Houses).
5. Chloroxylenol, reagent grade (Eastman Organic Chemicals).
(b) Nonionic Surfactants

Three n-alkyl polyoxyethylene surfactants (Glover Chemicals Ltd., Leeds, England) of the general formula \( \text{CH}_3(\text{CH}_2)_m(\text{OCH}_2\text{CH}_2)_n\text{OH} \):

- Cetomacrogol 1000, BPC, where \( m \) may be 15 or 17 and \( n \) may be 19 to 23, assuming \( m = 17 \) and \( n = 23 \), the molecular weight was taken as 1300;
- Texofor A16, where \( m = 15 \) and \( n = 16 \);
- Texofor A60, where \( m = 15 \) and \( n = 60 \). Since Texofor A16 and A60 were from the same batch as used by Simons and Rhodes (1971), the molecular weight of Texofor A16 and A60 was calculated using the mean molecular formula determined by the authors using NMR technique. The mean molecular formula of Texofor A16 was the same as claimed by the manufacturer, i.e., \( \text{CH}_3(\text{CH}_2)_{15}(\text{OCH}_2\text{CH}_2)_{16}\text{OH} \), and thus the calculated value of the mean molecular weight was 946. The mean molecular formula of Texofor A60 was \( \text{CH}_3(\text{CH}_2)_{15}(\text{OCH}_2\text{CH}_2)_{77}\text{OH} \), and the calculated value of the molecular weight was 3630. Polysorbate 80 (Atlas Chemical Industries) of the general formula.

\[
\begin{align*}
H_2\text{CH}_{2}\text{O} & \quad \text{CH}_2\text{C} - \text{C}_{17}\text{H}_{33} \\
\quad \text{CH}_2\text{CH}_2\text{O} \quad \text{CH}_2\text{O} \quad \text{CH}_2\text{O} & \quad \text{CH}_2\text{O} \quad \text{CH}_2\text{O}
\end{align*}
\]

where \( n_1 + n_2 + n_3 = n \), assuming \( n = 20 \), the molecular weight was taken as 1308.
(c) Light Liquid Paraffin, B.P.C. (British Drug Houses).
(d) Sodium Chloride, reagent grade (Mallinckrodt Chemical Works).
(e) Phosphomolybdic Acid (J.T. Baker Chemical Co.).
(f) Barium Chloride, Analar grade (J.T. Baker Chemical Co.).
(g) Nitrogen, G grade.
(h) Hydrochloric Acid, reagent grade (Mallinckrodt Chemical Works).
(i) Trypticase Soy Broth (BBL, Division of Bioquest).
(j) Trypticase Soy Agar (BBL, Division of Bioquest).
(k) Bacto Peptone (Difco Laboratories).
(l) Deionized Glass Distilled Water.
(m) Peptone Water

Sodium Chloride - 8.5 g.
Bacto Peptone - 1.0 g.
Distilled Water, Q.S. - 1 L.

C. Temperature

Unless otherwise stated a temperature of 25 ± 0.05° was used in all the studies.

D. Analysis of Surface-Active Agents

(a) Polarographic analysis

Quantitative analysis of cetomacrogol was made using a polarograph. The analysis was based on the damping of the polarographic maxima by surface-active agents (Vavruch, 1950; Jehring, 1966). Potassium chloride solution (0.002N) gives a very pronounced oxygen maximum. A comparison of the heights of the maxima in the presence and absence of cetomacrogol (Fig. 4) and reference to a calibration curve (Fig. 5) permitted the determination of
Fig. 4. Polarographic current voltage curves.

(a) Oxygen maximum of potassium chloride solution (N/500).

(b) Suppression of oxygen maximum by surface-active agent. $h$, height of oxygen maximum.
Fig. 5. Calibration curve for the polarographic determination of cetomacrogol. (Different symbols represent separate experiments.)
surfactant down to 1.0 mg l\(^{-1}\). Since the method is not specific for cetomacrogol, the results are expressed as apparent cetomacrogol concentrations.

To prepare a calibration curve, 100 ml each of 0.02N KCl solution and 0.5% surfactant solution were prepared in deionized glass-distilled water. One milliliter of 0.5% surfactant solution was transferred to a 100 ml volumetric flask and made up to volume with distilled water, so that the final concentration of surfactant was 0.005 %. Five milliliters each of 0.02N solution was pipetted to eleven 50 ml volumetric flasks and to the first ten volumetric flasks 1-10 ml of 0.005 % surfactant was added respectively. Finally all the volumetric flasks were made up to volume with distilled water. Thus the final concentration of surfactant in the first ten volumetric flasks ranged from 1-10 mg l\(^{-1}\) respectively and the final normality of KCl in all the flasks was 0.002N. All eleven solutions were subjected to polarographic analysis and the height of the oxygen maximum was measured for each solution. The heights of oxygen maxima of 0.002N KCl solutions containing various concentrations of surfactant (1-10 mg l\(^{-1}\)) were subtracted from the height of oxygen maximum of 0.002N KCl solution respectively. The percentage suppression of oxygen maximum was calculated for each solution.

The height of oxygen maximum, h, (Fig. 4) depends very much on the conditions of analysis, such as atmospheric pressure, temperature, electrolyte concentration, diameter of capillary, drop time, etc. Since it is impossible to control atmospheric pressure, it was necessary to plot a new calibration curve each time.
(b) Phosphomolybdic acid test

This method is based on the precipitation of nonionic-surfactants of the ethylene-oxide type by phosphomolybdic acid in barium chloride-hydrochloric acid medium (Oliver and Preston, 1949; Stevenson, 1954).

To detect the presence of nonionic surfactants in the solution of the aqueous compartment of a dialysis cell qualitatively, an aliquot of the aqueous solution (0.5 ml) was diluted with 5 ml of glass distilled water in a test tube. Five drops of hydrochloric acid (1:4), 5 drops of barium chloride (10% w/v) and five drops of phosphomolybdic acid (10% w/v) were added and the test tube was well stirred using a vortex mixer. The presence of nonionic surfactant was detected by the formation of a yellowish or yellowish green precipitate.

E. Analysis of the Preservatives in Aqueous and Surfactant Solutions

Aliquots of solutions were appropriately diluted with water (chlorocresol) or 0.01N HCl (benzoic acid), and the preservative concentrations were determined by UV spectrophotometry. The absorbance of chlorocresol and benzoic acid were measured at 280 nm, and 230 nm, respectively.

Where a comparison was made between the binding curves for the interaction of a preservative with a nonionic surfactant in the absence and presence of another preservative, the single preservatives were analyzed using a UV absorbancy-ratio method. This approach was used in order to avoid differences in binding curves due to analytical artifacts.

F. Analysis of Preservative Mixtures in Aqueous and Surfactant Solutions

Binary mixtures of preservatives were analyzed using the
absorbancy ratio method ("Q" analysis) of Pernarowski et al. (1961a,b). The same terminology and symbology has been observed in this work as used by the authors.

(a) The spectral characteristics of the individual preservatives of the binary mixture were determined (Figs. 6, 8, 10 and 12). (b) The isoabsorptive point was located by recording the spectrum of a solution of one preservative relative to a solution of another preservative (i.e., the second solution was the "blank"). The initial concentrations of both the preservative solutions were identical. The wavelength at which an absorbancy reading of zero was observed represented an isoabsorptive point. (c) The two wavelengths chosen for the analysis were the isoabsorptive point and the wavelength at which one of the two preservatives exhibited maximum absorption. (d) The "Qo" values (Eq. 50) for a number of preservatives mixtures were determined. The absorptivity value (a4) of both preservatives at the isoabsorptive point were also determined. For a given preservative combination, value of a4 for both the preservatives were similar, indicating the exactness in the determination of the isoabsorptive wavelength. (e) "Qo" vs fraction of one of the components in the preservative mixture (Fy) was plotted (Eq.50), and the data was subjected to least square fitting to ascertain the equation of the resulting straight line (Figs. 7, 9, 11 and 13). (f) The numerical constants obtained from the "Q" curve were substituted into the Eq.51 and 52. (g) The concentration of individual preservatives in an unknown binary mixture were calculated by substituting the values of Qo, A3 and a4 into Eq.51 and 52.

\[ Q_o = (Q_y - Q_x) F_y + Q_x \]  \hspace{1cm} \text{(Eq.50)}

\[ C_y = \frac{A_3}{a_4} \cdot \frac{Q_o - Q_x}{Q_y - Q_x} \]  \hspace{1cm} \text{(Eq.51)}
Fig. 6. Spectrophotometric curve for benzoic acid (---) and sorbic acid (-----) in 0.01N HCl using a Hitachi Coleman spectrophotometer. Concentration of benzoic acid and sorbic acid = 2.0 mg l⁻¹. Absorptivity value of preservatives at isoabsorptive wavelength, 236.4 nm = 73.5.
Fig. 7. Q curve for benzoic acid and sorbic acid. Points experimental, line fitted using least squares method. Slope = 0.641. Intercept = 0.613. Correlation coefficient = 0.999.
Fig. 8. Spectrophotometric curves for chlorocresol (-----) and methyl paraben (-----) in water using Beckman DBGT spectrophotometer. Concentration of chlorocresol and methyl paraben = 5.0 mg l⁻¹. Absroptivity value of preservatives at isoabsorptive wavelength, 236 nm = 30.4.
Fig. 9. Q curve for chlorocresol and methyl paraben. Points experimental, line fitted using least squares method. Slope = 1.164. Intercept = 0.505. Correlation coefficient = 0.999.
Fig. 10. Spectrophotometric curves for chlorocresol (-----) and propyl paraben (------) in water using Hitachi Coleman spectrophotometer. Concentration of chlorocresol and propyl paraben = 5.0 mg l⁻¹. Absorptivity value of preservatives at isoabsorptive wavelength, 233.2 = 27.0.
Fig. 11. Q curve for chlorocresol and propyl paraben. Points experimental, line fitted using least squares method. Slope = 1.318. Intercept = 0.509. Correlation coefficient = 0.999.
Fig. 12. Spectrophotometric curve for chloroxylenol (-----) and methyl paraben (--.--.) in water using Hitachi Coleman spectrophotometer. Concentration of chloroxylenol and methyl paraben = 5.0 mg l\(^{-1}\). Absorptivity value of preservative at isoabsorptive wavelength, 232 nm = 28.39.
Fig. 13. Q curve for chloroxylenol and methyl paraben. Points experimental, line fitted using least squares method. Slope = 0.952. Intercept = 0.556. Correlation coefficient = 0.999.
\[ Cx = \frac{A_3}{a_4} - Cy \]  

(Eq. 52)

where:

- \( Q_o \) = Absorbancy ratio of preservative mixture (X + Y).
- \( Q_x \) = Absorbancy ratio of preservative X.
- \( Q_y \) = Absorbancy ratio of preservative Y.
- Absorbancy ratio = \((\text{absorbance at } \lambda_{\text{max}} \text{ of preservative } Y)/(\text{absorbance at isoabsorptive point})\).
- \( F_y \) = Fraction of preservative 'Y' in a preservative mixture (X + Y).
- \( C_x \) = Concentration of preservative X.
- \( C_y \) = Concentration of preservative Y.
- \( A_3 \) = Absorbance at isoabsorptive wavelength.
- \( a_4 \) = Absorptivity value of preservative X or Y at isoabsorptive wavelength.

G. Permeability of Cellophane Membranes to Nonionic Surfactants

(a) Equilibrium dialysis

Dialysis cells similar to those described by Patel and Foss (1964) were used. The two chambers of the cells were separated by cellophane or silicone rubber membranes. Twenty milliliters of a surfactant solution were pipeted into one chamber and 20 ml of distilled water was pipeted into the other. The cells were tumbled in a temperature-controlled water bath. When the surfactant was cetomacrogol, equal volumes of the solutions were pipeted at 12-hour intervals from both chambers of the dialysis cell and analyzed for surfactant concentration. For Texofor surfactants \((A_3, A_{45}, A_{60})\), the solution of the aqueous compartment of the dialysis cell was analyzed qualitatively using the phosphomolybdic acid test for the presence of nonionic
surfactant at the end of the fourth day.

(b) Dynamic dialysis

Twenty-five milliliters of 10% cetomacrogol was transferred to a Fisher cellophane bag suspended in 200 ml of distilled water in a jacketed beaker. The solution in the jacketed beaker was stirred with a magnetic stirrer, while the cetomacrogol solution in the cellophane bag was stirred with a glass stirrer. One hundred milliliters of the solution was pipetted from the jacketed beaker at 12-hour intervals and analyzed for cetomacrogol. The volume of solution in the jacketed beaker was immediately made up to 200 ml with fresh distilled water, so as to maintain sink conditions.

(c) Ultrafiltration

A Visking cellophane membrane was cut and fitted into an Amicon ultra-filtration cell. Thirty-five milliliters of 1.8% cetomacrogol solution was placed in the cell and a pressure of 40 lb/sq inch was applied until complete filtration of the liquid was attained. The filtrate was analyzed for cetomacrogol.

H. Binding of Chlorocresol with Silicone Rubber in Equilibrium Dialysis

The silicone membrane was boiled in distilled water and subsequently washed with several changes of distilled water. The membrane was placed between the compartments of the dialysis cell. Twenty milliliters of chlorocresol solution (of varying concentrations) was pipetted into one compartment and 20 ml of water was pipetted into the other. The cells were tumbled until the concentrations of chlorocresol in both compartments were the same (four days). The chlorocresol in both chambers was analyzed spectrophotometrically
at 280 nm. and the percentage recovery was calculated to estimate membrane
binding.

I. Interaction of Preservatives with Nonionic Surfactants

(a) Equilibrium dialysis technique

Two chambered dialysis cells similar to those described by Patel
and Foss (1964) were used. Silicone rubber (phenols and parabens), nylon
(benzoic acid and sorbic acid) and cellophane (chlorocresol) were used as
dialysis membranes. An aqueous solution of preservative in the surfactant
was placed in one side of the cell and water or water plus preservative was
placed in the other. For the benzoic acid and sorbic acid study, 0.01N HCl,
instead of water, was used as the solvent. Two glass beads were added to each
compartment to ensure continuous mixing. The cells were rotated in a water
bath maintained at constant temperature. The preservative in both chambers
was analyzed spectrophotometrically at equilibrium (four days for chloro­
cresol and 7 days for parabens, benzoic acid and sorbic acid). For the inter­
action of chlorocresol with nonionic surfactants, the volume of solution in
each chamber of the dialysis cell was measured at the end of the fourth day.

(b) The diafiltration technique

(I) The diafiltration apparatus: The apparatus used in this study is
described with reference to Fig. 3.

The Amicon reservoir tank (12 liter capacity), with a maximum
pressure capacity of 100 psi, is made of stainless steel (epoxy coated) and
fitted with a fill-port pressure relief valve, and inlet and output
connectors. The reservoir was kept in a temperature controlled water bath.

The Amicon Ultrafiltration Cell (Model 52, 50 ml capacity, 43 mm diameter membrane) was seated in a water-jacketed beaker maintained at constant temperature, and the contents stirred by the use of a magnetic stirrer. Details of cell structure and materials of construction are given elsewhere (Amicon Publication No. 403 A).

The ultrafiltration membrane used in this study was UM05. This membrane is negatively charged and is said to prevent the passage of molecules of molecular weight greater than 600.

An Amicon C/D (concentration/dialysis) Selector was connected to a nitrogen cylinder, reservoir, and the ultrafiltration cell as shown in Fig. 3. This allowed gas or liquid to flow from the reservoir to the cell as desired and thus the solution in the cell was concentrated or dialyzed respectively.

(II) Procedure for the determination of benzoic acid-cetomacrogol interaction using the diafiltration technique:

(1) The ultrafiltration cell was charged with cetomacrogol solution.

(2) The reservoir was filled with benzoic acid solution in 0.01N HCl at the maximum concentration of interest.

(3) An Amicon C/D Selector, with the valve in the 'GAS' position, was connected with the reservoir, ultrafiltration cell and the pressure source (N₂ cylinder), thus permitting gas to pass from the reservoir tank to the cell. With the pressure relief valves on the tank and the cell both in the open position, the pressure valve of the nitrogen cylinder was opened slowly. After closing both pressure valves, the cylinder pressure valve was opened
until the desired pressure was achieved on the cylinder gauge. A time of two minutes was allowed for the pressure to equalize in the tank and the cell. The C/D selector switch was shifted to the 'LIQUID' position, and the pressure relief valve on the cell was slightly opened to allow the solution to begin to flow into the tubing connected to the cell. Extreme care was taken in opening the pressure relief valve, because if the valve was opened too much, solution flow was excessive, and a volume increase occurred in the cell.

(4) The ultrafiltrate was collected automatically by an Isco Fraction Collector. Samples were collected on a volume basis. This procedure involved the use of a volume collecting device but checks on volumes delivered indicated that the device tended to be inaccurate. Hence, all fractions collected were checked independently for volume.

(5) The concentration of benzoic acid in each successive fraction, \([D_f]\), was determined spectrophotometrically at 230 nm.

(6) The concentration of preservative bound to the surfactant, \([D_b]\), for a given free preservative concentration in the ultrafiltrate, \([D_f]\), was calculated using the following equations (see appendix 1):

\[
A_b = \sum A_t - \sum A_f - A'_f 
\]  \hspace{1cm} \text{(Eq.53)}

\[
A_b = \sum V_f[D_t] - \sum V_f[D_f] - V_m[D_f] 
\]  \hspace{1cm} \text{(Eq.54)}

\[
[D_b] = (A_b/V_m) \cdot 1000 
\]  \hspace{1cm} \text{(Eq.55)}

where:

\[A_t\] = Amount of preservative entering the cell.

\[A_b\] = Amount of preservative bound to surfactant.
\[ A_f = \text{Amount of preservative in a fraction of ultrafiltrate.} \]
\[ A'_f = \text{Amount of preservative free in the cell.} \]
\[ [D_t] = \text{Concentration of preservative in the reservoir.} \]
\[ [D_b] = \text{Concentration of preservative bound to surfactant.} \]
\[ [D_f] = \text{Concentration of free preservative.} \]
\[ V_m = \text{Volume of the surfactant solution in the cell.} \]
\[ V_f = \text{Volume of a fraction of ultrafiltrate.} \]

(7) The final volume of the cetomacrogol solution was recorded at the end of the experiment.

J. Interaction of Preservative Mixtures with Cetomacrogol

The equilibrium dialysis technique was used to study the interaction of preservative mixtures with cetomacrogol. The dialysis membrane was either nylon (benzoic acid and sorbic acid mixture) or silicone rubber (phenols and parabens mixtures). Twenty milliliters of aqueous cetomacrogol solution plus preservative (D) or competitor (C) or both was placed in one side of the two-chambered dialysis cell and 20 ml of water or water plus preservative (D) or competitor (C) or both was placed in the other. For the benzoic acid and sorbic acid study, 0.01N HCl, instead of water, was used as the solvent. The experiments were so designed that in each study the concentration of preservative (D) varied while the concentration of the competitor (C) was kept constant. The time required to reach equilibrium ranged from four to seven days. The concentration of preservative (D) and competitor (C) in both chambers was analyzed spectrophotometrically using the absorbancy-ratio method.
K. Interaction of Chlorocresol with Mixtures of Nonionic Surfactants

Methodology similar to that used for the interaction of preservatives with nonionic surfactants using equilibrium dialysis was utilized to study interaction of chlorocresol with surfactant mixtures. The dialysis membrane was silicone rubber. The surfactant solution in the dialysis cell was replaced with an aqueous solution of surfactant mixture.

L. Distribution of Chlorocresol in Liquid Paraffin-Water Systems

Equal volumes of aqueous chlorocresol solution and liquid paraffin were pipetted into glass-stoppered flasks and agitated using a wrist-action shaker for about one hour at room temperature. The flasks were transferred to a temperature controlled shaker bath and agitated till equilibrium was reached (three days). The aqueous phase was separated by centrifugation of the oil-water mixture and analyzed spectrophotometrically for chlorocresol concentration. The concentration of chlorocresol in the oil phase was calculated by subtracting the amount of chlorocresol in the aqueous phase from the total amount of chlorocresol added.

M. Distribution of Chlorocresol in Liquid Paraffin-Water-Cetomacrogol Systems

Liquid paraffin and cetomacrogol solutions were mixed in various ratios and passed through hand powered homogenizers, at least five times, to ensure the formation of stable emulsions. For microbiological work the emulsions were prepared aseptically in a laminar flow hood using sterile liquid paraffin and cetomacrogol solutions. The homogenizers were sterilized by autoclaving at 121° for 30 minutes.
For emulsions containing chlorocresol, prior to homogenization, the liquid paraffin and cetomacrogol solutions containing chlorocresol were mixed and agitated in a water bath maintained at a constant temperature for about 3 days, a time sufficient for the preservative to equilibrate between various phases of the emulsion.

An emulsion containing various amounts of chlorocresol was pipetted into one compartment of a dialysis cell fitted with a silicone rubber membrane. An aqueous solution containing various amounts of chlorocresol was pipetted into the other compartment. The cells were equilibrated (about 4 days) and the concentration of chlorocresol in the aqueous compartment was analyzed spectrophotometrically at 225 nm.

N. Microbiological Procedures

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

(b) **Culture media**: 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 (each 1500 ml) were sterilized by autoclaving at 121° for 35 minutes.

(c) **Sterilization of experimental solutions**: Aqueous chlorocresol solutions and cetomacrogol solutions with and without chlorocresol were sterilized by passing the solution through sterile Millipore 0.45 μm HA filters. Light liquid paraffin was sterilized by dry heat at 160° for one hour.
(d) **Sterilization of Millipore filtration equipment:** A Millipore 6 place sterility test manifold unit, Millipore funnels and Millipore 0.45/μ HAEG filters were sterilized according to specifications of the manufacturer (Millipore Catalogue No. MRP-4).

(e) **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 hours at 37° in an incubator. A sample of 0.2 ml of this culture was transferred to 50 ml of fresh trypticase soy broth and incubated at 37° for 12 hours. This final **E. coli** suspension was used for the construction of a standard curve and for the preparation of standard **E. coli** suspensions for experimental work. The purity of the culture was checked using Gram's staining procedure and examining the growth on MacConkey agar.

(f) **Construction of standard curve for E. coli:** A series of dilutions of **E. coli** suspension were made in trypticase soy broth and the absorbance measured at 550 nm. using a double beam spectrophotometer. Trypticase soy broth was used as blank in the absorbance measurements. An aliquot from each dilution was appropriately diluted with 0.85% saline and viable counts were made using the pour plate technique.

(g) **Preparation of standard E. coli suspension:** An aliquot of the culture was diluted with trypticase soy broth and the absorbance measured at 550 nm. From the absorbance, the dilution-factor and with reference to the standard curve (Fig. 14), the concentration of **E. coli** in the culture was determined. The culture was diluted with 0.85% saline to produce the desired number of **E. coli** per ml of suspension.
Fig. 14. Standard curve for E. coli using spectrophotometric technique. Viable counts of E. coli made using pour-plate technique. Slope of the regression line = 2.195 x 10^-6. Correlation coefficient = 0.995.
(h) **Viable count method using pour-plate technique**: Samples of 0.5 ml were withdrawn from the cultures and appropriately diluted into sterilized 0.85% saline so that 50–200 colonies per plate would result. From these dilutions, aliquots of 1 ml were pipeted into each of three petriplates and to each plate 12 ml of sterile trypticase soy agar, kept at 45° in water bath, was poured. The plates were rotated to disperse the culture in agar and incubated for 48 hours at 37°. The colonies were counted using a colony counter.

(i) **Viable count method using membrane filtration technique**: A Millipore 6 place Sterility Test Manifold unit with 47 mm edge-hydrophobic Millipore filters (HAEG, 0.45μ) was used. Details of the Millipore filtration assembly setup and operation are given elsewhere (Millipore Catalogue No. MRP-4). All operations were performed under a laminar flow hood.

Samples of 0.5 ml were appropriately diluted with sterile normal saline to produce 100 to 200 colonies per Millipore filter. From these dilutions aliquots of 1 ml were pipeted into Millipore funnels containing approximately 10 ml of peptone water and vacuum was applied immediately to draw the sample through the filter. The filter was washed 5 times with 25 ml portions of peptone water, and each time vacuum was applied immediately. Upon completion of filtration, the filter was transferred to trypticase soy agar plates, and incubated at 37° for 24 hours. The colonies were counted using a colony counter. For chlorocresol treated *E. coli* the plates were incubated for another 12 hours, and colonies recounted to check for the emergence of new colonies.
(j) **Bactericidal activity of chlorocresol in water:** Aqueous chlorocresol solutions (41.5 ml) were inoculated with 0.5 ml of standard *E. coli* suspension so that each ml of solution contained $10^6$ organisms. Decrease in preservative concentration due to the addition of 0.5 ml of culture was taken into account in the calculation of initial chlorocresol concentrations. The chlorocresol solutions were agitated in a water bath maintained at 25°, samples were withdrawn at given time intervals and viable counts made using the membrane filtration technique. Controls consisting of distilled water were similarly run with each experiment to check the viability of the organisms during the experimental period.

(k) **Bactericidal activity of chlorocresol in cetomacrogol solutions:** Cetomacrogol solutions of varying concentration were made in distilled water. The total concentration of chlorocresol, $[D_t]$, giving the desired free preservative concentration in the aqueous phase $[D_f]$, was calculated using Eq.28. All cetomacrogol solutions had the same $[D_f]$ but different $[D_t]$. Corrections were made in the initial cetomacrogol and chlorocresol concentrations to account for the dilution of final surfactant solution due to the addition of 0.5 ml of *E. coli* suspension.

Cetomacrogol solutions (41.5 ml) containing chlorocresol were agitated in a water bath for about 3 hours to equilibrate the preservative between the aqueous and micellar phases of the surfactant solution at given temperature. At equilibrium, the cetomacrogol solutions were inoculated with 0.5 ml of standard *E. coli* culture so that each ml of surfactant solution contained $10^6$ organisms. The cetomacrogol solutions were sampled at given time intervals and viable counts were made using the membrane
filtration technique. Controls consisting of aqueous cetomacrogol solutions were run with each experiment to check the viability of the organisms during the experimental period.

(1) Bactericidal activity of chlorocresol in liquid-paraffin emulsions stabilized with cetomacrogol: Liquid paraffin emulsions of varying oil-water ratios were prepared aseptically (see method for the preparation of emulsions) using sterile liquid paraffin and cetomacrogol solutions. The total chlorocresol concentration, $[D]$, required in an emulsion to get the desired free preservative concentration in the aqueous phase, $[D_f]$, was calculated using Eq.48. Corrections were made in the initial cetomacrogol and chlorocresol concentrations to account for the dilution of the final emulsion due to the addition of 0.5 ml of $E. coli$ suspension.

Freshly homogenized liquid paraffin emulsions (41.5 ml) containing chlorocresol were agitated in a water bath for about 3 hours to equilibrate the preservative between the various phases of the emulsion. The emulsions were inoculated with 0.5 ml of standard $E. coli$ culture so that each ml of emulsion contained $10^6$ organisms. The emulsions were sampled at given time intervals and viable counts were made using the membrane filtration technique. Controls consisting of blank liquid paraffin emulsions were similarly run with each experiment to check the viability of the organisms during the experimental period.
RESULTS AND DISCUSSION

A. Permeability of Membranes to Nonionic Surfactants

(a) Permeability of membranes to Texofor surfactants

Qualitative tests of the solution of the aqueous compartment of the dialysis cell showed that the Fisher cellophane membrane was permeable to Texofors $A_{23}$ (cetomacrogol), $A_{30}$, $A_{45}$ and $A_{60}$. The silicone rubber was totally impermeable to the same surfactants.

(b) Permeability of membranes to cetomacrogol

Figure 15 shows the fraction of cetomacrogol dialyzed through Fisher and Visking cellophane membranes as a function of time in equilibrium dialysis. Both membranes were permeable to cetomacrogol at approximately the same rate.

Figure 16 shows the permeability of Fisher cellophane membrane to cetomacrogol using dynamic dialysis under sink conditions. The rate of permeation decreases with increase in time. This is possibly due to rapid permeation of low molecular weight fractions, followed by a slow diffusion of the molecules of higher molecular weight.

Ultrafiltration of cetomacrogol solution (1.8%) through Visking cellophane membrane showed that 2.7% of the total surfactant passed through the membrane.

B. Volume Change due to Osmosis in Dialysis Studies

Increase in the volume of solution in the surfactant chamber of
Permeability of cellophane membranes to cetomacrogol in equilibrium dialysis. Initial cetomacrogol concentrations: \( \square, 38.46 \times 10^{-3}; \bigcirc, 76.92 \times 10^{-3} \text{ moles l}^{-1} \). Open and closed symbols represent Fisher and Visking cellophane membranes respectively.
Fig. 16. Permeability of Fisher cellophane membrane to cetomacrogol in dynamic dialysis under sink conditions. Initial cetomacrogol concentration was $76.92 \times 10^{-3}$ moles $\text{l}^{-1}$. The fraction dialyzed was not corrected for changes in surfactant concentration due to osmosis.
the equilibrium dialysis cell and the cellophane bag used in dynamic dialysis technique showed that osmosis had occurred.

Table 3 shows the volume changes in an equilibrium dialysis study of the interaction of chlorocresol with some Texofor surfactants when Fisher cellophane was used as the semipermeable membrane. Fifteen to forty percent of the water was removed from the aqueous compartment of the dialysis cell, thus causing appreciable dilution of the solution in the surfactant compartment.

C. Interaction of Preservatives with Nonionic Surfactants

(a) Equilibrium dialysis technique

(i) Interaction of chlorocresol with Texofor surfactants: Table 4 shows a comparison between the binding results obtained using silicone rubber and Fisher cellophane membranes in equilibrium dialysis studies involving the interaction of chlorocresol with Texofor $A_{30}$, $A_{45}$ and $A_{60}$. For a given initial surfactant concentration the total amount of chlorocresol in the dialysis cell was the same for both silicone rubber and cellophane membranes. However, the values of $r$ obtained using cellophane membranes are lower than those obtained using silicone rubber membranes. Since the cellophane membrane was permeable to the surfactants, and osmosis occurred during dialysis (Table 3), the observed decrease in the values of $r$ can be ascribed both to osmosis and surfactant permeation through the membrane. This aspect will be discussed further in the following section.

(ii) Interaction of chlorocresol with cetomacrogol: The binding results in the form of a Scatchard plot (Scatchard, 1949) are shown in
TABLE 3. Volume change due to osmosis in equilibrium dialysis using Fisher cellophane as a semipermeable membrane.

<table>
<thead>
<tr>
<th>Texofor</th>
<th>Initial Surfactant Concentration %</th>
<th>Volume in Surfactant Compartment ml</th>
<th>Volume in Aqueous Compartment ml</th>
</tr>
</thead>
<tbody>
<tr>
<td>A₃₀</td>
<td>5.0</td>
<td>22.8</td>
<td>16.6</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>25.2</td>
<td>14.2</td>
</tr>
<tr>
<td>A₄₅</td>
<td>5.0</td>
<td>24.1</td>
<td>15.1</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>26.5</td>
<td>12.5</td>
</tr>
<tr>
<td>A₆₀</td>
<td>5.0</td>
<td>24</td>
<td>15.2</td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>27.4</td>
<td>11.9</td>
</tr>
</tbody>
</table>

1. Initial volumes in the aqueous and surfactant compartments were 20 ml.

2. Volumes were measured at the end of the 4th day.

3. Volumes were determined using pipets, therefore, complete recovery of fluids was not possible. Thus, the volumes shown in the table represent approximate values.
TABLE 4. Comparison of "r" for a given total amount of chlorocresol in equilibrium dialysis using silicone rubber and Fisher cellophane as semipermeable membranes.

<table>
<thead>
<tr>
<th>Texofor</th>
<th>Initial Surfactant Concentration %</th>
<th>( r = [D_b]/[M] )</th>
<th>Silicone Membrane</th>
<th>Fisher Cellophane Membrane</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>A30</td>
<td>5.0</td>
<td>1.68</td>
<td>1.47</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1.75</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td>A45</td>
<td>5.0</td>
<td>11.73</td>
<td>1.37</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1.89</td>
<td>1.38</td>
<td></td>
</tr>
<tr>
<td>A60</td>
<td>5.0</td>
<td>1.76</td>
<td>1.43</td>
<td></td>
</tr>
<tr>
<td></td>
<td>10.0</td>
<td>1.98</td>
<td>1.39</td>
<td></td>
</tr>
</tbody>
</table>

* The surfactant concentration, \([M]\), was not corrected for dilution due to osmosis, or loss due to permeation through the dialysis membrane.
Figure 17. The binding of chlorocresol with cetomacrogol is independent of surfactant concentration when silicone rubber is used as the dialysis membrane. Similar independence of surfactant concentration has been reported previously for the binding of several other preservatives with cetomacrogol when nylon was used as the dialysis membrane (Kazmi and Mitchell, 1970). The curve in Fig. 17 was characterized according to Eq. 25 on the assumption that two classes of binding sites are involved in the interaction.

\[ \frac{[D_b]}{[M]} = r = \frac{n_1K_1[D_f]}{1+K_1[D_f]} + \frac{n_2K_2[D_f]}{1+K_2[D_f]} \]  

(Eq. 25)

The experimental data are indicated by points while the solid lines were fitted according to the n and K values computed from Eq. 25 using a non-linear regression program (see appendix 2).

Figure 17 shows that when cellophane dialysis membrane was used, the binding is apparently not independent of cetomacrogol concentration and a series of curves are obtained. Theoretically such results indicate that an increase in the concentration of cetomacrogol results in a decrease in the number of binding sites on the cetomacrogol molecule. However, since the results obtained with silicone rubber membrane are independent of surfactant concentration, it is suggested that changes in cetomacrogol concentration due to dilution as a result of osmosis and/or to permeation of surfactant through the cellophane membrane provide a more likely explanation. Permeation of sufficient cetomacrogol into the "surfactant-free" chamber of the dialysis cell would lead to an increase in the apparent value of \([D_f]\) due to micellar interaction with the chlorocresol and, therefore,
Fig. 17. Scatchard plot for the interaction of chlorocresol with cetomacrogol. Initial cetomacrogol concentrations: O, 7.69 x 10^{-3}; ▼, 15.3 x 10^{-3}; □, 23.07 x 10^{-3}; ○, 38.46 x 10^{-3}; △, 76.92 x 10^{-3} moles l^{-1}. Open and closed symbols represent data obtained using silicone and Fisher cellophane membranes respectively.
decrease the $r/[D_f]$ ratio for a given $r$ value. Moreover, loss of surfactant by permeation through the membrane would also decrease $r$ due to the uncorrected decrease in the value of $[M]$ and the decrease in $[D_b]$. Thus, a plot of $r/[D_f]$ versus $r$ for different concentrations of cetomacrogol would result in a series of curves each representing a given surfactant concentration. Dilution of cetomacrogol as a result of osmosis would produce a similar displacement of the binding curves.

The surfactant concentration required to produce the observed displacement of the binding curves was calculated from a rearrangement of Eq. 25.

$$[M] = \frac{[D_b] (1 + K_1[D_f] + K_2[D_f] + K_{12}[D_f])^2}{[D_f] (n_1K_1 + n_2K_2 + n_1K_1K_2[D_f] + n_2K_1K_2[D_f])}$$

(Eq. 56)

where $[D_b]$ and $[D_f]$ are the experimental values for cellophane membrane (Fig. 17) and $n_1$, $K_1$, $n_2$, $K_2$ are the binding constants obtained using silicone rubber membrane. The data in Table 5 shows that the changes in surfactant concentration (i.e., the differences between the initial and calculated cetomacrogol concentrations, $M_1 - M_3$) were considerably greater than could be accounted for solely by loss of surfactant as a result of permeation through the cellophane membrane, $M_4$. However, when the binding curves are replotted using surfactant concentrations corrected for volume changes in the chambers of the dialysis cell, $M_2$, the discrepancies between the curves determined using silicone rubber membrane and cellophane membrane is markedly reduced (Fig. 18).

Since the difference between $M_2$ and $M_3$ is of the same order of
TABLE 5. Change of Surfactant Concentration in Equilibrium Dialysis with Cellophane as a Semipermeable Membrane

<table>
<thead>
<tr>
<th>Cetomacrogol Concentration Moles $1^{-1} \times 10^3$</th>
</tr>
</thead>
<tbody>
<tr>
<td>$M_1$</td>
</tr>
<tr>
<td>Initial</td>
</tr>
<tr>
<td>38.46</td>
</tr>
<tr>
<td>76.92</td>
</tr>
</tbody>
</table>

(a) Each value represents the mean of five readings.
(b) Values were obtained by substituting $n_1 K_1$ and $n_2 K_2$ calculated from the binding curve obtained using silicone rubber membrane (Fig. 17), and $[D_b]$ and $[D_2]$, obtained using cellophane membrane (Fig. 17), into Eq.56. Each value represents the mean of five readings.
(c) From Figure 15.
Fig. 18. Scatchard plot for the interaction of chlorocresol with cetomacrogol. Upper binding curve obtained using silicone rubber membrane as in Fig. 17. Cellophane membrane data corrected for changes in surfactant concentrations due to osmosis; initial concentrations as in Fig. 17; corrected concentrations shown in Table 5.
magnitude as $M_4$, the residual displacement of the binding curves can be attributed to the decrease in $[M]$ due to permeability of the cellophane membrane to cetomacrogol.

Hence, it can be concluded that the observed displacement of the binding curves obtained in the equilibrium dialysis study using cellophane membranes is due both to osmosis and to permeability of the membrane to the surfactant, with dilution of surfactant as a result of the osmotic differential across the membrane being the major factor. It is apparent that the use of cellophane as a membrane will introduce appreciable errors into interaction and transport studies involving nonionic surfactants unless corrections are made for osmosis of the experimental design is such that osmosis is avoided.

(b) Diafiltration technique

Figure 19 is a Scatchard plot (Scatchard, 1949) for the interaction of benzoic acid with cetomacrogol. The binding curve derived from the equilibrium dialysis data is independent of cetomacrogol concentration. This is in agreement with the benzoic acid-cetomacrogol interactions reported in the literature (Mitchell and Brown, 1966; Donbrow, Azaz and Hamburger, 1970; Kazmi and Mitchell, 1971). However, a plot of diafiltration data give two separate binding curves for 1% and 5% cetomacrogol concentrations respectively. An increase in cetomacrogol concentration results in a downward shift of the binding curve. For a given cetomacrogol concentration the binding curve is reasonably reproducible. Thus the diafiltration results indicate that the simple Law of Mass Action does not adequately describe the benzoic acid-cetomacrogol interaction, and the binding is not independent of
Fig. 19. Scatchard plot for the interaction of benzoic acid with ceto-macrogol. Cetomacrogol concentrations: ○, $7.69 \times 10^{-3}$; ○, $38.46 \times 10^{-3}$ moles $l^{-1}$. Open and closed symbols represent data obtained using equilibrium dialysis and equilibrium ultrafiltration techniques respectively.
macromolecule concentration. This is very unlikely. Similar dependence of binding curves of macromolecule concentration have been reported for drug-protein interactions using the diafiltration technique (Blatt, Robinson and Bixler, 1968; Ryan and Hanna, 1971; Crawford, Jones, Thompson and Wells, 1972; Palmer, 1972; Barnes, Mitchell, Palmer and Pernarowski, 1973).

Attempts have been made to explain the anomalous binding results obtained using diafiltration technique in drug-protein interaction studies. Blatt, Robinson and Bixler (1968) explained their results in terms of polarization (or caking) of drug molecules at the membrane. To overcome this problem they suggested using the lowest possible pressures and low macromolecule concentrations or large porosity membranes. The possibility of preservative polarization cannot be ruled out in this study because a high pressure (9.1 kg/m$^2$) was used to enhance the flux rate of the aqueous phase through UM05 membrane. Unlike drug-protein interaction studies, the use of large porosity membranes to alleviate preservative polarization in preservative-surfactant interaction studies is not feasible here. Possible surfactant permeation through the membrane will introduce appreciable errors into the binding parameters. Even the Amicon UM05 (molecular weight cutoff = 500) did not hold the cetomacrogol (molecular weight = 1300) completely. Qualitative tests of the ultrafiltrate showed that traces of surfactant passed through the membrane. The magnitude of the errors introduced into the binding parameters due to permeability of cetomacrogol through Fisher cellophane membrane in an equilibrium dialysis study were pointed out in the previous section. A similar explanation can be given for the downward shift of the binding curves in the diafiltration studies of benzoic acid-cetomacrogol.
interactions. Increasing the concentration of cetomacrogol in the cell would result in the permeation of a greater amount of surfactant in the ultrafiltrate, and thus higher value of \([D_f]\) due to micellar solubilization of the preservative. This effect will decrease the \(r/[D_f]\) ratio, for a given \(r_c\), and cause a downward shift of the binding curve.

Drug binding and/or rejection by the diafiltration apparatus can also affect the binding results (Blatt, Robinson and Bixler, 1968; Ryan and Hanna, 1971; Palmer, 1972). Attempts to separate the effects of drug binding and rejection in diafiltration studies were unsuccessful (Blatt, Robinson and Bixler, 1968; Palmer, 1972). The phenomenon of membrane rejection was demonstrated by Ryan and Hanna (1971). Ultrafiltration of testosterone solution was carried out using an Amicon UM05 membrane. Simultaneous sampling of the cell content and the ultrafiltrate revealed that cell testosterone levels rose above the reservoir concentration. The authors ascribed this observation to the membrane-rejection phenomenon. Palmer (1972) demonstrated the binding of bishydroxycoumarin and phenylbutazone with various parts of the diafiltration apparatus. Bishydroxycoumarin was bound with the diafiltration apparatus to a greater extent than phenylbutazone. Binding of drugs and preservatives to plastics and synthetic membranes is a common problem (Patel and Kostenbauder, 1958; Kapadia, Guess and Autian, 1964; Ootegehem and Herbots, 1969; Schoenwald and Belcastro, 1969; Chiou and Smith, 1970; Cho, 1970; Patel and Nagabhushan, 1970; Flynn and Roseman, 1971; Kakemi et al., 1971; Kazmi, 1971; McCarthy, 1972; Nasim et al., 1972; Jacob and Gilbert, 1973a,b; Fig. 36). The diafiltration data for the interaction of benzoic acid with cetomacrogol gave higher values of \(K\) than the value of \(K\) obtained using equilibrium dialysis (Fig. 19). It is
possible that an increase in the value of $K$ using diafiltration could be due to binding of benzoic acid with materials of the diafiltration apparatus. If this is true, then values of $n$ for diafiltration binding curves should be higher than the values of $n$ obtained using equilibrium dialysis technique. However, since the reverse is the case, the higher values of $K$ for the diafiltration curves must be due to an effect other than binding of benzoic acid with diafiltration apparatus.

Ryan and Hanna (1971) suggested that the membrane properties may change during diafiltration. A decreased flow rate with increase in protein concentration was reported (Ryan and Hanna, 1971; Palmer, 1972). It was suggested that the decrease in the flow rate may be due to clogging of the membrane pores with increasing concentrations of the protein. Recently, Palmer et al. (1973) reported flux rate changes in the ultrafiltration of dilute aqueous solutions of surface-active and nonsurface-active solutes through commercially available cellulose acetate and polysalt complex (Amicon UM05) membranes. Triton X-100, sodium dodecylbenzenesulfonate, alkyl(dimethyl)benzylammonium chloride (Hyamine 3500), and carbowax 600 were chosen as the model nonionic surfactant, anionic surfactant, cationic surfactant, and nonionic symmetrical solute respectively. Flux declines resulting from specific solute-membrane interactions were observed for Triton X-100 with cellulose acetate membranes and for the ionic surface-active agents with polysalt membranes. Although small flux declines were observed for the ionic surfactants with cellulose acetate membranes and for the nonionic surfactant with polysalt complex membranes, these small flux-limiting effects were within the range of those observed for the nonsurface-active carbowax 600. Interaction of cetomacrogol and Triton X-100 with
Millipore VS membrane was demonstrated using equilibrium dialysis technique (Kazmi, 1971; Kazmi, unpublished results). In the present study flux rate changes due to possible specific or non-specific interaction of surfactant with the membrane should have little effect on the benzoic acid-cetomacrogol binding results because samples of ultrafiltrate were collected on a volume rather than time basis.

Ryan and Hanna (1971) considered the possibility of conformational changes in the bovine serum albumin (BSA) during diafiltration. The albumin solution was washed in the diafiltration cell for 12 hours with buffer. A subsequent equilibrium dialysis experiment indicated that the washed BSA had a decreased affinity for testosterone. Palmer (1972) suggested that alteration in the binding characteristics, either through conformational changes or by polymerisation and/or denaturation could be caused by effects of pressure or stirring stress on the protein molecule. Unlike proteins, nonionic surfactants are very stable, and it is quite unlikely that the observed displacement of the binding curves in the diafiltration studies involving benzoic acid-cetomacrogol interaction could be caused due to conformational changes in the surfactant molecules.

Palmer (1972) observed changes in the volume of macromolecule in the cell during diafiltration studies. Similar observations were made in this work. This is due to an initial drop of cell pressure because of the large difference in the volumes of reservoir and the cell. A pressure drop in the cell will cause an overflow of preservative solution from the reservoir into the cell resulting in dilution of the surfactant solution. The uncorrected value of $[M]$ in the binding calculations will produce a downward displacement of the binding curves (Palmer, 1972), as observed in this work.
(Fig. 19). This effect is analogous to dilution of surfactant due to osmosis in the equilibrium dialysis study (C,a). It is suggested that dilution of surfactant during diafiltration is the major cause of the discrepancy between the diafiltration and the equilibrium dialysis binding results.

It is concluded that the anomalous binding curves obtained using the diafiltration technique are due to inherent technical artifacts of the method. Unless these are understood for each drug-macromolecule system, diafiltration is not a suitable technique for studying drug-macromolecule interactions. The difficulties in the diafiltration method overweigh its various advantages over the more commonly used techniques, such as equilibrium dialysis. Therefore, the diafiltration method was not used further for studying preservative-surfactant interactions. Although equilibrium dialysis is a slow technique and requires numerous separate experiments to fully characterize the binding curve, it is thermodynamically sound and, if carefully used, the binding results are free from most of the technical artifacts.

D. Interaction of Preservative Mixtures with Cetomacrogol

The preservative combinations were selected on the following grounds.

1. Preservative combinations in which both the preservatives have weak association with the surfactant molecule, e.g., benzoic acid and sorbic acid.

2. Preservative combinations in which both the preservatives have strong association with the surfactant molecule, e.g., chlorocresol and propyl paraben.
3. Preservative combinations in which one preservative has weak association and the other has strong association with the surfactant molecule, e.g., chlorocresol and methyl paraben; chloroxylenol and methyl paraben.

Figure 20 is a Scatchard plot for the interaction of chlorocresol with cetomacrogol in the absence and presence of a constant concentration of methyl paraben. Increasing the concentration of methyl paraben results in a downward displacement of the binding curve (curves B and C). This indicates that there is a possible competition between the chlorocresol and methyl paraben for the same binding sites on a surfactant molecule in a micelle. The experimental data are indicated by points, while the solid lines are theoretical. Curve A was calculated using n and K values for chlorocresol computed from Equation 25. Curves B and C were generated by substituting the values of n, K_d, K_c (obtained from independent binding studies for the individual preservatives) and the experimental [D_f] and [C_f] into Eq.30 (see appendix 3). The same method was used for calculating the theoretical binding curves shown in Figures 21, 23, 24 and 26. Figure 20 shows reasonable agreement between the theoretical and the experimental values, particularly in view of the fact that small normal variations in the values of the four association constants in Eq.30 can have rather marked influences on the shape and position of a theoretically generated plot.

Figure 21 is similar to Fig. 20, and shows the inhibition of the binding of chlorocresol with cetomacrogol in the presence of a constant concentration of propyl paraben. Curve B' is a theoretical binding curve for the competitive interaction (Eq.30), whereas curve B is obtained experimentally. No correlation was obtained between the experimental and the
Fig. 20. Scatchard plot for the interaction of chlorocresol with cetomacrogol in absence and presence of methyl paraben. Cetomacrogol concentration = 7.69 x 10^{-3} moles 1^{-1}. Methyl paraben concentration: 0, 0.0; □, 8.54 x 10^{-3}; ○, 13.14 x 10^{-3} moles 1^{-1}. Points experimental, curves B and C calculated using Eq.30.
Fig. 21. Scatchard plot for the interaction of chlorocresol with cetomacrogol in absence and presence of propyl paraben. Cetomacrogol concentration = 7.69 x 10^{-3} moles l^{-1}. Propyl paraben concentration: ○, 0.0; ●, 8.3 x 10^{-3} moles l^{-1}. Points experimental, curve B' calculated using Eq. 30. Binding parameters for propyl paraben derived from Fig. 22.
Fig. 22. Scatchard plot for the interaction of propyl paraben with cetomacrogol. Cetomacrogol concentration = $7.69 \times 10^{-3}$ moles l$^{-1}$. 
theoretical curves.

Figure 23 shows the interaction of methyl paraben with cetomacrogol in the absence and presence of a fixed concentration of chlorocresol. Values of n and K for methyl paraben alone were obtained from Fig. 22. Increasing the concentration of chlorocresol results in downward displacement of the binding curve (curves B and C). However, unlike Figure 20, increasing the concentration of competitor results in a decrease in the value of n and an increase in the value of K. Since in a simple competition, of the type described by Eq. 30, n should remain constant and K changes, Fig. 23 suggests that the competition between methyl paraben and chlorocresol for the binding sites in cetomacrogol molecule is of a complex nature. Disagreement between the experimental (curves B and C) and the theoretical (curves B' and C') binding curves supports this view. Similar results were obtained for the interaction of methyl paraben with cetomacrogol in the presence of chloroxylenol (Fig. 24, values of n and K for chloroxylenol alone were obtained from Fig. 25) and the interaction of benzoic acid with cetomacrogol in the presence of sorbic acid (Fig. 26, values of n and K for sorbic acid were obtained from Fig. 27).

Correlation between the theoretical and the experimental values will be obtained if the following conditions are observed:

I The preservative and the competitor share exactly the same locus of solubilization in the surfactant micelle.

II Interaction of a preservative with the surfactant neither alters the nature nor the number of binding sites in the surfactant micelle.

The first condition requires an examination of the locus of
Fig. 23. Scatchard plot for the interaction of methyl paraben with cetomacrogol in absence and presence of chlorocresol. Cetomacrogol concentration = $7.69 \times 10^{-3}$ moles l$^{-1}$. Chlorocresol concentration: $\bigcirc$, 0.0; $\square$, $1.19 \times 10^{-3}$; $\bigcirc$, $2.24 \times 10^{-3}$ moles l$^{-1}$. Points experimental, curves $B'$ and $C'$ calculated using Eq.30.
Fig. 24. Scatchard plot for the interaction of methyl paraben with cetomacrogol in absence and presence of chloroxylenol. Cetomacrogol concentration = 7.69 x 10^{-3} moles l^{-1}. Chloroxylenol concentration: 0, 0.0; 8.9 x 10^{-3} moles l^{-1}. Points experimental, curve B' calculated using Eq.30. Binding parameters for chloroxylenol derived from Fig. 25.
Fig. 25. Scatchard plot for the interaction of chloroxylenol with cetomacrogol. Cetomacrogol concentration = $7.69 \times 10^{-3}$ moles $1^{-1}$.
Scatchard plot for the interaction of benzoic acid with cetomacrogol in absence and presence of sorbic acid. Cetomacrogol concentration = $7.69 \times 10^{-3}$ moles l$^{-1}$. Sorbic acid concentration = 0, 0.0; $4.46 \times 10^{-3}$; $10.7 \times 10^{-3}$ moles l$^{-1}$. Points experimental, curves B' and C' calculated using Eq.30. Binding parameters for sorbic acid derived from Fig. 27.
Fig. 27. Scatchard plot for the interaction of sorbic acid with cetomacrogol. Cetomacrogol concentration = $7.69 \times 10^{-3}$ moles l$^{-1}$. 
solubilization of the preservatives, used in this study, in the cetomacrogol micelle. The locus of solubilization generally depends on the polarity of the solute and the HLB of the surfactant (Corby and Elworthy, 1971a).

Jacob et al. (1971) demonstrated, using a NMR technique, that phenol was mainly located in the polyoxyethylene region of the cetomacrogol micelle. Mulley and Metcalf (1956) suggested from UV spectroscopy studies that chloroxylenol was located in the polyoxyethylene region of the surfactant micelle. Higuchi and Lach (1954) also hypothesized that compounds like phenol would form hydrogen bonds with polyoxyethylene groups of nonionic macromolecule. Patel (1967) studied the interaction of a number of phenols, including chlorocresol, with cetomacrogol and polysorbate 80. An increased binding affinity of p-chlorophenol over phenol for both macromolecules was attributed to its greater capacity to undergo hydrogen bond formation. Thus substitution of a chlorine atom in the benzene ring increased the proton donating power of phenol. Hence, from these studies it is apparent that the locus of solubilization of chlorocresol and chloroxylenol is largely in the oxyethylene chain and involves hydrogen bonding between the acidic hydrogen of the phenol and the electrophilic oxygen of the ethylene oxide chain.

Corby and Elworthy (1971b) identified the sites of solubilization of p-hydroxybenzoic acid and its esters in a cetomacrogol micelle using UV, NMR, viscometry and solubility techniques. p-Hydroxybenzoic acid was wholly solubilized deep in the oxyethylene layer of the micelle. Ethyl p-hydroxybenzoate was solubilized mostly in the oxyethylene layer adjacent to the core, while some solubilization occurred within the core. Butyl p-hydroxybenzoate was solubilized mainly at the oxyethylene-hydrocarbon junction, with
the phenyl ring in the oxyethylene region and the butyl chain in the core. Some solubilizate was wholly present in the core. Thus the locus of solubilization of p-hydroxybenzoates depends on the polarity of the molecule. Since methyl and propyl parabens fall between p-hydroxybenzoic acid and butyl paraben, it is logical to assume that methyl paraben will be solubilized mostly in the oxyethylene layer while propyl paraben will be located near or at the oxyethylene-hydrocarbon junction.

Donbrow and Rhodes (1964, 1966, 1967) showed from their UV and NMR spectroscopic studies that benzoic acid was located at the junction of the hydrocarbon nucleus and the palisade layer (ethylene oxide chain) of the cetomacrogol micelle, with the lipophilic benzene ring enclosed within the nucleus and the hydrophilic carboxylic acid group protruding into the palisade layer. According to these authors, benzoic acid so located would lack mobility because of the presence of the polyethylene oxide chain of the surfactant molecules. The location could also allow hydrogen bond formation between the acidic hydrogen atom of benzoic acid and the electrophilic ether oxygen atom of the innermost palisade layer of the cetomacrogol micelle. Since sorbic acid lacks a phenyl ring and is less lipophilic than benzoic acid, it will be principally located in the palisade layer. The interaction between sorbic acid and nonionic macromolecules, such as polysorbate 80 and cetomacrogol, involves hydrogen bond formation between acidic hydrogen and the electrophilic oxygen of the polyoxyethylene layer (Blaug and Ahsan, 1961a). Thus the locus of solubilization of benzoic acid and sorbic acid in the cetomacrogol micelle is not exactly the same.

It is concluded that none of the preservative combinations used in this study have exactly the same locus of solubilization in the cetomacrogol
micelle. Thus simple competition, as described by Eq. 30, is not possible.

Another possible explanation for the disagreement between the theoretical and the experimental binding curves comes from studies concerning the effect of solutes on micellar molecular weight (Mn). As indicated in the Literature Survey (B, e), various authors have demonstrated that the interaction of solutes with surfactants can cause micellar perturbation resulting in a change of micellar molecular weight (Mn). A change in Mn can be due to a change in the aggregation number of the micelles, or inclusion of solubilizate into the micelle. Changes in the aggregation number of a micelle will affect the total number of binding sites available for the interaction. These studies were made using a single solute. Hence, the complexity involved in interpreting the effects of more than one solute on Mn is readily apparent.

In this work, curvature in the Scatchard plot, for the interaction of a preservative with the nonionic surfactant, was assumed to be due to the existence of two entirely different classes of binding sites in the surfactant molecule of a micelle, that both sites were independent and that sites within the class were equivalent. However, a curvature in the Scatchard plot may also be due to, what biochemists term, negative cooperativity, i.e., uptake of solute within the micelle, which progressively alters the interaction between the binding sites and the solute and leads to a change in both the number of sites available for interaction and a decrease in the association constant. In addition to the factors discussed above, the observed displacement of binding curves (Figs. 20, 21, 23, 24 and 26) may be due to cooperative or "anticooperative" interactions between two preservatives.
Although the reasons for the disagreement between the theoretical and the experimental results are not clear, these findings may have considerable practical significance. For example, reduction in the degree of interaction of one or both components of a preservative mixture may result in an increased free concentration of preservative, for a given concentration added, and consequently in enhanced antimicrobial activity. The net result would be an apparent synergism between the two preservatives. On the other hand, if the extent of binding of one or both compounds is increased, the free concentration would then be reduced. Consequently, the preservative activity would be less than that anticipated from binding studies of the individual component.

E. Interaction of Chlorocresol with Mixtures of Some Nonionic Surfactants

Figure 28 is a Scatchard plot for the binding of chlorocresol with Texofor $A_{16}$ (curve A), Texofor $A_{60}$ (curve F) and various mixtures of Texofor $A_{16}$ and Texofor $A_{60}$ (curves B to E). Curves A and F are independent of surfactant concentration. The values of the binding parameters (n's and K's) for curve F are of greater magnitude than curve A. An increase in the value of n indicates that an increase in the ethylene oxide chain results in an increase in the number of binding sites in the surfactant molecule. This suggests that the locus of solubilization of chlorocresol is largely associated with ethylene oxide chain of the surfactant molecule. An increase in K shows that an increase in ethylene oxide chain length results in an increase in the extent of solubilization of preservative per mole of surfactant. The binding curves for Texofor $A_{16}$ and Texofor $A_{60}$ mixtures are independent of surfactant concentration only when the molar ratio of Texofor $A_{16}$ to Texofor
Fig. 28. Scatchard plot for the interaction of chlorocresol with Texofor A\textsubscript{16}, Texofor A\textsubscript{60} and mixtures of Texofor A\textsubscript{16} and Texofor A\textsubscript{60}.

A - Texofor A\textsubscript{16}: concentration of Texofor A\textsubscript{16} (moles l\textsuperscript{-1}) : O, 10.57 \times 10^{-3}; \Large{\triangle}, 31.71 \times 10^{-3}; \Large{\square}, 52.85 \times 10^{-3}. B to E - Texofor A\textsubscript{16} + Texofor A\textsubscript{60}: concentration of Texofor A\textsubscript{16} and A\textsubscript{60} in a mixture (moles l\textsuperscript{-1}): x, 50.89 \times 10^{-3}; A\textsubscript{16} + 2.75 \times 10^{-3}, A\textsubscript{60}; o, 10.57 \times 10^{-3} A\textsubscript{16} + 2.75 \times 10^{-3}, A\textsubscript{60}; \bullet, 10.57 \times 10^{-3}, A\textsubscript{16} + 13.26 \times 10^{-3}, A\textsubscript{60}; \Large{\diamond}, 5.28 \times 10^{-3}, A\textsubscript{16} + 6.63 \times 10^{-3}, A\textsubscript{60}; \Large{\blacktriangle}, 2.11 \times 10^{-3}, A\textsubscript{16} + 5.51 \times 10^{-3}, A\textsubscript{60}. F - Texofor A\textsubscript{60}: concentration of Texofor A\textsubscript{60} (moles l\textsuperscript{-1}): O, 2.75 \times 10^{-3}; \Large{\triangle}, 8.26 \times 10^{-3}; \Large{\square}, 13.77 \times 10^{-3}.
A_{60} was kept constant (curve D). Increasing the molar ratio of Texofor A_{16} to Texofor A_{60} results in a shift of the binding curves towards curve A and a consequent decrease in the values of n and K. Similarly, increasing the molar ratio of Texofor A_{60} to Texofor A_{16} results in the shift of the binding curves towards curve F and an increase in the value of n and K. This indicates that mixing the two surfactants in various molar ratios results in the formation of mixed micelles of different composition and binding characteristics. Where interaction of a solute involves a heterogeneous mixture of macromolecules, the value of n in the Scatchard treatment represents an average of the total number of binding sites of all the monomers of varying molecular weights (Karush, 1950). Based on this reasoning, the value of n for the binding of chlorocresol with various mixtures of Texofor A_{16} and Texofor A_{60} should be the mean of the total number of binding sites of Texofor A_{16} and Texofor A_{60} monomers in a mixed micelle.

Figure 29 is a Scatchard plot for the binding of chlorocresol with cetomacrogol and polysorbate 80. The binding curves are independent of surfactant concentration and there is no significant difference between the magnitude of the binding parameters for cetomacrogol and polysorbate 80. Similarly, the binding curves of the surfactant mixtures are almost independent of the molar ratio of mixing. This is presumably due to the close similarity in the number of ethylene oxide units of cetomacrogol and polysorbate 80; These observations further support the view that the locus of solubilization of chlorocresol is largely in the polyoxyethylene region of the micelle.
Fig. 29. Scatchard plot for the interaction of chlorocresol with cetomacrogol, polysorbate 80 and mixtures of cetomacrogol and polysorbate 80. A - Cetomacrogol; concentration of cetomacrogol (moles l⁻¹): same as given in Fig. 17. B - Polysorbate 80; concentration of polysorbate 80 (moles l⁻¹): ○, 7.64 x 10⁻³; △, 22.93 x 10⁻³; □, 38.22 x 10⁻³. Concentration of cetomacrogol (C) and polysorbate 80 (P) in a mixture (moles l⁻¹): ●, 7.69 x 10⁻³, C + 7.64 x 10⁻³, P; ◇, 23.11 x 10⁻³, C + 7.64 x 10⁻³, P; ■, 7.69 x 10⁻³, C + 22.97 x 10⁻³, P.
Figures 30 and 31 show plots of $[D_t]$ versus $[D_f]$ for surfactants mixed in various molar ratios. The curves were calculated by substituting the values of n's and K's, determined experimentally for each surfactant, into Eq.37 and solving for $[D_t]$ at given $[D_f]$ values (see appendix 4). Agreement between the calculated curves and the experimental values confirms that the binding behavior of surfactant mixtures can be predicted using Eq.37 and the binding parameters of the individual surfactants.

$$[D_t] = [D_f] \left\{ \frac{n_1 K_1 [M_I]}{1 + K_1 [D_f]} + \frac{n_2 K_2 [M_I]}{1 + K_2 [D_f]} + \frac{n_1' K_1' [M_{II}]}{1 + K_1' [D_f]} + \frac{n_2' K_2' [M_{II}]}{1 + K_2' [D_f]} \right\}$$

(Eq.37)

The classification of nonionic surfactants in terms of their HLB values is of great practical importance in formulation. Wedderburn (1958) suggested that the binding of a preservative with a nonionic surfactant is a function of the HLB of the surfactant and the physico-chemical properties of the preservative. Equation 37 indicates that the extent of preservative binding in a surfactant mixture is a simple summation of the binding observed using the individual surfactants. Since the HLB values of surfactant mixtures are also obtained by addition of the individual surfactant HLB values, a simple relationship to be expected between HLB values and solute-surfactant binding. HLB values of individual surfactants and surfactant mixtures were calculated using Eqs.57 and 58, respectively (Griffin, 1954).

$$\text{HLB of a surfactant} = \frac{E}{5} \quad \text{(Eq.57)}$$

$$\text{HLB of a surfactant mixture} = (f) \frac{E_1}{5} + (1-f) \frac{E_2}{5} \quad \text{(Eq.58)}$$
Fig. 30. Variation of free preservative concentration $[D_f]$ with total preservative concentration $[D_t]$ for the interaction of chlorocresol with mixtures of Texofor $A_{16}$ and Texofor $A_{60}$. Concentration of Texofor $A_{16}$ and Texofor $A_{60}$ in a mixture (moles l$^{-1}$): $\times$, $5.089 \times 10^{-3}$, $A_{16} + 2.75 \times 10^{-3}$, $A_{60}$; $\bullet$, $10.57 \times 10^{-3}$, $A_{16} + 2.75 \times 10^{-3}$, $A_{60}$; $\circ$, $10.57 \times 10^{-3}$, $A_{16} + 13.26 \times 10^{-3}$, $A_{60}$; $\blacklozenge$, $2.11 \times 10^{-3}$, $A_{16} + 5.51 \times 10^{-3}$, $A_{60}$. Points experimental, curves calculated using Eq.37.
Fig. 31. Variation of free preservative concentration $[D_f]$ with total preservative concentration $[D_t]$ for the interaction of chlorocresol with mixtures of cetomacrogol and polysorbate 80. Concentration of cetomacrogol (C) and Polysorbate 80 (P) in a mixture (moles $l^{-1}$): \(\bullet\), $7.69 \times 10^{-3}$ C + $7.64 \times 10^{-3}$, P; \(\Delta\), $23.11 \times 10^{-3}$, C + $7.64 \times 10^{-3}$, P; \(\ldots\), $7.69 \times 10^{-3}$, C + $22.97 \times 10^{-3}$, P. Points experimental, curves calculated using Eq.37.
Where:

\[
E = \text{Weight per cent ethyleneoxide in a surfactant molecule.}
\]

\[
E_1 = \text{Weight per cent ethylene oxide in a surfactant molecule of type I.}
\]

\[
E_2 = \text{Weight per cent ethylene oxide in a surfactant molecule of type II.}
\]

\[
f = \text{Fraction of a surfactant in surfactant mixture.}
\]

Figures 32 and 33 show that there is a linear relationship between HLB and $[D_r]$, where $[D_r]$ values were calculated using Eq. 37 for given values of $[D_f]$. For the interaction of chlorocresol with Texofor $A_{16}$, $A_{60}$, Fig. 32 shows that an increase in HLB results in a decrease in the value of $[D_r]$, i.e., in practical terms, a smaller total preservative concentration is required to maintain a given concentration of free preservative in the aqueous phase as the composition of the surfactant mixture is changed to increase the HLB. Such results indicate that when surfactant concentrations are expressed as equivalents of ethylene oxide per cent, there is an apparent decrease in the efficiency of solubilization with increase in ethylene oxide chain length. Similar binding characteristics have been reported for the interaction of aldehydes with polyoxyethylene ethers of varying ethylene oxide chain length (Mitchell and Wan, 1964), and the interaction of benzoic acid derivatives (Goodhart and Martin, 1962) and barbiturates (Gouda, Ismail and Motawi, 1970) with polyoxyethylene stearates of varying ethylene oxide chain length using solubility techniques. The decrease in the efficiency of solubilization with increase in the number of ethylene oxide units was explained (Mitchell and Wan, 1964) in terms of a decrease in the micellar molecular weight and in the number of surfactant molecules per micelle as the ethylene oxide chain is lengthened.

In contrast to Texofors $A_{16}$ and $A_{60}$, the interaction of chlorocresol
Fig. 32. $[D_f]$ versus HLB at constant $[D_f]$ for the interaction of chlorocresol with Texofor A$_{16}$, Texofor A$_{60}$ and mixtures of Texofor A$_{16}$ and Texofor A$_{60}$. Total concentration of surfactant or surfactant mixtures = 1% (w/v). Concentration of free chlorocresol $[D_f]$: A, $1.0 \times 10^{-3}$; B, $2.0 \times 10^{-3}$; C, $3.0 \times 10^{-3}$ moles l$^{-1}$. HLB Texofor A$_{16} = 14.88$; HLB Texofor A$_{60} = 18.67$. Curves calculated using Eq.37.
Fig. 33. \([D_t] \) versus HLB at constant \([D_f] \) for the interaction of chlorocresol with cetomacrogol, polysorbate 80 and mixtures of cetomacrogol and polysorbate 80. Total concentration of surfactant or surfactant mixture = 1% (w/v). Concentration of free chlorocresol \([D_f] \): A, \(0.5 \times 10^{-3}\); B, \(1.0 \times 10^{-3}\); C, \(1.5 \times 10^{-3}\) moles l\(^{-1}\). HLB cetomacrogol = 15.57. HLB polysorbate 80 = 13.46. Curves calculated using Eq.37.
with cetomacrogol, polysorbate 80 and mixtures of cetomacrogol and polysorbate 80 is virtually independent of HLB for a given \([D_f]\), Fig. 33. Since cetomacrogol and polysorbate 80 have approximately the same number of ethylene oxide units, Fig. 33 supports the suggestion that solubilization is associated essentially with the ethylene oxide chain.

Kazmi and Mitchell (1971) described a three-chambered dialysis method for determining the distribution of a preservative in an emulsion and discussed the advantages of this direct approach over determining the necessary factors for substitution into a mathematical model. One of the problems with the model approach is that most emulsions contain more than one surfactant and the degree of binding will depend on the nature and composition of the surfactant mixture. However, the present work shows that it is unnecessary to determine the binding parameters for each surfactant mixture since the degree of interaction of the mixture with a preservative can be predicted from a knowledge of the parameters of the individual surfactants comprising the mixture.

F. Distribution of Chlorocresol in Liquid Paraffin-Water Systems

Figure 34 shows the concentration of chlorocresol in the oil phase \([D_o]\), plotted as a 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 liquid paraffin and water obeys the simple partition law. The slope of the line gives the partition coefficient, \(K^O_w\). The value of \(K^O_w\) obtained from the slope was 1.67 which is close to the value of 1.53 reported in the literature (Bean et al., 1965). A small discrepancy between the observed and the literature values of \(K^O_w\) may be due to the fact that light liquid paraffin varies from one batch to another.
Fig. 34. 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 liquid paraffin and water. Points experimental, line fitted using least squares method. Slope = 1.67.
G. Calculation of Total Concentration of Chlorocresol Required in Cetomacrogol Solutions to Produce the Desired Concentration of Free Preservative in the Aqueous Phase

Figure 35 shows the interaction of chlorocresol with various concentrations of cetomacrogol (derived from Fig. 17, silicone rubber data) represented in the form of \([D_t]\) versus \([D_f]\). There is close agreement between the experimentally determined values and the curves predicted using Eq.28. The values of the binding parameters (n's and K's) for the substitution into Eq.28 were derived in a similar manner to that described earlier (C(a)2).

\[
[D_t] = [D_f] \left\{ 1 + \frac{n_1 K_1[M]}{1 + K_1[D_f]} + \frac{n_2 K_2[M]}{1 + K_2[D_f]} \right\} \quad \text{(Eq.28)}
\]

Hence, Eq.28 is valid and enables a calculation to be made of the total concentration of preservative required in a surfactant solution to produce the desired concentration of free preservative in the aqueous phase.

H. Calculation of Concentration of Chlorocresol Required in a Liquid Paraffin Emulsion to Produce the Desired Concentration of Free Preservative in the Aqueous Phase

(a) Estimation of free chlorocresol concentration in a liquid paraffin emulsion

Analysis of the concentration of chlorocresol in the aqueous compartment of the two-chambered dialysis cell enables the free chlorocresol concentration, \([D_f]\), to be determined. From \([D_f]\), amount of chlorocresol bound to the dialysis membrane (Fig. 36) and the total amount of chlorocresol
Fig. 35. Variation of free preservative concentration $[D_f]$, with total preservative concentration, $[D_t]$, for the interaction of chlorocresol with cetomacrogol. Cetomacrogol concentrations (%): $\bullet$, 1.0; $\blacktriangle$, 2.0; $\square$, 3.0; $\circ$, 5.0; $\triangle$, 10.0. Points experimental, curves calculated using Eq.28.
Fig. 36. Binding of chlorocresol with silicone membrane. Points experimental, line fitted using least squares method.
added, \( [D_\circ] + [D_b] \) can be determined, although it is not possible to separate these quantities. Where it is desirable to differentiate between \( [D_\circ] \) and \( [D_b] \), a three chambered dialysis technique, as described by Kazmi and Mitchell (1971b), can be used (see page 62 and 141).

(b) Figure 37 is a plot of the total preservative concentration, \( [D] \), versus the concentration of free preservative in the aqueous phase, \( [D_f] \), for the distribution of chlorocresol between light liquid paraffin-water-cetomacrogol systems of a fixed oil-water ratio. There is close agreement between the experimentally determined values and the curve predicted using Eq. 48. Close agreement was also found between \( [D_f] \) predicted using Eq. 48 and the observed values for the distribution of chlorocresol between liquid paraffin-water-cetomacrogol systems of varying oil-water ratios (Table 6). Thus, equation 48 is valid and permits a calculation to be made of the total concentration of the preservative required in an emulsion to provide the desired concentration of free preservative in the aqueous phase.

\[
[D] = \left\{ [D_f] \left[ 1 + n_1 K_1 M / (1 + K_1 [D_f]) + n_2 K_2 M / (1 + K_2 [D_f]) + K_2^2 q \right] \right\} / (q + 1)
\]

(Eq. 48)

I. Correlation of Physico-Chemical Data with Antimicrobial Activity

In the previous sections (G and H), physico-chemical models for predicting the required preservative concentration in surfactant solutions and emulsions were evaluated. The implicit assumption underlying the development of these models is that antimicrobial activity is largely a function of the concentration of free preservative in the aqueous phase and that preservative bound to surfactant micelles or partitioned into the oil
Fig. 37. Variation of free chlorocresol concentration, \( [D_f] \), in the aqueous phase of the emulsion with total chlorocresol, \([D]\), for an O/W emulsion containing 50% v/v liquid paraffin emulsified with 3% w/v cetomacrogol. Points experimental, curve calculated using Eq.48.
TABLE 6. Chlorocresol Concentration in Liquid Paraffin Emulsions Stabilized with Cetomacrogol

<table>
<thead>
<tr>
<th>Oil/Water ratio</th>
<th>Moles l⁻¹ x 10³</th>
<th>Standard Deviation 10⁵</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td>[D] Calculated using Eq.48</td>
<td>[D_r] Required</td>
</tr>
<tr>
<td>0.2</td>
<td>27.15</td>
<td>2.45</td>
</tr>
<tr>
<td>0.5</td>
<td>22.54</td>
<td>2.45</td>
</tr>
<tr>
<td>1.0</td>
<td>17.93</td>
<td>2.45</td>
</tr>
</tbody>
</table>

*Mean of three observations.

Cetomacrogol concentration = 23.1 x 10⁻³ moles l⁻¹
phase is biologically inactive. Good correlations were obtained between the predicted and the observed values of free preservative concentration in the aqueous phase, \([D_f]\), using Eq.28 for surfactant solutions and Eq.48 for emulsions.

The present work is an attempt to correlate the physico-chemical data with the observed antimicrobial activity in order to evaluate the validity of the hypothesis that antimicrobial activity is largely a function of the concentration of free preservative and is essentially independent of factors such as surfactant concentration and oil-water ratio. The death rate of \(E. coli\) was taken as a measure of preservative activity.

(a) Comparison between Millipore and Pour-Plate Techniques

The death rate of micro-organisms in a given solution is generally studied by counting the number of surviving organisms as a function of time. Pour-plate technique or roll tube method are generally used for the viable counting of micro-organisms. These techniques involve dilution of the sample in normal saline or \(\frac{1}{2}\) strength Ringer's solution and subsequent plating on agar. A few difficulties arise with the use of these techniques for studying the death rate of micro-organisms in systems containing preservatives. Large dilutions are required to reduce the concentration of the preservative which would otherwise inhibit the growth of micro-organisms upon plating. This is a serious limitation, especially when the death rate is followed up to 100% mortality. Since at high mortality levels large dilutions are not possible, the chances of carry over of preservative to the growth medium are great. The Millipore method obviates these problems by rinsing the test filter with sterile fluid after sample filtration. In
the Millipore procedure, the test filter is a special type having a hydrophobic rim approximately 3 millimeters wide. This prevents intrusion of the sample fluid into the area under the sealing edge of the filter holder. No matter how large a sample is used, all growth-inhibiting residuals are within immediate reach of the rinse fluid, and are diluted to the point where they have no discoverable effect on culturing. Thus, using the Millipore technique, it is possible to follow the death rate to any desired level of mortality without limiting the sample size or without any danger of residual preservative inhibiting the growth of micro-organisms.

Figure 38 shows that there was a good correlation between the Millipore method and the pour-plate technique for the enumeration of *E. coli*. Thus the Millipore technique gave viable counts comparable with the well established pour-plate method.

(b) Bactericidal Activity of Chlorocresol in Water

Figure 40 is a plot of the logarithm of the number of organisms surviving versus time (semi log plot) for the bactericidal activity of chlorocresol in water against *E. coli*. For a given chlorocresol concentration a curvilinear relationship, instead of straight line, was obtained. This suggests that the death rate of *E. coli* in the aqueous chlorocresol solutions does not follow first order kinetics. Similar relationships were obtained for the death rate of *E. coli* in solutions of chlorocresol in cetomacrogol and liquid paraffin emulsions. However, when the same data is represented in the form of probit % survivors versus time plot (probit plot), a straight line relationship (Fig. 41) results. This means that the extinction rate of *E. coli* follows a normal distribution.
Fig. 38. Comparison between Millipore filtration and pour-plate techniques for the enumeration of *E. coli*. Different symbols represent separate experiments. Correlation coefficient = 0.95.
Fig. 39. Semilogarithmic plot of the number of organisms/ml as a function of time for the survival of *E. coli* in, A - water, B - aqueous cetomacrogol solutions; concentration of cetomacrogol as in Fig. 40, C - liquid paraffin emulsions; values of q and [M] as in Fig. 45.
Fig. 40. Semilogarithmic plot of the number of organisms/ml as a function of time for the bactericidal activity of chlorocresol in water against *E. coli*. Chlorocresol concentration (%): □, 0.02; ○, 0.03; ★, 0.035.
Fig. 41. Probit % survivors as a function of time for the bactericidal activity of chlorocresol in water against E. coli. Chlorocresol concentration (%): □, 0.02; ○, 0.03; ×, 0.035.
Probit plots offer many advantages over semilog plots, e.g., a straight line is easier to fit to a set of points than a nonlinear relationship; it is easier to perform statistical analyses of the data; the slope of probit plot seems to be little influenced by small changes in the concentration of organisms in the inoculum (Kavanagh, 1963), whereas a small change in the inoculum size produces marked change in the slope of a semilog plot; extrapolation of the straight line in the probit plot can give information about the time required to achieve 100% mortality with much more accuracy than extrapolation of the curve in the semilog plot. Thus, in the present work the probit plot is considered as a method of choice for the graphical presentation of the data on the death kinetics of E. coli in aqueous solutions, surfactant solutions and liquid paraffin emulsions containing chlorocresol.

Figure 41 is a plot of the probit % survivors versus time for the bactericidal activity of chlorocresol in water against E. coli. The symbols represent experimental data, while lines were fitted using Wang calculator programmed for linear regression analysis (GLICKSTAT, designed by Dr. Glick, Department of Mathematics, U.B.C.). Increasing the concentration of chlorocresol from 0.02% to 0.03% makes little change in the death rate of E. coli. However, at a concentration of 0.035% chlorocresol, there is a marked increase in the death rate. Since 0.035% chlorocresol produced 99% mortality within 6-8 hours, a time suitable from the viewpoint of experimental design, this concentration was selected as a reference standard to which the bactericidal activity of chlorocresol in aqueous cetomacrogol solutions and liquid paraffin emulsions could be compared (see sections c and d). The results of the control experiments are shown in Fig. 39(A).
The viability of *E. coli* in water remained almost unchanged during the experimental period of eight hours.

(c) Bactericidal Activity of Chlorocresol in Aqueous Solutions of Cetomacrogol

Figure 42 shows probit % survivors plotted as a function of time for the bactericidal activity of chlorocresol in water (curve A) and aqueous solutions of cetomacrogol against *E. coli*. The aqueous solution contained 0.035% chlorocresol and the surfactant solutions contained sufficient total preservative, calculated from Eq. 28, to provide a free concentration of 0.035% chlorocresol in the aqueous phase. The symbols represent experimental data and the lines were fitted using the linear regression analysis program.

The slopes of the lines in Fig. 42 were compared using *t* tests, without assumption of equal variances (Eq. 59), and using modified Welch degrees of freedom (Eq. 60). Test of significance between the two slopes were performed using the null hypothesis of equality against a one sided alternative.

\[
t = \frac{b_1 - b_2}{\sqrt{S_{b1}^2 + S_{b2}^2}}
\]

where \(b_1\) and \(b_2\) = slopes of curves 1 and 2 respectively.

\[
\frac{1}{\text{D.F.}} = \frac{\theta^2}{N_1 - 2} + \frac{(1 - \theta)^2}{N_2 - 2}
\]

(Eq. 59) (Eq. 60)
Fig. 42. Probit % survivors as a function of time for the bactericidal activity of chlorocresol in aqueous cetomacrogol solutions against E. coli. Cetomacrogol concentration (%): A, 0.0 (from Fig. 41); B, O, 1.0; C, □, 3.0; D, Δ, 5.0. Total preservative concentration, [Dₜ] %: A, 0.035; B, 0.1743; C, 0.4528; D, 0.7314. Initial free preservative concentration, [Dᵢ], for all solutions = 0.035 %. Points experimental, lines fitted using least squares method.
where
\[ D.F. = \text{modified Welch degrees of freedom.} \]
\[ N_1 \text{ and } N_2 = \text{Number of observations for curves 1 and 2 respectively.} \]
\[ \varrho = \frac{(S_{b1}^2/N_1)}{(S_{b1}^2/N_1) + (S_{b2}^2/N_2)} \]

Table 7 gives the summary of the statistical analysis of the slopes of the curves in Fig. 42. Curves B, C and D (surfactant solutions) are significantly different from curve A (water). Curves C and D (3% and 5% cetomacrogol solutions respectively) are significantly different from Curve B (1% cetomacrogol solution). However, the difference between curves C and D is nonsignificant.

Since the aqueous and cetomacrogol solutions have the same initial \([D_f]\) and only \([D_t]\) varies, a significant difference between the slopes of the surfactant and aqueous curves suggests that the antimicrobial activity in cetomacrogol solutions is perhaps not simply a function of the concentration of preservative in the aqueous phase, and that some additional factors may be involved in controlling the antibacterial activity. The results and statistical analysis appear to show that:

1. the probit-survivor curves for the surfactant solutions are significantly different from those in water.
2. the slope of the probit-survivor curve is not independent of surfactant concentration.

In view of these findings it is necessary to examine the assumption underlying both the development of the mathematical models and
### TABLE 7. Comparison of Slopes in Fig. 42 Using t Test.

<table>
<thead>
<tr>
<th>Curve</th>
<th>$t$ Calculated using Eq.59</th>
<th>Modified Welch Degrees of Freedom (Eq.60)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td><strong>Comparison of curves B, C, and D with curve A</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>B</td>
<td>2.47</td>
<td>27.62</td>
<td>0.01</td>
</tr>
<tr>
<td>C</td>
<td>6.12</td>
<td>29.86</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>D</td>
<td>5.23</td>
<td>30.42</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td><strong>Comparison of curves C and D with curve B</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>3.77</td>
<td>43.75</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td>D</td>
<td>2.93</td>
<td>43.29</td>
<td>&lt; 0.005</td>
</tr>
<tr>
<td><strong>Comparison of curve C with curve D</strong></td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>C</td>
<td>0.70</td>
<td>43.88</td>
<td>&lt; 0.3</td>
</tr>
</tbody>
</table>
the experimental design.

(1) Comparison between Aqueous and Surfactant Solutions of Preservative

Although there is a significant difference between the slopes of the aqueous and surfactant curves, comparison with Fig. 41 shows that all the surfactant curves fall between the values of the slopes for 0.030% and 0.035% aqueous chlorocresol solutions, i.e., a relatively small difference in $[D_f]$ will produce a significant change in the slope. This concentration difference should be compared with the large difference between $[D_f]$ for each surfactant solution, i.e., B, 0.1743%; C, 0.4528%; D, 0.7324%. It is apparent from Fig. 41 that the slope of the probit-survivor curve is very sensitive to changes in $[D_f]$, in the concentration range chosen for these experiments. Therefore, any factor producing a change in $[D_f]$ will have a significant effect on the slope of the probit-survivor curve.

Two factors may change $[D_f]$ in surfactant solutions relative to a solution in water:

I. Depletion of preservative due to interaction with microorganisms or foreign materials (see Tables III and IV in Bean, 1972), volatilization, chemical decomposition (Nair and Lach, 1959), or even metabolism by the micro-organisms (Sokolski et al., 1962) will be significantly greater from a solution in water than from a solubilized system where the micelles will act as a reservoir of preservative. Figure 43 shows a direct relationship between the % loss of chlorocresol from the water and the aqueous phase of cetomacrogol (see appendix 5 for the calculation of this parameter), when equal amounts of chlorocresol are removed from water and the surfactant solution. On the percentage basis, for a given loss in
Fig. 43. Comparison between percent loss of chlorocresol from water and aqueous phase of cetomacrogol when equal amount of preservative is removed from water and cetomacrogol solution. Cetomacrogol concentration (%): A, 0.1; B, 0.5; C, 1.0; D, 2.0; E, 3.0; F, 4.0; G, 5.0.
the concentration of preservative from water, the loss in the concentration of preservative from the aqueous phase of the surfactant is smaller and is a function of the surfactant concentration. This is apparent from the decrease in the slope of curves with increase in the surfactant concentration. A slope approaching unity means that the ability of the system to resist changes in \([D_f]\) is decreasing in the direction of water which offers no resistance to change. If the ability of a system to resist changes in \([D_f]\) is defined as its **capacity** then the inverse of the slope of a given curve in Fig. 43 becomes a numerical expression for capacity. Allawala and Riegelman (1953) consider saturation solubility of a solute in a given system as a measure of capacity. Therefore, an increase in solubility results in an increase of capacity. Basically, both definitions are similar because saturation solubility of the preservative increases with increase in surfactant concentration. Similarly, the inverse of the slope in Fig. 43 increases with increase in surfactant concentration (Fig. 44). However, the definition of Allawala and Riegelman (1953) is qualitative in nature and does not give a numerical value to capacity. Figure 44 shows a linear relationship between the capacity and the surfactant concentration, \([M]\), for the interaction of chlorocresol with Texofor A_{16}, Texofor A_{23} (cetomacrogol) and Texofor A_{60}. For a given surfactant concentration the capacity decreases with an increase in the ethylene oxide chain. This is because the efficiency of solubilization decreases with an increase in the ethylene oxide units (see page 138). At zero surfactant concentration the capacity of the system is equal to 1.0. Thus, from Figs. 43 and 44, it is evident that the capacity of the system is an important factor which should be taken into consideration in any study involving a comparison of the preservative activity between two unlike systems such as water and surfactant solutions.
Fig. 44. Capacity as a function of surfactant concentration [M], for the interaction of chlorocresol with A—Texofor A$_{16}$, B—Texofor A$_{23}$ (cetomacrogol), and C—Texofor A$_{60}$. 
II. A second but less likely factor which may change $[D_f]$ is the adsorption of surfactant monomer on the microbial surface. This will reduce the free surfactant concentration. Re-establishment of the micelle-monomer equilibrium will lead to the liberation of some micellar preservative, an increase in $[D_f]$ and hence antimicrobial activity.

Hence, a comparison of antimicrobial activity in surfactant solutions with solutions in water may lead to confusion unless it can be shown that $[D_f]$ remains the same throughout the experiment. Previous studies have shown, as expected from the capacity concept, that surfactant solutions are slightly more active than aqueous solutions containing the same $[D_f]$ (Mitchell, 1964; Humphreys, Richardson and Rhodes, 1968; Brown, 1968; Bradshaw, Rhodes and Richardson, 1972). However, in the present work, the results in Fig. 42 show that the surfactant solutions are less active than the aqueous solution.

In view of the difficulties which arise in attempting to compare two unlike systems, such as aqueous and surfactant solutions, it is better to compare the antimicrobial activity of the preservative solubilized in various surfactant concentrations having the same initial value of $[D_f]$. Changes in $[D_f]$ due to interaction of preservative or surfactant with the micro-organism, or other factors, will be relatively less and any enhancement or diminution of activity can then be attributed to the presence of surfactant. This approach was used in designing the experiments for the present study (Fig. 42).
(2) Comparison between Surfactant Solutions of Preservative

A significant difference between the slopes of curve B and the slopes of curves C and D suggests that increasing the concentration of cetomacrogol results in a decrease in antimicrobial activity. However, there is no significant difference between the slopes of C and D and this indicates that increasing the concentration of cetomacrogol between 3%-5% produces little further change in activity. The small decrease in activity with increase in the cetomacrogol concentration could be due to the following factors:

I. **Stimulation of the growth of E. coli by cetomacrogol:** Stimulation of the growth of micro-organisms by nonionic surfactants is not an uncommon phenomenon. Dubos and Davis (1946) reported that polysorbate 80 encourages the growth of tubercle bacilli in culture media which they attribute to the surface activity of the nonionic surfactant. Englar (1950) and Williams et al. (1947) reported that nonionics stimulate the growth of certain micro-organisms, while Bolle and Mirimanoff (1950) found that all the nonionics they tested (carbowax 1500, some crills, spans, and tweens) possessed a stimulating action on the development of mould mycelia. de Navarre and Bailey (1956) and de Navarre (1957b) showed that fungal mycelial growth in media containing nonionics was more luxuriant than in plain media. However, in the present work, the possibility of the stimulating effect of cetomacrogol on the growth rate of *E. coli* is ruled out because the results of control experiments (Fig. 39,B) showed a slight decrease, instead of enhancement, of the growth of the organism in the surfactant solutions.

II. **Protection of E. coli by cetomacrogol from the lethal effect of**
chlorocresol: The possibility of this effect is very likely because there are evidences which suggest that very low concentrations of nonionic surfactants protect micro-organisms from the lethal effect of antimicrobial agents. Beckett et al. (1958a,b; 1959) have shown that addition of cetomacrogol, in as low a concentration as possible without affecting complex formation, reduced the uptake of hexylresorcinol from solutions by *E. coli* at all concentration levels of the phenol studied. According to these authors, cetomacrogol appeared to prevent or interfere with the interaction of phenol with the cytoplasmic membrane of the bacteria. Judis (1962) has also shown that the site of action of phenolic disinfectants in *E. coli* is at the cytoplasmic membrane, and Tween 80 protected *E. coli* from the lethal effects of chloroxylenol.

Wedderburn (1958) observed changes in Gram-staining characteristics of some bacteria after contact with nonionics. Gram-positive organisms turned Gram-negative after a period of contact with the nonionic and, on subculture, reverted back to Gram-positive. Similarly Gram-negative organisms upon contact with nonionics became Gram-positive for a period and, when transferred to media without nonionic, once again turned Gram-negative. This phenomenon was also observed by N. and A. Delmotte (1956). In the same study Wedderburn (1958) demonstrated that some organisms, including *E. coli*, were rendered more resistant to certain germicides after contact with nonionics. This resistance varied not only for different nonionics but also for different organisms. These phenomena were said to be due to the protective effect of nonionics on the cell wall of the micro-organisms.

From the observations quoted above it appears that the protection
offered by nonionics to micro-organisms against the lethal effect of the
antimicrobial agents is related to the mechanism of action of the preser-
vatives. Only the effect of those antimicrobial agents which exert their
lethal effect through direct action on the cytoplasmic membrane of the
organisms will be inhibited.

In contrast to the inhibitory effects of nonionic surfactants on
the antimicrobial activity of preservatives through protective action on
micro-organisms, several authors have demonstrated that nonionics potentiate
the antimicrobial activity of preservatives. However, in addition to the
various factors discussed in the literature survey an apparent synergistic
effect above CMC could be merely an artifact due to the use of an oversimpli-
fied physico-chemical model. In the present work Eq.28 was used to predict
the total concentration of preservative required in the surfactant solution
in order to achieve the desired concentration in the aqueous phase. A good
correlation between theoretically generated plots and experimentally deter-
mined values (Fig. 35) showed that Eq.28 was valid and adequately described
the interaction.

However, equation 61 has been used extensively for calculating the
required preservative concentration in surfactant solutions (Patel and
Kostenbauder, 1958; Pisano and Kostenbauder, 1959; Blaug and Ahsan, 1961a,b;
Bahal and Kostenbauder, 1964; Patel and Foss, 1965; Ashworth and Heard,
1966; Patel, 1967; Bean, Konning and Malcolm, 1969)

$$\frac{[D_L]}{[D_F]} = R = 1 + k[M] \quad (Eq.61)$$

where k represents the binding capacity of the surfactant. For a given
surfactant concentration the R value, or binding or solubilization constant,
is assumed to be independent of \([D_f]\). The total preservative concentration, \([D_t]\), is calculated by multiplying the concentration of preservative required for antimicrobial action with the R values at an appropriate surfactant concentration, \([M]\).

Equation 61 is in fact a special case of Eq. 28 and, for a given macromolecule concentration, the R value will be independent of \([D_f]\) only under two conditions:

I. When \([D_f] \to 0\)
\[
R = 1 + n_1 K_1[M] + n_2 K_2[M] \quad \text{(Eq. 62a)}
\]
or
\[
R = 1 + k'[M] \quad \text{(Eq. 62b)}
\]

II. When \([D_f]\) is constant, as in the case of saturated solutions
\[
R = 1 + \frac{n_1 K_1[M]}{1 + K_1[D_f]} + \frac{n_2 K_2[M]}{1 + K_2[D_f]} \quad \text{(Eq. 63a)}
\]
or
\[
R = 1 + k''[M] \quad \text{(Eq. 63b)}
\]

Hence, \(k'\) (or \(k''\)) does not fully characterize the interaction. Table 8 shows a comparison between Eq. 28 and 62 using the appropriate binding parameters for the interaction of chlorocresol with cetomacrogol. The values of total preservative concentration \([D_t]\), calculated using Eq. 62 are approximately three times higher than the \([D_t]\) values obtained using Eq. 28. Thus, it is not valid to assume that R is independent of \([D_f]\) in practical situations.

An apparent synergism reported by Pisano and Kostenbauder (1959)
on the basis of comparison between the MIC determined experimentally and
the MIC calculated using Eq.61 might be due to a higher calculated value of
total preservative concentration than to real synergism.

**TABLE 8. Comparison Between Equation 28 and 62 for the Interaction
of Chlorocresol with Cetomacrogol**

<table>
<thead>
<tr>
<th></th>
<th>Total Preservative Concentration</th>
<th></th>
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<th></th>
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</thead>
<tbody>
<tr>
<td></td>
<td>[Dₜ]</td>
<td>[Dₜ']</td>
<td>[Dₜ']</td>
<td></td>
</tr>
<tr>
<td></td>
<td>Calculated using Eq.28</td>
<td>Calculated using Eq.62</td>
<td></td>
<td></td>
</tr>
<tr>
<td>[M]</td>
<td>[Dₕ]</td>
<td></td>
<td></td>
<td></td>
</tr>
<tr>
<td>7.69</td>
<td>2.45</td>
<td>12.22</td>
<td>32.44</td>
<td>8.67</td>
</tr>
<tr>
<td>23.08</td>
<td>&quot;</td>
<td>31.76</td>
<td>92.42</td>
<td>10.35</td>
</tr>
<tr>
<td>38.46</td>
<td>&quot;</td>
<td>51.29</td>
<td>152.4</td>
<td>10.8</td>
</tr>
</tbody>
</table>

*[^Dₕ'] is the concentration of free preservative calculated for a
given [Dₜ'].*

Table 8 shows that the values of free preservative concentration,
[Dₕ'], corresponding to [Dₕ'], are approximately four times higher than the
value of [Dₕ'] and, moreover, are not constant. Thus, in the present work
an apparent synergism between preservative and surfactant would have been
observed if the total preservative concentration were calculated using Eq.62.
The values of [Dₕ'] were calculated using Eq.64 which is a rearrangement of
Eq.28. The roots of Eq.64 were calculated using Bairstow's method (U.B.C.
computer program, ZPOLY).
\[
K_1K_2[D_f^T]^3 + \left[K_1 + K_2 + n_1K_1K_2[M] + n_2K_1K_2[M] - K_1K_2[D_t^T]\right][D_f^T]^2 + \\
\left[1 + n_1K_1[M] + n_2K_2[M] - K_1[D_t^T] - K_2[D_t^T]\right][D_f^T] - [D_f^T] = 0 \quad (\text{Eq. 64})
\]

(d) Bactericidal Activity of Chlorocresol in Liquid Paraffin Emulsions Stabilized with Cetomacrogol

Figure 45 shows probit % survivors plotted as a function of time for the bactericidal activity of chlorocresol in water (curve A) and liquid paraffin emulsions of varying oil-water ratios (curves B, C and D) against \textit{E. coli}. The symbols represent experimental data, while the lines are fitted using the least squares method. The initial value of \([D_f^T]\) is the same for all the curves, although the total preservative concentration, \([D]\), varies.

The results of control experiments are shown in Fig. 39(C). The viability of \textit{E. coli} in liquid paraffin emulsions of varying oil-water ratio, \(q\), remained almost unchanged during the 8 hour interval.

A statistical analysis of the slopes of the curves in Fig. 45 was performed using a similar method to that described in the previous section for Fig. 42. The results are given in Table 9. Differences between the slopes of the curves A and B, B and C, C and D are nonsignificant. The difference between the slopes of the curve A and curves C and D is significant. Similarly there is significant difference between the slopes of curves B and D. Thus the difference between the slopes of any two adjacent curves is nonsignificant. However, there is a significant difference between the slopes of alternate curves. This suggests that the magnitude of deviation of the slopes of curves B, C and D from the slope of curve A is
Fig. 45. Probit % survivors of *E. coli* as a function of time for the bactericidal activity of chlorocresol in water (Curve A, from Fig. 41) and liquid-paraffin emulsions stabilized with cetomacrogol. Oil-water ratio, q, of the emulsions: B △, 1.0; C □, 0.5; D ○, 0.2. Cetomacrogol concentration = 3.0%. Initial free preservative concentration, [Dₗ], for water and the emulsions = 0.035%. Total preservative concentration, [Dₜ], %: A, 0.035; B, 0.2557; C, 0.3214; D, 0.3871. Points experimental, lines fitted using least squares method.
<table>
<thead>
<tr>
<th>Curve</th>
<th>t Calculated using Eq.59</th>
<th>Modified Welch Degrees of Freedom (Eq.60)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td></td>
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<td></td>
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<td></td>
<td></td>
</tr>
</tbody>
</table>

Comparison of curves B, C and D with curve A

<table>
<thead>
<tr>
<th>Curve</th>
<th>t Calculated using Eq.59</th>
<th>Modified Welch Degrees of Freedom (Eq.60)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>B</td>
<td>1.67</td>
<td>28.14</td>
<td>$&lt; 0.1$</td>
</tr>
<tr>
<td>C</td>
<td>3.83</td>
<td>31.33</td>
<td>$&lt; 0.005$</td>
</tr>
<tr>
<td>D</td>
<td>5.53</td>
<td>25.24</td>
<td>$&lt; 0.005$</td>
</tr>
</tbody>
</table>

Comparison of curves B and C with curve B

<table>
<thead>
<tr>
<th>Curve</th>
<th>t Calculated using Eq.59</th>
<th>Modified Welch Degrees of Freedom (Eq.60)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>C</td>
<td>1.28</td>
<td>30.02</td>
<td>$&lt; 0.2$</td>
</tr>
<tr>
<td>D</td>
<td>2.11</td>
<td>24.37</td>
<td>$&lt; 0.025$</td>
</tr>
</tbody>
</table>

Comparison of curve C with curve D

<table>
<thead>
<tr>
<th>Curve</th>
<th>t Calculated using Eq.59</th>
<th>Modified Welch Degrees of Freedom (Eq.60)</th>
<th>Probability</th>
</tr>
</thead>
<tbody>
<tr>
<td>D</td>
<td>0.91</td>
<td>36.33</td>
<td>$&lt; 0.2$</td>
</tr>
</tbody>
</table>
small. Hence, antimicrobial activity of chlorocresol in liquid paraffin emulsions is largely a function of \( [D_f] \). The small differences between the slopes of curves B, C and D also supports this view. A significant difference between the curves B and D suggests that the oil-water ratio does affect the antimicrobial activity of the preservative, and increasing the oil-water ratio results in an increase in the antimicrobial activity. However, the effect is of small magnitude.

Recently Bean, Konning and Malcolm (1969) and Patel and Romanowski (1970) used mathematical models to predict the required preservative concentration and reported satisfactory correlations using microbiological techniques. Some aspects of their work are open to criticism for the following reasons:

I. The binding parameters used to characterize the interaction between preservative and surfactant do not fully describe the interaction. This aspect is discussed in the previous section (criticism regarding Eq.61). Furthermore Patel and Romanowski (1970) used cellophane membrane in their study of the preservative-surfactant interaction using the equilibrium dialysis technique. In an earlier part of the discussion (C) it was shown that using cellophane membrane in interaction studies introduced appreciable errors into the binding parameters due to osmosis and permeation of the surfactant through the dialysis membrane. Thus, the validity of the binding results used by Patel et al. in their mathematical model is doubtful. Bean et al. (1969) also used the equilibrium dialysis technique for studying preservative-surfactant interaction. However, they did not report the type of dialysis membrane used.

II. The microbiological experimental technique of Patel et al. (1970)
is open to criticism because the micro-organisms were not in direct contact with the emulsion. The work of Bean et al. (1962) on simple oil-water dispersions suggests that antimicrobial activity is not simply a function of the concentration of free preservative and that preservative at the oil-water interface plays a part. However, the results of the present study show that this effect is of small magnitude.

III. Patel and Romanowski (1970) compared minimum inhibitory concentration (MIC) of a preservative in an emulsified system with the concentration of preservative calculated using a mathematical model (Eq.44) and a value of MIC determined in a growth promoting medium. This assumes that the MIC determined in a growth promoting medium parallels the MIC in the aqueous phase of the emulsion.

The choice of the MIC as the concentration of free preservative required in the aqueous phase of the system, \([D_f]\), is, of course, a rough approximation of the amount actually needed to preserve a complex disperse system. MIC values are normally determined in a nutrient growth medium and depend on factors such as oxygen tension, interfacial tension, osmotic pressure, degree of aeration, etc., as well as the nutritive value of the medium. The nutritive value of the aqueous phase of an emulsified system is unlikely to be as great as that of nutrient broth or other growth media, although, as pointed out by Tenenbaum (1967), the nutritive quality of "cosmetic and pharmaceutical formulations often ranges from water solutions to high protein soups" and is frequently overlooked when attempting to preserve a product. However, in most cases the nutritive value of the preparation will be less than that of the growth medium in which the MIC was determined and the \([D_f]\) value will therefore exceed minimum concentration required to inhibit growth.
IV. Bean et al. (1969) used an extinction time method (literature survey, 1(b)2) for studying the antimicrobial activity of preservatives in emulsified systems. There are a few weaknesses in the extinction time method when applied to emulsion systems. For example, it was difficult to distinguish between the turbidity due to the growth of micro-organisms from turbidity due to oil droplets. However, the authors overcame this problem by the use of indicator broth. A more serious disadvantage of this method is the carry-over of an appreciable amount of preservative, associated with the oil droplets or the surfactant micelles, to the growth medium. This residual preservative can inhibit the growth of micro-organisms. In the present work the problem of carry-over was avoided by the use of the Millipore filtration technique.

Bean, Richards and Thomas (1962) demonstrated that on dispersing liquid paraffin in an aqueous phenol solution greater antimicrobial activity was observed than was observed without the oil. Investigation of this system indicated that both micro-organisms and phenol were in higher concentration at the interface than in the bulk aqueous phase and the increased activity of the dispersion was attributed to this interfacial adsorption. Since the interfacial area of oil-water dispersions stabilized with emulsifiers is very much larger than simple oil-water dispersions, the interfacial effect on the antimicrobial activity of a preservative in a stabilized oil-water dispersion should be much more significant than demonstrated by Bean and others. Figure 45 shows that, although the difference between the slopes of curves B (q = 1.0) and D (q = 0.2) is significant, the magnitude of the difference is small. Hence, the effect of oil-water interface and the close proximity between micro-organisms and preservative rich
oil droplets on the antimicrobial activity of preservatives in emulsified systems is of minor importance.

Hence, a realistic test of the theory that preservative action depends on $[D_f]$ is to compare the viability of a given micro-organism in emulsions with the same $[D_f]$ and varying $[D]$ values. Equal biological response in emulsions with differing oil-water ratios would then be an indication that the antimicrobial activity in emulsified systems is mainly a function of $[D_f]$, and that the preservative in the oil phase or bound with surfactant micelles is biologically inactive. This approach was used in the design of the experiments in the present work (Fig. 45).

In the previous section, the concept of capacity was developed for the surfactant systems. This concept will now be extended to oil-water dispersions and emulsions (oil-water dispersions stabilized with a surfactant).

(i) Oil-water dispersions: Figure 46 shows a direct relationship between the percent loss of chlorocresol from water and the aqueous phase of hypothetical oil-water dispersions (see appendix 6 for the calculation of this parameter) when an equal amount of preservative is removed from water and the oil-water dispersion. For a given percent loss of preservative from water, the percent loss from the aqueous phase of oil-water dispersion is a function of the oil-water partition coefficient ($K^O_w$) and the oil-water ratio ($q$). The percent loss from the aqueous phase of oil-water dispersion increases with an increase in $q$ when $K^O_w$ is equal to 0.1; it is independent of $q$ when $K^O_w$ is equal to 1.0, and decreases with an increase in $q$ when $K^O_w$ is equal to 10.0. As before, the capacity of a system to resist changes in $[D_f]$ is inversely proportional to the slope of a given line. A slope equal to or
Fig. 46. Comparison between percent loss of chlorocresol from water and aqueous phase of an hypothetical oil-water dispersion when equal amounts of preservative are removed from water and the O/W dispersion. $K^O_W = 0.1$; oil-water ratio, q: A', 0.1; B', 0.2; C', 0.4; D', 0.6; E', 1.0; F', 2.0; G', 3.0; H', 5.0; $K^O_W = 1.0$; q: X, 0.0 to $\infty$. $K^O_W = 10.0$; q: A, 0.1; B, 0.2; C, 0.4; D, 0.6; E, 1.0; F, 5.0.
greater than one means that the system offers no resistance to changes in $[D_f]$. Figure 47 shows a direct relationship between capacity (the reciprocal of the slope of a given line in Fig. 46) and $K_w^O$ for various values of $q$. For a given $q$, the capacity increases when $K_w^O$ is more than 1.0 and decreases when $K_w^O$ is less than 1.0. When $K_w^O$ is equal to 1.0, the capacity is independent of $q$ (see Fig. 48) and its value is unity (the capacity of water is also equal to unity).

Figure 48 shows a curvilinear relationship between capacity and $q$ for various values of $K_w^O$. The capacity increases with an increase in $q$ when $K_w^O$ is more than 1.0. The capacity decreases with an increase in $q$ when $K_w^O$ is less than 1.0. The capacity is independent of $q$ when $K_w^O$ is equal to 1.0. Hence, from Figs. 47 and 48 it is apparent that both $K_w^O$ and $q$ control the capacity of an oil-water dispersion.

(ii) Emulsions: Figure 49 shows a direct relationship between the percent loss of chlorocresol from water and the aqueous phase of hypothetical emulsions (see appendix 7 for the calculation of this parameter) when an equal amount of preservative is removed from water and the emulsion. For a given percent loss of preservative from water, the percent loss from the aqueous phase of the emulsion is a function of $q$, $K_w^O$ and $[M]$. As before, the capacity of a system to resist changes in $[D_f]$ is inversely proportional to the slope of a given line. A slope equal to or greater than one means that the system offers no resistance to changes in $[D_f]$. For a given oil-water ratio the value of slope is a function of $[M]$ and $K_w^O$. Figure 49 shows that for a given $[M]$ an increase in $q$ results in a decrease in the value of
Fig. 47. Capacity as a function of oil-water partition coefficient, $K_w^O$, for the distribution of chlorocresol in hypothetical oil-water dispersions. Oil-water ratio: A, 0.1; B, 0.2; C, 0.4; D, 0.6; E, 0.8; F, 1.0; G, 2.0; H, 3.0; I, 4.0; J, 5.0.
Fig. 48. Capacity as a function of oil-water ratio, \( q \), for the distribution of chlorocresol in hypothetical oil-water dispersions. \( K^O_w \): A, 0.1; B, 0.5; C, 1.0; D, 3.0; E, 5.0; F, 7.0; G, 10.0.
Fig. 49. Comparison between percent loss of chlorocresol from water and aqueous phase of an hypothetical oil-water emulsion stabilized with cetomacrogol when equal amount of preservative are removed from water and the emulsion. $K_w^0 = 0.1; \ q: A', 0.1; \ B', 1.0; \ C, 5.0. \ K_w^0 = 1.0; \ q: X_1, 0.1; \ X_2, 1.0; \ X_3, 5.0. \ K_w^0 = 10.0; \ q: A, 0.1; \ B, 0.2; \ C, 0.4; \ D, 0.6; \ E, 1.0; \ F, 5.0. \ Cetomacrogol \ concentration = 0.1\%.$
the slope when $K^O_w$ is equal to 10.0. For the same $[M]$, increasing $q$ results in an increase in the value of the slope when $K^O_w$ is equal to 0.1 and 1.0. Thus, unlike surfactant and oil-water systems where capacity is determined only by $[M]$ and $K^O_w$, $q$, respectively, in emulsified systems the capacity is a resultant of the interaction between the parameters $[M]$, $K^O_w$ and $q$. Figure 50 shows a curvilinear relationship between capacity (the reciprocal of the slope of a given line in Fig. 49) and $q$ for various $K^O_w$ at a constant value of $[M]$. The intercept gives the capacity of the aqueous phase (containing surfactant) in the absence of the oil phase. At a fixed $[M]$, increasing $q$ decreases the capacity up to a certain value of $K^O_w$ (curves A, B and C), beyond that value an increase in $q$ results in an increase in capacity (curves D, E and F). This is because in emulsified systems the overall capacity of the system is determined by two factors - the capacity of the oil phase and the capacity of the aqueous phase containing the surfactant. The capacity of the oil phase is governed by $K^O_w$ while the capacity of the aqueous phase is controlled by the surfactant concentration. If the capacity of the oil is less than the capacity of the aqueous surfactant phase, then an increase in $q$ will result in a decrease in the overall capacity of the system (curves A, B and C). This aspect is further illustrated in Fig. 51 for the liquid paraffin emulsions used in this study. Conversely, if the capacity of the aqueous phase is less than the capacity of the oil phase, then an increase in $q$ will result in an increase in the capacity (curves D, E and F). As the capacity of the aqueous phase approaches the capacity of water, e.g., with very dilute surfactant solutions, an increase in $q$ will result in an increase in the capacity for all values of $K^O_w$ greater than 1.0 (compare Figs. 48, 50 and 52).
Fig. 50. Capacity as a function of oil-water ratio, q, for the distribution of chlorocresol in hypothetical O/W emulsions stabilized with cetomacrogol. Cetomacrogol concentration = 1.0%. $K^O_w$: A, 0.1; B, 1.0; C, 3.0; D, 5.0; E, 7.0; F, 10.0.
Fig. 51. Capacity as a function of oil-water ratio, $q$, for the distribution of chlorocresol in liquid-paraffin emulsions stabilized with cetomacrogol. Cetomacrogol concentration = 3.0%. $K_w^0$, liquid-paraffin = 1.67.
Fig. 52. Capacity as a function of oil-water ratio, $q$, for the distribution of chlorocresol in hypothetical O/W emulsions stabilized with cetomacrogol. Cetomacrogol concentration = 0.1%. $K_w^0$: A, 0.1; B, 1.0; C, 3.0; D, 5.0; E, 7.0; F, 10.0.
The capacity of a system clearly affects its ability to withstand microbial contamination. High capacity systems are able to resist losses in the concentration of preservative due to such factors as:

I. adsorption onto or complexation with the container and closure surfaces

II. adsorption, absorption of chemical reaction with contaminants including micro-organisms

III. chemical decomposition.

It is apparent that the overall effectiveness of a preservative in a complex disperse system will depend on the capacity of the system in addition to the concentration of free preservative. The magnitude of the capacity factors' contribution to the apparent overall antimicrobial activity of a preservative will vary according to the testing procedure adopted. Thus, the effect of capacity is more likely to become apparent in procedures which involve sampling for micro-organisms over prolonged time periods, e.g., days, weeks or months compared with hours in the present work, and challenge tests in which the preparation is repeatedly inoculated with micro-organisms over a long storage period. Such procedures allow time for the preservative to re-equilibrate between the various phases following any depletion and for this re-equilibration to exert its effect on the antimicrobial action. The object of the present work was to test the hypothesis that antimicrobial activity depends mainly on the concentration of free preservative in the aqueous phase and to evaluate the effect of factors such as changes in surfactant concentration and oil-water ratio. The experimental conditions chosen were such that capacity factors are unlikely to have a significant effect on the results. However, incomplete understanding of the capacity factor and its significance is a probable reason for much of the controversy
in the literature regarding the evaluation of preservative effectiveness in solubilized and emulsified systems.

The concept of capacity, as illustrated quantitatively in this work, not only emphasizes its importance in the design of experiments for the evaluation of theoretical models, but it can be used in the formulation of effective preservative systems. The graphical methods used for relating the capacity and the various physicochemical parameters, such as $K^0_w$, $[M]$ and $q$, can help in selecting an appropriate preservative which will provide high capacity without conferring undue toxicity to the given formulation.

In view of the number of variables involved in the preservation of solubilized and emulsified systems, it is realized that physico-chemical methods cannot replace a final microbiological evaluation of the product for its ability to withstand microbial contamination. However, physico-chemical methods do provide a logical first step in estimating the required preservative concentration, and thus help in avoiding much 'trial and error' formulation which is common where purely microbiological techniques are employed.
SUMMARY

1. Permeability of Membrane to Nonionic Surfactants

The permeability of two brands of cellophane membranes and a silicone membrane to a nonionic surfactant, cetomacrogol, was investigated using equilibrium dialysis, dynamic dialysis, and an ultrafiltration technique. The permeability of Fisher cellophane membrane to three n-alkyl polyoxyethylene surfactants was tested using the equilibrium dialysis technique. The results show that cellophane membranes are permeable to the nonionic surfactants, while silicone membrane is impermeable to the same surfactants.

2. Osmosis in Dialysis Studies Involving Nonionic Surfactants

Equilibrium and dynamic dialysis studies showed increase in the volume of the surfactant solution due to osmosis when cellophane was used as a semipermeable membrane. No osmotic effect was observed using silicone membranes.

3. Interaction of Preservatives with Nonionic Surfactants

(a) Equilibrium dialysis

Cellophane and silicone membranes were compared in an equilibrium dialysis study of the interaction of chlorocresol with n-alkyl polyoxyethylene surfactants. Appreciable errors were introduced into the binding parameters when cellophane was used as the dialysis membrane. These errors are due both to osmosis and to permeability of the membrane to the surfactants, with dilution of surfactant as a result of the osmotic differential across the membrane being the major factor. It is suggested that the use of cellophane
as a membrane can introduce appreciable errors into interaction studies involving nonionic surfactants unless corrections are made for osmosis. Silicone rubber is a satisfactory membrane for these studies.

(b) Diafiltration technique

The diafiltration technique was compared with equilibrium dialysis for the interaction of benzoic acid with cetomacrogol. The results were expressed in the form of Scatchard plot. When the equilibrium dialysis technique was used, binding of the benzoic acid with cetomacrogol was independent of the surfactant concentration. However, the results obtained using the diafiltration technique showed a dependence of the binding curves on the surfactant concentration. This anomalous behavior is attributed to various technical artifacts of the method and not to changes in the characteristics of the surfactant molecules during diafiltration.

4. Interaction of Preservative Mixtures with Cetomacrogol

Interaction of various binary preservative mixtures with cetomacrogol was studied using the equilibrium dialysis technique. The binding of a preservative with cetomacrogol was altered in the presence of another preservative. This observation is similar to the phenomena of competitive binding of drugs with macromolecules. However, no correlation was found between the binding behavior of preservative mixtures predicted using the theory of competitive binding and the observed values. The disagreement between the predicted values and the experimental results is explained in terms of the locus of solubilization of preservatives in a surfactant micelle, and changes in the number as well as behavior of binding sites in a surfactant micelle upon interaction with the preservatives.
5. Interaction of Chlorocresol with Mixtures of Some Nonionic Surfactants

The interaction of chlorocresol with binary mixtures of nonionic surfactants was studied using the equilibrium dialysis technique. An attempt was made to predict the binding behavior of surfactant mixtures using binding parameters which characterize the interaction of a preservative with individual surfactants. Good agreement was found between the predicted and the experimental values. In addition it was shown that binding is a direct function of the HLB value of nonionic surfactants. Since HLB is an additive property, the binding of a preservative with a surfactant mixture is an additive function of the binding of the preservative with the individual surfactants.

6. Distribution of Chlorocresol in Liquid Paraffin-Water Systems

The distribution of chlorocresol between liquid paraffin and water was studied using a shake-out method. The distribution obeyed a simple partition law.

7. Calculation of Total Concentration of Chlorocresol Required in Cetomacrogol Solutions to Produce the Desired Concentration of Free Preservative in the Aqueous Phase

The validity of Eq.28 was checked using the equilibrium dialysis technique. Good correlation was found between predicted and experimentally determined values.

8. Calculation of Total Concentration of Chlorocresol Required in Liquid Paraffin Emulsions to Produce the Desired Concentration of Free Preservative in the Aqueous Phase
The validity of Eq. 48 was checked for the distribution of chlorocresol between liquid paraffin-water-cetomacrogol systems using the equilibrium dialysis technique. Good correlation was found between predicted and experimentally determined values.

9. Correlation of Physico-Chemical Data with Antimicrobial Activity

(a) Comparison between Millipore and pour-plate techniques

The Millipore filtration method was compared with a pour-plate technique for the viable counting of E. coli. Good correlation was found between the two techniques.

(b) Bactericidal activity of chlorocresol in water

The bactericidal activity of chlorocresol in water against E. coli was studied using a viable count method. The results were expressed as log number of organisms surviving, or probit % survivors as a function of time. Graphical presentation of the data using probit plots is recommended because the latter give straight line relationships. The bactericidal activity of chlorocresol in water against E. coli is a function of the concentration of the preservative. Increasing the concentration of chlorocresol from 0.02% to 0.03% makes little change in the death rate of E. coli. However, a concentration of 0.035% chlorocresol produced 99% mortality within 6-8 hours.

(c) Bactericidal activity of chlorocresol in aqueous solutions of cetomacrogol

The bactericidal activity of chlorocresol in various concentrations of cetomacrogol against E. coli was studied using the viable count method.
Statistical analysis of the probit plot of the data indicated that the bactericidal activity of chlorocresol in cetomacrogol solutions was largely a function of the free preservative concentration in the aqueous phase. However, an increase in cetomacrogol concentration produced a significant but small decrease in antibacterial activity.

(d) Bactericidal activity of chlorocresol in liquid paraffin emulsions stabilized with cetomacrogol

The bactericidal activity of chlorocresol in liquid paraffin emulsions of varying oil-water ratios against E. coli was studied using the viable count method. Statistical analysis of the probit plot of the data indicated that the antibacterial activity of chlorocresol in liquid paraffin emulsions was largely a function of the concentration of free preservative in the aqueous phase. A significant but small increase in the antibacterial activity with increase in the oil-water ratio is possibly due to interfacial effects.
REFERENCES


Navarre, M.G. de (1957a). ibid., 8, 68-75.


APPENDICES

1. Sample Calculations for Estimating $[D_f]$ and $[D_b]$ Employing the Diafiltration Technique Using the Data for the Interaction of Benzoic Acid (BA) with Cetomacrogol (Fig. 19)

<table>
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<tr>
<th>Volume of filtrate $V_f$ ml.</th>
<th>Dilution factor</th>
<th>Absorbance 230 nm</th>
<th>Concentration of BA in the filtrate $[D_f]$ g.l. $^{-1} \times 10^2$</th>
<th>Amount of BA in the filtrate $= A_f$</th>
<th>Cumulative amount of BA bound $= \sum A_f$</th>
<th>Amount of BA free in the cell $= A_f'$</th>
<th>Amount of BA entering the cell $= \sum A_t$</th>
<th>Cumulative amount of BA entering the cell $= \sum A_t - A_f$</th>
<th>Amount of BA bound $= \frac{A_b}{V_m}$</th>
<th>Concentration of BA bound $= [D_b]$ g.l. $^{-1} \times 10^2$</th>
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<tbody>
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<td>2.02</td>
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<td>41.98</td>
<td>210.37</td>
<td>80.22</td>
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</table>

Volume of cetomacrogol in the cell, $V_m = 50$ ml.
Concentration of benzoic acid in the reservoir = 0.933 g.l.$^{-1}$
Absorptivity of benzoic acid in 0.01N HCl at 230 nm. = 91.76.
Concentration of cetomacrogol = 1.0%.
2. Calculation of the Binding Parameters for the Interaction of a Preservative with a Nonionic Surfactant

(a) For a given \([M]\), determine experimentally \([D_b]\) and \([D_f]\) for various values of \([D_c]\).

(b) Calculate \(r = \frac{[D_b]}{[M]}\).

(c) Calculate values of \(n's\) and \(K's\) by fitting the values of \(r\) and \([D_f]\), with the aid of a nonlinear regression program (Meyer and Guttman, 1968), to the Eq. 25

\[
  r = \frac{n_1 K_1 [D_f]}{1 + K_1 [D_f]} + \frac{n_2 K_2 [D_f]}{1 + K_2 [D_f]} \quad \text{(Eq. 25)}
\]

(d) The regression program requires experimental values of \(r\) and \([D_f]\), and a rough estimate of the values of \(n's\) and \(K's\). The more accurate these estimates are, the fewer the iterations required for the computer calculation.

(e) The parameters calculated by the program include: fitted values of \(n_1, K_1, n_2\) and \(K_2,\) fitted values of \(r \in (r)\) for a given \([D_f]\), and \(Cr/[D_f]\).

(f) Table 10 gives a listing of the computer program and a sample of the results computed using the data for the interaction of chlorocresol with cetomacrogol (Fig. 17, silicone rubber data).
FLTRAN IV C COMPILER  SSCE  C4-06-74  14:15:47  PAGE 0001

0001  FUNCTION SSCE(A1,A2,A3,A4,X)
0003  DIMENSION I(10),CA(10),TA(10)
0004  DIMENSION X1(15),X2(15),Y(15),D(15)
0005  CONTINUE
0006  RETURN
0007  CALL X1=A1*CA(1)+A1
0008  10 CONTINUE
0009  SSCE=0.
0010  CALL 2C X1=A2
0011  FY=FY+CALC((TA(1),TA(2),TA(3),TA(4),X1,K))
0012  SSCE=SSCE+FY*FY
0013  CONTINUE
0014  RETURN
0015  FNE

TOTAL MEMORY REQUIREMENTS CCC33E BYTES

COMPILE TIME = 0.1 SECONDS
SUBROUTINE DVFV(K,F,Y,P)

DIMENSION DW(155),A(155),X(155),Y(155)

CC033
CALL Y(1),X(1),ZERG,YNIT

CC04
CALCY(A(1),A(2),A(3),A(4),X)=A(1)*X/11.+A(1)*A(2)*A(3)*A(4)*X/(1.+A(2)*X)

0055
CC 5 i=1,K+2

CC06
TERV=1.+A(1)*X(I)

0077
CV(I)=TEMV*(A(I)+1)*(A(I)-A(I+1)*X(I+1)*X(I))

0088
LV(I)=CV(I)/TERV

CC09
5 CV(I+1)=P(I)*X(I)/TERV

0010
FY+Y(K)-CALCY(A(1),A(2),A(3),A(4),X(I))

0011
RETURN

CC12
END

TOTAL MEMORY REQUIREMENTS 0032A BYTES

CCFILE TIME = 0.1 SECONDS
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$SIGACFF
3. Interaction of Preservative Mixtures with Cetomacrogol

(a) Calculation of a theoretical binding curve using Eq.30

(i) Determine the interaction of a preservative (D) with cetomacrogol. Calculate binding parameters \( n_1, n_2, K_{d1} \) and \( K_{d2} \) using the method described in appendix 2.

(ii) Determine the interaction of another preservative (C), the competitor, with cetomacrogol. Calculate binding parameters \( n_1', n_2', K_{c1} \) and \( K_{c2} \) using the method described in appendix 2.

(iii) Determine the binding of a variable concentration of D with cetomacrogol in presence of a constant concentration of C. Calculate values of \([D_f], [D_b], [C_f] \) and \([C_b] \).

(iv) Substitute values of \( n_1, n_2, K_{d1}, K_{d2}, K_{c1}, K_{c2}, [D_f], \) and \([C_f] \) into Eq.30 and calculate \( r \).

(v) Calculate the value of \( r/[D_f] \).

(vi) Plot \( r/[D_f] \) versus \( r \) (Scatchard plot).

(b) Sample calculations for the interaction of preservative mixtures with cetomacrogol using the data for the interaction of chlorocresol with cetomacrogol in absence and presence of a constant concentration of methyl paraben (Fig. 20, curves A and B).
(i) Interaction of chlorocresol with cetomacrogol (Fig. 20, curve A)

<table>
<thead>
<tr>
<th>moles $l^{-1} \times 10^3$</th>
<th>$[D_b]$</th>
<th>$[D_f]$</th>
<th>$r = \frac{[D_b]}{[M]}$</th>
<th>$r/[D_f]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>14.86</td>
<td>4.58</td>
<td></td>
<td>1.93</td>
<td>421.49</td>
</tr>
<tr>
<td>11.03</td>
<td>3.24</td>
<td></td>
<td>1.43</td>
<td>443.07</td>
</tr>
<tr>
<td>10.44</td>
<td>2.88</td>
<td></td>
<td>1.36</td>
<td>471.03</td>
</tr>
<tr>
<td>8.27</td>
<td>2.01</td>
<td></td>
<td>1.07</td>
<td>534.66</td>
</tr>
<tr>
<td>8.33</td>
<td>2.00</td>
<td></td>
<td>1.08</td>
<td>540.45</td>
</tr>
<tr>
<td>5.92</td>
<td>1.18</td>
<td></td>
<td>0.77</td>
<td>652.43</td>
</tr>
<tr>
<td>5.91</td>
<td>1.15</td>
<td></td>
<td>0.76</td>
<td>666.48</td>
</tr>
<tr>
<td>3.19</td>
<td>0.45</td>
<td></td>
<td>0.41</td>
<td>920.44</td>
</tr>
</tbody>
</table>

$M = 7.69 \times 10^{-3}$ moles $l^{-1}$

$n_1 = 0.5334$  
$n_2 = 243.27$  

$K_{d1} = 2549.5$  
$K_{d2} = 1.2789$

(ii) Interaction of methyl paraben with cetomacrogol (Fig. 23, curve A).

<table>
<thead>
<tr>
<th>moles $l^{-1}$</th>
<th>$[C_b]$</th>
<th>$[C_f]$</th>
<th>$r = \frac{[C_b]}{[M]}$</th>
<th>$r/[C_f]$</th>
</tr>
</thead>
<tbody>
<tr>
<td>8.26</td>
<td>11.63</td>
<td></td>
<td>1.07</td>
<td>92.33</td>
</tr>
<tr>
<td>6.89</td>
<td>9.10</td>
<td></td>
<td>0.89</td>
<td>98.54</td>
</tr>
<tr>
<td>5.77</td>
<td>6.72</td>
<td></td>
<td>0.75</td>
<td>111.64</td>
</tr>
<tr>
<td>4.34</td>
<td>4.95</td>
<td></td>
<td>0.56</td>
<td>114.14</td>
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<tr>
<td>3.19</td>
<td>3.19</td>
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<tr>
<td>1.80</td>
<td>1.54</td>
<td></td>
<td>0.234</td>
<td>151.91</td>
</tr>
</tbody>
</table>

$M = 7.69 \times 10^{-3}$ moles $l^{-1}$

$n_1' = 0.7276$  
$n_2' = 69.47$  

$K_{c1} = 166.62$  
$K_{c2} = 0.7412$
(iii) Interaction of chlorocresol with cetomacrogol in presence of a constant concentration of methyl paraben (Fig. 20, curve B)

<table>
<thead>
<tr>
<th>Experimental values</th>
<th>Values calculated using Eq.30</th>
</tr>
</thead>
<tbody>
<tr>
<td>moles $1^{-1} \times 10^3$</td>
<td>$r = \frac{[D_b]}{[M]}$</td>
</tr>
<tr>
<td>$[D_b]$</td>
<td>$[D_f]$</td>
</tr>
<tr>
<td>10.38</td>
<td>3.11</td>
</tr>
<tr>
<td>9.39</td>
<td>2.64</td>
</tr>
<tr>
<td>8.10</td>
<td>2.18</td>
</tr>
<tr>
<td>6.86</td>
<td>1.69</td>
</tr>
<tr>
<td>4.69</td>
<td>1.04</td>
</tr>
<tr>
<td>3.10</td>
<td>0.63</td>
</tr>
</tbody>
</table>

$M = 7.69 \times 10^{-3}$ moles $1^{-1}$
Total methyl paraben concentration = $8.54 \times 10^{-3}$ moles $1^{-1}$

N.B. All calculations were performed using IBM 370 computer.

(c) Analysis of antagonism between the two different types of small molecules for binding with a macromolecule using Scatchard plot

Interaction between a small molecule and macromolecule can be expressed by Eq.64 which is a rearrangement of Eq.24 and is known as the Scatchard equation (Scatchard, 1949). A plot

$$\frac{r}{[D_f]} = nK - rK$$  \hspace{1cm} (Eq.64)

of Eq.64 gives a straight line with a negative slope (Fig. 53,a). Extrapolation of the line to $r$ axis gives the value of the maximum number of free binding sites, $n$, available per mole of macromolecule for the interaction. The slope of the line gives the intrinsic association constant, $K$. The intrinsic association constant is defined as the equilibrium constant...
Fig. 53. Analysis of antagonism using Scatchard plot. (a) Interaction in absence of competitor. (b) Competitive antagonism. (c) Non-competitive antagonism. (d) Mixed competitive-noncompetitive antagonism. A - binding of D with M in absence of C. B and C - binding:of D with M in presence of a constant C. The value of C for curve B is less than curve C.
for the association reaction between 1 mole of free drug and 1 mole of unoccupied binding site to form 1 mole of complex. Curvature in this plot is an indication of the electrostatic interactions between the binding sites of the macromolecule, the negative or positive cooperative effects in the binding, or the involvement of more than one class of binding sites in the interaction. However, in the present work, curvature in the Scatchard plot was assumed to be due to the presence of two classes of binding sites and hence the binding data was characterized using Eq.25. For the sake of simplicity in the present discussion only one class of binding sites is assumed.

Antagonism between a small molecule, D, and another small molecule, the competitor, C, for the binding with a macromolecule, M, can be classified into three types.

Case I. **Competitive antagonism:** The molecule C is said to be competitive with the molecule D if it combines reversibly with the same binding sites of the macromolecule as the molecule D. However, if the combination of C with M induced a conformational change that altered the binding energy of the complex MD, the antagonism would be classified as competitive even if C and D combined with M independently at different sites. The effect of the competition, in any case, is a reduction of the apparent affinity of D for M in the presence of C, as predicted by the law of mass action (Eq.30).

In Scatchard plot, competitive inhibition is exhibited by reduction of the slope and hence the association constant, $K$, with increasing concentrations of the competitor, C (Fig. 53,b). However, n remains constant for all values of C.
Case II. **Noncompetitive antagonism**: The molecule C is said to be noncompetitive if it inactivates the binding sites for D so that the effective complex with the molecule D cannot be formed, regardless of the concentration of D. The molecule C might combine irreversibly with M at the same site where D ordinarily combines, giving rise to apparent noncompetitive interaction. The essential point in noncompetitive antagonism is that the molecule D has no influence upon the degree of antagonism.

In the Scatchard plot, noncompetitive antagonism is exhibited by the reduction in the value of n with increasing concentrations of the competitor, C (Fig. 53,c). However, the slope, and hence the association constant, K, remains constant for all values of C.

Case III. **Mixed competitive-noncompetitive antagonism**: This antagonism is of a complex nature and their mechanisms are not well defined. In the Scatchard plot, this antagonism is exhibited by both changes in the values of n and K with increase in the concentration of C (Fig. 53,d).
4. Sample Calculations for the Interaction of Chlorocresol with Mixtures of some Nonoionic Surfactants (Fig. 30, lowest curve)

(a) Binding parameters for the interaction of chlorocresol with Texofor A16:

\[ n_1' = 0.5377; \quad K_1' = 2122.4 \]
\[ n_2' = 141.53; \quad K_2' = 1.647 \]

(b) Binding parameters for the interaction of chlorocresol with Texofor A60:

\[ n_1 = 0.4319; \quad K_1 = 4974.3 \]
\[ n_2 = 148.4; \quad K_2 = 3.161 \]

(c) Interaction of chlorocresol with mixtures of Texofor A16 and A60:

<table>
<thead>
<tr>
<th>[Df]</th>
<th>[Dc]</th>
<th>[Dc']</th>
</tr>
</thead>
<tbody>
<tr>
<td>3.694</td>
<td>17.19</td>
<td>18.21</td>
</tr>
<tr>
<td>2.667</td>
<td>13.34</td>
<td>13.99</td>
</tr>
<tr>
<td>2.039</td>
<td>10.87</td>
<td>11.37</td>
</tr>
<tr>
<td>1.422</td>
<td>8.347</td>
<td>8.719</td>
</tr>
<tr>
<td>0.847</td>
<td>6.047</td>
<td>6.099</td>
</tr>
<tr>
<td>0.358</td>
<td>3.308</td>
<td>3.473</td>
</tr>
</tbody>
</table>

Concentration of Texofor A16, \([M_1] = 2.114 \times 10^{-3}\) moles \(l^{-1}\)

Concentration of Texofor A60, \([M_{III}'] = 5.51 \times 10^{-3}\) moles \(l^{-1}\)

N.B. All calculations were performed using IBM 370 computer.
5. Calculation of Percent Loss of Preservative From the Aqueous Phase of Surfactant Solution for a Given Loss of Preservative From Total System

1. Let $[D_t]$ and $[D_f]$ be the initial total and free preservative concentrations respectively.

2. Let loss in the concentration of preservative from the surfactant solution = $[D_{t1}]$.

3. Concentration of Preservative remaining in the surfactant solution = $[D_t] - [D_{t1}] = [D'_t]$.

4. Concentration of free preservative in the aqueous phase, $[D'_f]$, for a given total preservative concentration, $[D'_t]$, was calculated using Eq.64 which is a rearrangement of Eq.28.

$$K_1K_2[D'_f]^3 + (K_1 + K_2 + n_1K_1K_2[M] + n_2K_1K_2[M] - K_1K_2[D'_t]) [D'_f]^2 +$$

$$+ (1 + n_1K_1[M] + n_2K_2[M] - K_1[D'_t] - K_2[D'_t]) [D'_f] - [D'_t] = 0 \quad (Eq.65)$$

The roots of Eq.64 were calculated using Bairstow's method (U.B.C. computer program, ZPOLY).

5. Percent preservative loss from the aqueous phase of surfactant = \[
\frac{100([D_f] - [D'_f])}{[D_f]}.
\]
6. Calculation of Percent Loss of Preservative From the Aqueous Phase of an Emulsion for a Given Loss of Preservative From Total System

A similar method is used as given in Appendix 5 for a surfactant solution, except \([D'_f]\) for a given \([D'_t]\) is calculated using Eq.65 (Bean, Heman-Ackah and Thomas, 1965).

\[
[D'_f] = \frac{[D'_t](q + 1)}{K^o_w q + 1}
\]

(Eq.66)
7. Calculation of Percent Loss of Preservative From the Aqueous Phase of an Emulsion Stabilized with a Surfactant – For a Given Loss of Preservative From Total System.

A similar method is used as given in Appendix 3 for a surfactant solution, except \([D_f']\) for a given \([D_t']\) is calculated using Eq. 66 which is a rearrangement of Eq. 48. The roots of Eq. 65 were calculated using Bairstow's method (U.B.C. computer program, ZPOLY).

\[
(K_1 K_2 + K_w^0 K_1 K_2) [D_f']^3 + (K_1 + K_2 + n_1 K_1 K_2^2 [M] + n_2 K_1 K_2 [M] + K_w^0 q + K_2 + n_1 K_2 [M] + n_2 K_2 [M] + K_w^0 q - K_1 [D_f'] - K_1 [D_t'] - K_2 [D_t'] - [D_t']^2 + (1 + n_1 K_1 [M] + n_2 K_2 [M] + K_w^0 q - K_1 [D_f'] - K_1 [D_t'] - K_2 [D_t'] - [D_t'] [D_f'] - [D_t'] (q + 1) = 0
\]

(Eq. 67)