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Interaction of preservatives with cetomacrogol

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The interaction of a number of commonly used preservatives (benzoic acid, *p*-hydroxybenzoic acid, methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate and chloroxylenol with the non-ionic surfactant cetomacrogol was examined and a comparison made of various methods of expressing this interaction. It is suggested that the Scatchard equation is the most satisfactory equation for describing the binding data. Binding parameters determined from a Scatchard plot in the concentration range of free preservative appropriate for antimicrobial activity can be used to calculate the total concentration of preservative required in the surfactant system.

Interaction of preservatives with surfactants leads to a loss of antimicrobial activity. It is generally accepted that preservative solubilized or bound within the micelles is inactive and, although the micelles act as a reservoir of preservative, the antimicrobial activity depends largely on the concentration of unbound or free preservative (Allawala & Riegelman, 1953; Pisano & Kostenbauder, 1959; Mitchell, 1964). Hence the physico-chemical parameter(s) used to express the interaction should permit calculation of the total preservative concentration required to provide a concentration of free preservative adequate to inhibit microbial growth.

The interaction of preservatives, drugs and other solutes with various macromolecules such as surfactants, polymers and proteins has been studied extensively. Methods used to express the interaction with proteins are well established (Goldstein, 1949; Klotz, 1953; Meyer & Guttman, 1970) and have been applied successfully to polymers such as methylcellulose and polyvinylpyrolidone (Eide & Speiser, 1967a,b; Cho, Mitchell & Pernarowski, 1971). Results for the interaction between solute and surfactant, however, have been presented in a variety of ways depending essentially on the particular theory adopted to explain the mechanism of interaction. In this paper, some of these methods are compared using results obtained in studies of the interaction between several commonly used preservatives and the non-ionic surfactant cetomacrogol.

MATERIALS AND METHODS

Materials. Benzoic acid, *p*-hydroxybenzoic acid, methyl *p*-hydroxybenzoate, propyl *p*-hydroxybenzoate, chloroxylenol and cetomacrogol were as described previously (Mitchell, 1964; Mitchell & Brown, 1966; Brown, 1968).

Solubility and equilibrium dialysis. The experimental methods have been described before (Mitchell & Brown, 1966) except that a 0·0005 inch nylon membrane (Capran 77C, Allied Chemical Corporation, Morristown, New Jersey) was used in the dialysis technique and glass beads were added to each compartment to ensure continuous mixing. The interaction of cetomacrogol with benzoic acid was studied at 30° in citrate-phosphate buffer pH 3·0 and ionic strength 0·2. The amount of benzoic acid on both sides of the membrane at equilibrium was analysed spectrophotometrically at

273 nm. Data for the interaction of the other preservatives with cetomacrogol in unbuffered aqueous solution were derived from previous work (Mitchell, 1964; Mitchell & Brown, 1966; Brown, 1968).

RESULTS AND DISCUSSION

Interaction as a partition phenomenon

One of the earliest attempts to express solubilization quantitatively was due to McBain & Hutchinson (1955). They suggested that the formation of micelles, and in particular the occurrence of a hydrocarbon region in the centre of the micelles, justifies the treatment of micelle formation as a phase separation. Solubilization may be regarded therefore as the distribution of solute between water and the micellar phase. McBain & Hutchinson expressed this:

$$K_m = \frac{\text{mol micellar solute/mol micellar surfactant}}{\text{mol free solute/mol water}} \quad \cdots \quad (1)$$

where K_m is the apparent partition coefficient for the distribution of solute between the micelles and aqueous phases. This approach has been used by Evans (1964) and Mitchell & Brown (1966). However (1) does not include the volumes of the aqueous or micellar phases and the values of K_m cannot therefore be compared with classical oil-water partition coefficients. An estimate of micellar volume can be made from the partial molar volume of the surfactant and K_m expressed according to equation (2) (Donbrow & Rhodes, 1963; Mitchell & Broadhead, 1967)

$$K_m = \frac{D_b/v}{D_f/(1 - v)} \quad \cdots \quad (2)$$

where D_b is the amount of solute in the micellar phase, D_f is the amount of solute in the aqueous phase, v is the volume of the micellar phase and $1 - v$ is the volume fraction of the aqueous phase. Apparent partition coefficients calculated according to equation (2) for various preservatives in cetomacrogol solutions are shown in Fig. 1. The K_m values are not constant but depend on the free drug concentration.

A major problem associated with the application of (2) is that the value assigned to the volume of the micelles is somewhat arbitrary since the volume could be (a) the hydrocarbon core of the micelles, (b) the entire micelle or (c) the entire micelle including bound and trapped water. Humphreys & Rhodes (1968) attempted to overcome this problem in a study of the solubilization of benzoic acid in a series of non-ionic surfactants, by extrapolating the solubility curves to 100% w/w surfactant. This value was taken to represent the solubility of the solute in the micellar phase, S_m, and

$$K_m = S_m/S_w \quad \cdots \quad (3)$$

where S_w is the solubility in the aqueous phase.

This technique will normally entail a very large extrapolation to 100% w/w surfactant and like all methods based on solubility measurements is, in effect, a one-point method. It cannot be assumed that the value of K_m obtained from equation (3) will be applicable to under-saturated systems. Moreover it has been shown that the solubilization process of benzoic acid is not governed by the distribution law (Donbrow & Rhodes, 1964; Donbrow, Molyneux & Rhodes, 1967; Donbrow, Azaz & Hamburger, 1970).

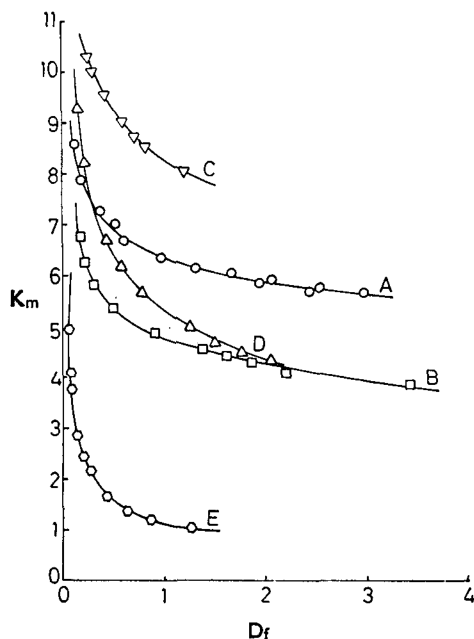

FIG. 1. Variation of apparent partition coefficient with free preservative concentration for the partition of preservative between micelles and aqueous phase of cetomacrogol. A. Benzoic acid at 30° ($D_f \times 10^2$, $K_m \times 10^{-1}$); cetomacrogol concentrations (mol/litre): 0·0077; 0·015; 0·031; B. *p*-Hydroxybenzoic acid at 25° ($D_f \times 10^2$, $Km \times 10^{-1}$); C. Methyl *p*-hydroxybenzoate at 25° ($D_f \times 10^2$, $K_m \times 10^{-1}$); D. Propyl *p*-hydroxybenzoate at 25° ($D_f \times 10^3$, $K_m \times 10^{-2}$); cetomacrogol concentrations (mol/litre): 0·01; 0·04; 0·06; 0·1 (Brown, 1968). E. Chloroxylenol at 20° ($D_f \times 10^3$; $K_m \times 10^{-3}$); cetomacrogol concentrations (mol/litre): 0·005; 0·01; 0·049; 0·096 (Mitchell & Brown, 1966).

Interaction as a "binding" phenomenon

An alternative and widely used method is to express interaction data according to equation (4) (Patel & Kostenbauder, 1958; Blaug & Ahsan, 1961 a,b; Bahal & Kostenbauder, 1964; Patel & Foss, 1965; Ashworth & Heard, 1966; Patel, 1967; Bean, Konning & Malcolm, 1969).

$$[D_t]/[D_f] = 1 + k\,[M] \quad \ldots \quad (4)$$

where $[D_t]/[D_f]$, represented by R, is the ratio of total solute concentration to the free solute concentration and [M] is the surfactant concentration. Plots of R as a function of surfactant concentration are normally presented as a single curve, the slope of which k, is taken as a measure of the binding capacity of the surfactant. The total preservative concentration is calculated by multiplying the concentration of free preservative required for antimicrobial activity by the R value at the appropriate surfactant concentration. However, as will be shown later (see Fig. 2) the R value at any given surfactant concentration is constant only under limited conditions.

Since the "partition" and "binding" approaches to solubilization are so widely used it is of interest to compare equations (2) and (4). Over a limited concentration range, the volume of the micellar phase, v, is directly proportional to the surfactant concentration, [M], i.e. $v = k'\,[M]$. Hence D_b/v in equation (2) can be written $[D_b]/k'[M]$ where $[D_b]$ is the concentration of solute in mol/litre. Similarly, for

relatively dilute solutions $D_f/(1 - v)$ is proportional to the concentration of free solute in mol/litre, $[D_f]$, and equation (2) can be rewritten:

$$\frac{[D_b]}{[D_f][M]} = k'K_m = k \quad \ldots \quad (5)$$

Since $[D_t] = [D_b] + [D_f]$, equation (4) can be rearranged into the same form as (5). Hence both the "partition" and simple "binding" approaches to solubilization depend on the same relation and a fit of data to either equation does not permit any assumptions to be made about the mechanism of the interaction. Although many authors have expressed solubilization in terms of a partition coefficient or as a binding constant, neither of these constants fully characterizes the interaction.

In contrast to the controversy surrounding methods used to describe the interaction of solute with surfactant, the fundamental concepts dealing with the interaction of solute with proteins are well established. The interaction can be expressed by equation (6) which is derived from the law of mass action:

$$r = \frac{nK\,[D_f]}{1 + K[D_f]} \quad \ldots \quad (6)$$

where r is the molar ratio of bound solute to total protein $[D_b]/[P_t]$, n is the maximum number of independent binding sites on the protein and K is the association constant. Garrett (1966) suggested that the binding of preservatives to macromolecules other than protein may be treated similarly i.e. $r = [D_b]/[M]$ where [M] is the concentration of any macromolecule including surfactant. An important difference between surfactants and other macromolecules is that interaction occurs between the solute and surfactant micelles rather than monomer surfactant molecules. Theoretically [M] in equation (6) should be the concentration of micelles, n the number of binding sites per micelle and K the association constant for reaction with the micelles. From a practical viewpoint however, it is more convenient to express [M] in terms of the surfactant concentration. The critical micelle concentration of commonly used non-ionic surfactants is sufficiently low for the monomer concentration to be neglected.

Equation (6) has the same form as the Langmuir equation which has led some authors to suggest that the mechanism of interaction between solute and surfactant is one of adsorption onto the surface of the micelle or some other site within the micelle (Donbrow & Rhodes, 1964; Donbrow, Molyneux & Rhodes, 1967). However, as pointed out by Goldstein (1949) and Klotz (1953) for solute-protein interaction, although the equations are similar it is not necessarily correct to assume that binding and adsorption are identical processes.

Fig. 2 shows the results plotted as the ratio of total preservative to free preservative, R, as a function of surfactant concentration according to equation (4). Contrary to the manner in which data are normally presented for this type of plot, the results cannot be represented by a single curve. Equation (4) is in fact a special case of equation (6) and a single curve will be obtained only under two conditions: (a) when $[D_f]^{\lim} \rightarrow O$ then $nK/(1 + K[D_f]) = nK$ and $R = 1 + k[M]$ where $k = nK$; (b) when $[D_f]$ is constant as in the solubility method, then $nK/(1 + K[D_f])$ = a constant, k'', and $R = 1 + k''[M]$. Hence k (or k'') does not fully characterize the interaction. A macromolecule or micelle has a limited binding capacity for solute molecules and a single value of k (or k'') will be obtained only over a limited range of free solute concentration. It is impossible to maintain $[D_f]$ constant using the equilibrium dialysis technique and Fig. 2 was constructed using calculated values of $[D_f]$. The

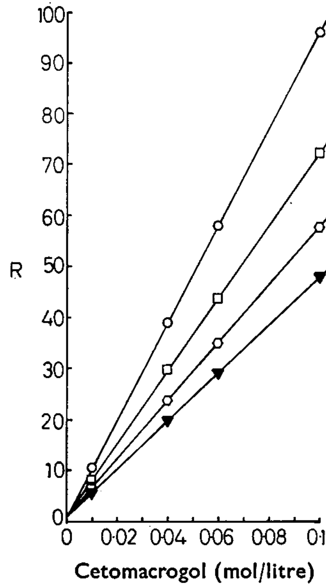

FIG. 2. Ratio of total: free propyl *p*-hydroxybenzoate as a function of cetomacrogol concentration at 25°. Concentration of free propyl *p*-hydroxybenzoate (mol/litre): ○, $0{\cdot}21 \times 10^{-3}$; □ $0{\cdot}56 \times 10^{-3}$; ⬡, $1{\cdot}23 \times 10^{-3}$; ▼, $2{\cdot}0 \times 10^{-3}$. Closed symbols represent solubility points.

slope decreases with increasing values of $[D_f]$ and the lowest limiting slope, corresponding to a solubility curve, represents the saturation-point in the Langmuir-type plot, Fig. 3.

The simplest way to express the binding data is a Langmuir-type plot of r versus $[D_f]$. Equation (6) is a segment of a rectangular hyperbola passing through the origin. If $[D_f]$ becomes infinite, the r value approaches n as a limit

$$\lim_{[D_f] \to \infty} r = n \quad \ldots \quad (7)$$

and at $r = n/2$

$$[D_f] = 1/K \quad \ldots \quad (8)$$

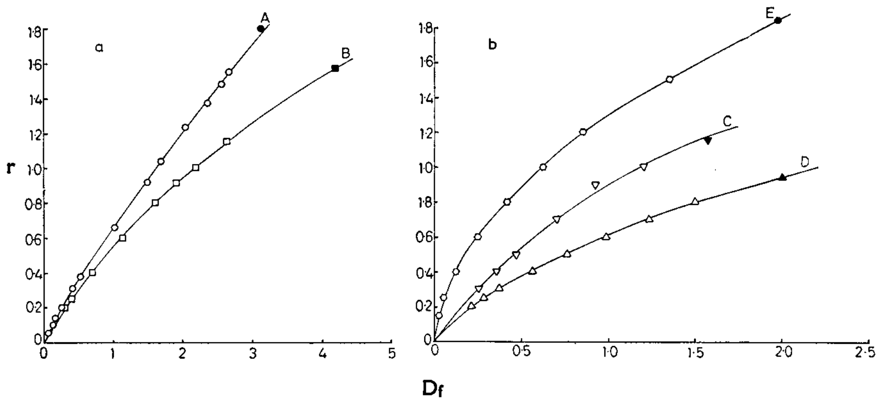

FIG. 3a, b. Langmuir-type plots for the interaction of preservatives with cetomacrogol solutions: A. Benzoic acid. B. *p*-Hydroxybenzoic acid. C. Methyl *p*-hydroxybenzoate. D. Propyl *p*-hydroxybenzoate. E. Chloroxylenol. Cetomacrogol concentrations and D_f values as in Fig. 1. Closed symbols represent solubility point.

Equations (7) and (8) indicate the importance of a wide concentration range of free solute in any binding study. Fig. 3a,b shows that at low concentrations the preservatives are more easily bound to cetomacrogol than at high concentrations. Only results obtained from solubility experiments show saturation of the binding sites. Hence binding parameters were not derived from these plots.

Equation (6) is normally rearranged into forms more convenient for graphical presentation of the results. Fig. 4a,b shows results for the interaction of some preservatives with cetomagrogol plotted according to the reciprocal form of the equation,

$$\frac{1}{r} = \frac{1}{n} + \frac{1}{nK\,[D_f]} \qquad (9)$$

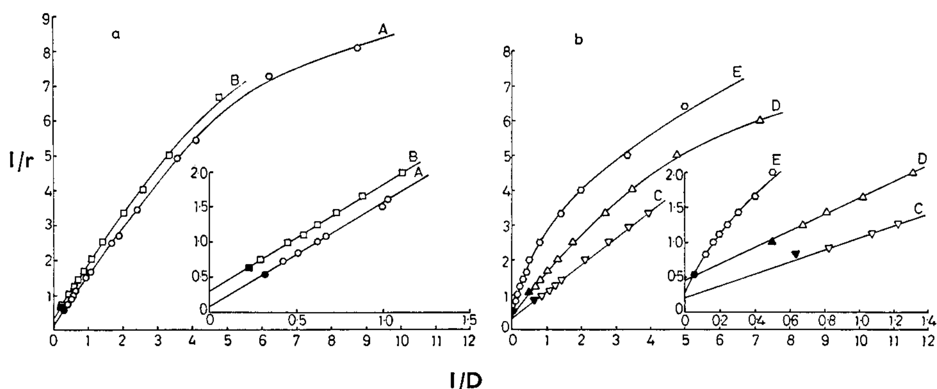

FIG. 4a, b. Double-reciprocal plot for the interaction of preservatives with cetomacrogol solutions: A. Benzoic acid ($1/D_f \times 10^{-2}$). B. *p*-Hydroxybenzoic acid ($1/D_f \times 10^{-2}$). C. Methyl *p*-hydroxybenzoate ($1/D_f \times 10^{-2}$). D. Propyl *p*-hydroxybenzoate ($1/D_f \times 10^{-3}$). E. Chloroxylenol ($1/D_f \times 10^{-4}$). Cetomacrogol concentrations as in Fig. 1. Closed symbols represent solubility points.

A line passing through the origin rather than an intercept corresponding to a limiting binding capacity has been taken as evidence that the mechanism of interaction is partitioning into the micelles rather than adsorption on a micellar surface or to specific sites on the macromolecule (Bahal & Kostenbauder, 1964). This plot, however, heavily weights those experimental points obtained at low concentrations of free drug and may lead to large errors on extrapolation to infinitely high free preservative concentrations. An alternative rearrangement of equation (6) is known as the Scatchard equation (Scatchard, 1949)

$$\frac{r}{[D_f]} = nK - rK \qquad (10)$$

which on plotting gives a more even weighting to the different points on the curve. The plot for each preservative shown in Fig. 5, has a definite curvature. In protein-binding studies, this is taken as evidence for the existence of more than one type of binding site. In the case of solute-surfactant interaction the binding sites within the micelles probably do not behave independently of one another as required by equation (6). It is possible that uptake of solute into the micelles progressively alters the

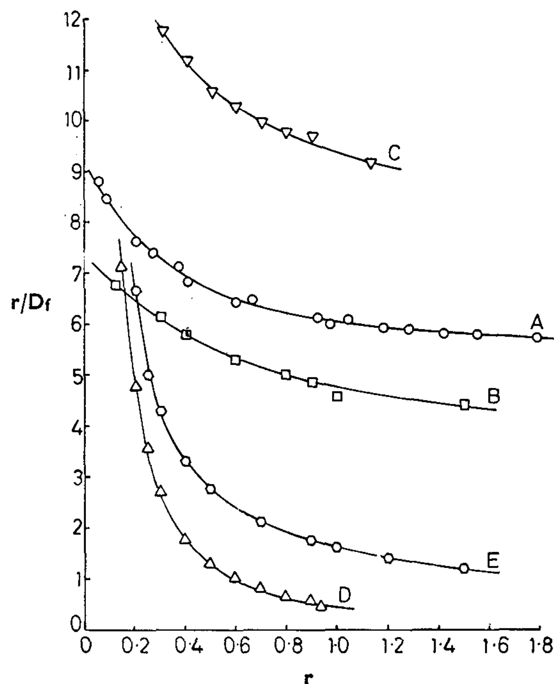

FIG. 5. Scatchard plot for the interaction of preservatives with cetomacrogol solutions: A. Benzoic acid ($r/D_f \times 10^{-1}$). B. *p*-Hydroxybenzoic acid ($r/D_f \times 10^{-1}$). C. Methyl *p*-hydroxybenzoate ($r/D_f \times 10^{-1}$). D. Propyl *p*-hydroxybenzoate ($r/D_f \times 10^{-3}$). E. Chloroxylenol ($r/D_f \times 10^{-3}$). Cetomacrogol concentrations as in Fig. 1.

interaction between the binding sites and solute leading to a change in both the number of sites available and the association constant. Hence to describe the interaction it is necessary to plot the curve over a wide range of $[D_f]$ and determine n and K values from the slope in the region of interest. In the case of preservatives, this is the concentration of free preservative required for antimicrobial activity e.g. a concentration equal to or greater than the minimum inhibitory concentration. Table 1 gives the minimum inhibitory concentrations for a number of preservatives and values of n and

Table 1. *Minimum inhibitory concentrations and binding parameters for the interaction of preservatives with cetomacrogol.*

Preservative	Minimum inhibitory concentration (%) (a)	n (Calculated using monomer molecular weight (e))	K (litre mol^{-1}) (Calculated using monomer molecular weight (e))	n (Calculated using micellar molecular weight)	K (litre mol^{-1}) (Calculated using micellar molecular weight)
Benzoic acid	0·1 (b)	4·6	16	371	16
Methyl *p*-hydroxybenzoate	0·15 (c)	5·2	22	445	22
Propyl *p*-hydroxybenzoate	0·06 (c)	2·2	343	176	341
Chloroxylenol	0·02 (d)	2·8	942	216	949

(a) Highest concentration quoted in each reference (b) Bandelin (1958) (c) Nowak (1963) (d) Aist Gucklhorn (1969) (e) taken as 1300 (f) from Attwood, Elworthy & Kayne (1969).

K for the interaction with cetomacrogol. Substitution of n, K, $[D_f]$ and [M] into equation (11)

$$[D_t] = \left[\frac{nK\,[D_f][M]}{1 + K[D_f]}\right] + [D_f] \quad \ldots \quad \ldots \quad \ldots \quad (11a)$$

$$= [D_f]\left[\frac{nK\,[M]}{1 + K\,[D_f]} + 1\right] \quad \ldots \quad \ldots \quad \ldots \quad (11b)$$

enables the required preservative concentration to be calculated.

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