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The colloidal properties of chlorhexidine and its interaction with some macromolecules

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Surface tension, conductivity and dye solubilization experiments show that, in aqueous solution, chlorhexidine diacetate forms micelles and has a molar critical micellar concentration (CMC) of 0·010–0·011 at 25°. Similarly, the digluconate salt has a molar CMC of 0·0066. Above its CMC, the freely soluble digluconate salt solubilizes the less soluble diacetate salt. The formation of micelles does not affect the rate of hydrolysis of chlorhexidine to *p*-chloroaniline. It is suggested that the micelles formed by chlorhexidine resemble those formed by many dyes rather than those of colloidal surfactants. Equilibrium dialysis experiments show that 1·0 and 3·0% polysorbate 80 inactivates 37·5 and 70·0% respectively of the chlorhexidine in a 0·10% solution of the diacetate salt.

A SIMILARITY in the mode of action between the potent antibacterial compound chlorhexidine (Hibitane) and the quaternary ammonium germicides has been pointed out by Hugo & Longworth (1964) and Rye & Wiseman (1964). Also it is well known that aqueous solutions of chlorhexidine froth markedly on gentle agitation. These two facts have prompted an investigation to see if chlorhexidine behaves as a typical surface-active agent, and, in particular, if it forms micelles and shows a critical micelle concentration (CMC). In addition, chlorhexidine, like other antibacterial compounds, is known to be less active in the presence of non-ionic surfactants. As no quantitative data were available, physicochemical studies of the degree of inactivation have been made.

SURFACE AND COLLOID PROPERTIES

Determinations of CMC were made using a commercial sample of the diacetate salt, but the digluconate solutions were prepared from recrystallized base and 1,5-gluconolactone solutions. Deionized water was used throughout.

Surface tension measurements were made using a Du Nouy tensiometer at room temperature in the usual way, except that surface-ageing effects made it necessary to form the surface 24 hr before measurement. The corrections of Harkins & Jordan (1930) were applied. The results are shown in Fig. 1. A Mullard conductivity bridge and a dipping electrode were used at 25° (±0·01°) for the conductivity measurements. These results are shown in Fig. 2. The standard procedure was followed for determining the CMC by dye solubilization (Rigg & Liu, 1953). Agitation was for 7 days at 25° (±0·1°). Satisfactory results could not be obtained for the digluconate salt but those for the diacetate are shown in Fig. 3. The CMC values obtained by the above methods are listed in Table 1. The presence of large aggregates in chlorhexidine diacetate solutions above the CMC and their absence below was demonstrated by the analytical ultracentrifuge.

The solubilization of chlorhexidine diacetate by the very soluble digluconate was determined by shaking excess diacetate salt with solutions

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of the digluconate of known concentration at 25° (±0·1°). Aliquots were filtered after equilibration (48 hr) and the total chlorhexidine content of the filtrate determined colorimetrically (Holbrook, 1958). The increase in chlorhexidine content must be due to the diacetate salt and Fig. 4 shows diacetate solubility at various concentrations of digluconate.

Experiments to determine the effect of chlorhexidine concentration on the rate of *p*-chloroaniline formation were also made. The *p*-chloroaniline contents of various concentrations of chlorhexidine diacetate, chosen to

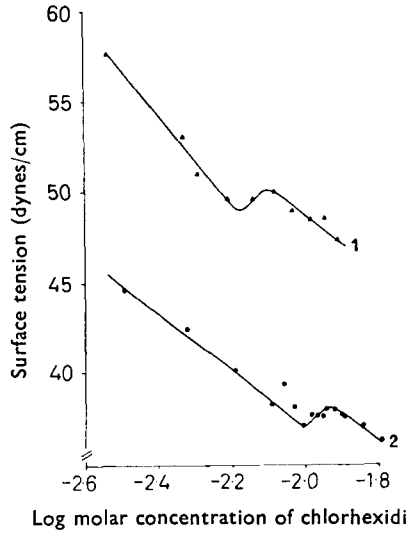

FIG. 1. Determination of CMC values of (1) chlorhexidine digluconate, (2) chlorhexidine diacetate, by surface tension measurements at room temperature

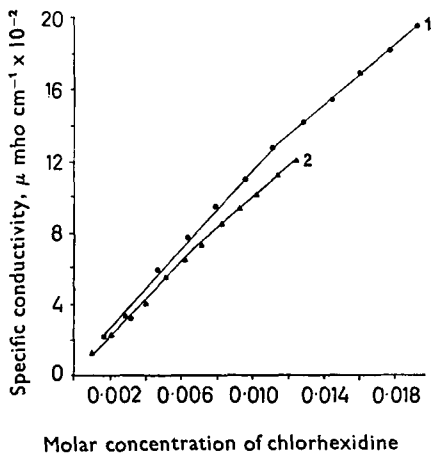

FIG. 2. Determination of CMC values of (1) chlorhexidine diacetate and (2) chlorhexidine digluconate by conductivity measurements at 25°.

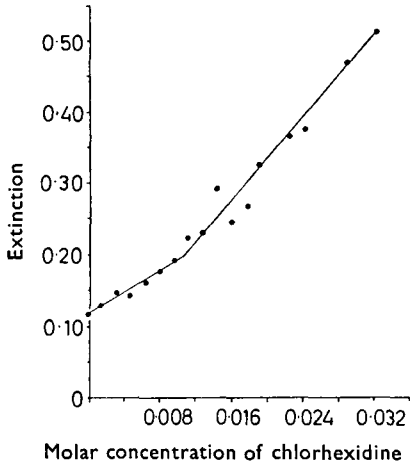

FIG. 3. Determination of CMC of chlorhexidine diacetate by dye solubilization at 25°.

be above and below the CMC (0·77%) at the temperature of the experiment (80°), were determined by a method based on that in the B.P. 1963. Two series of ampoules were filled with the solutions and stored at 80° for 64 and 136 hr respectively. The *p*-chloroaniline concentration was redetermined.

TABLE 1. THE CMC OF CHLORHEXIDINE DIGLUCONATE AND DIACETATE AS DETERMINED BY VARIOUS METHODS

Chlorhexidine salt	Method	CMC Molar	CMC % w/v
Diacetate	Surface tension	0·010	0·63
Diacetate	Conductivity	0·011	0·69
Diacetate	Solubilization	0·0105–0·011	0·66–0·69
Digluconate ..	Surface tension	0·0066	0·59
Digluconate ..	Conductivity	0·0066	0·59

INTERACTION OF CHLORHEXIDINE DIACETATE WITH MACROMOLECULES

To investigate the interaction between chlorhexidine diacetate and polysorbate (Tween) 80 (Honeywill-Atlas), Visking dialysis tubing (Scientific Instrument Centre) was suitable provided that the dialysis was not allowed to continue beyond the time necessary for equilibration of the chlorhexidine diacetate, thus keeping negligible the error caused by dialysis of the surfactant. Dialysis bags, just large enough to hold 20 ml of surfactant solution, were prepared and immersed in 20 ml of chlorhexidine diacetate solution contained in glass-stoppered jars. The jars were agitated at 25° for 5 hr for solutions containing up to 0·2% chlorhexidine diacetate and for 18 hr for stronger solutions. At equilibrium the chlorhexidine content on both sides of the membrane was determined either by measuring the extinction at 254 mμ after suitable dilution or colorimetrically (Holbrook, 1958). When the former method was used, correction for absorbance by the surfactant was necessary. The

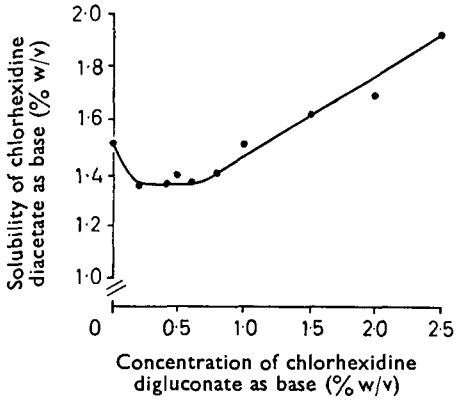

FIG. 4. Effect of chlorhexidine digluconate concentration on the solubility of chlorhexidine diacetate at 25°.

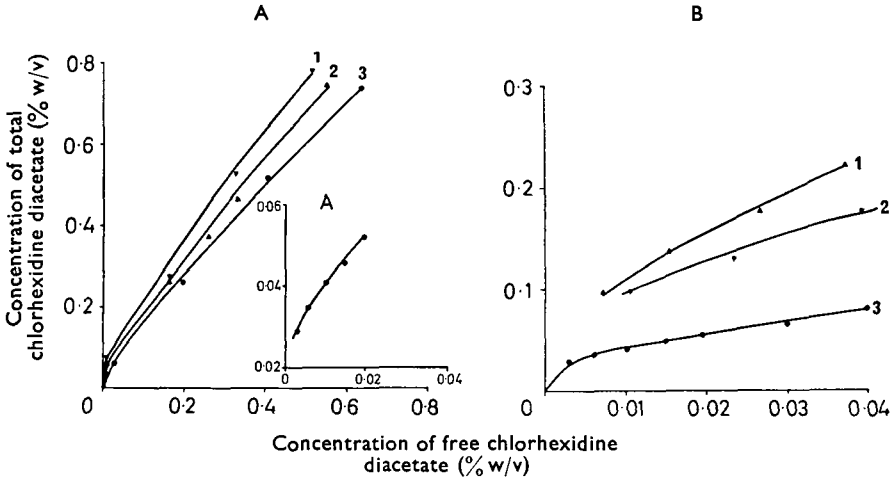

FIG. 5A. A plot showing the interaction of chlorhexidine diacetate with (1) 3%, (2) 2%, (3) 1% polysorbate 80 at 25°. A is a plot of the interaction of 1% polysorbate 80 at low chlorhexidine diacetate concentrations.

B. A plot showing the effect of various concentrations of sodium acetate on the interaction of chlorhexidine diacetate with polysorbate 80 at 25° and pH 5·8. (1) 0·25 M buffer, (2) 0·1 M buffer, (3) buffer absent.

results for various polysorbate 80 concentrations are in Fig. 5A which shows the amount of free chlorhexidine diacetate at various total concentrations. The dialysis experiments were repeated in the presence of 0·1 and 0·25M sodium acetate buffers respectively at pH 5·8 (this is the pH of unbuffered chlorhexidine diacetate and polysorbate 80 mixtures). The results are in Fig. 5B.

Because ethanol has a dis-aggregating effect on non-ionic surfactant micelles (Becher, 1965) the dialysis experiments were again repeated in the presence of various concentrations of ethanol. The solubility of

chlorhexidine diacetate in ethanol–water mixtures and the effect of ethanol concentration on the CMC of polysorbate 80, determined by the method of Becher (1962), are shown in Table 2.

TABLE 2. THE EFFECT OF VARIOUS CONCENTRATIONS OF AQUEOUS ETHANOL ON THE SOLUBILITY OF CHLORHEXIDINE DIACETATE AND THE CMC OF POLYSORBATE 80

Concentration of ethanol % v/v	Solubility of chlorhexidine diacetate at 25°	CMC polysorbate 80 at 20°
0	1·89% w/v	0·0078% w/v
10	2·40	0·0105
20	3·65	0·0145
30	—	0·0263
35	—	0·053
40	—	No micelles formed
50	14·40	—
60	18·40	—

Using the same dialysis technique, the interaction of chlorhexidine diacetate with both methylcellulose and polyvinylpyrrolidone was determined. At equilibrium, the concentrations of chlorhexidine on both sides of the membrane were essentially the same.

Discussion

SURFACE AND COLLOID PROPERTIES

Surface tension, conductivity and dye solubilization experiments all show that at a certain concentration, aqueous solutions of chlorhexidine exhibit a sharp change in the relevant physico-chemical property. The concentration at which this occurs depends on the counter-ion. This is strong evidence that aggregation of either single molecules or, possibly, small aggregations of molecules are starting to form micelles at this concentration. Confirmation of this comes from the analytical ultracentrifuge which demonstrated that only above these concentrations were large aggregates present. Chlorhexidine consists of a series of alternate hydrophilic and hydrophobic groups. In no way can it be considered to have an amphipathic character in the sense of having a polar head and a non-polar chain. It is suggested, therefore, that like polyvinyl alcohol, chlorhexidine should be considered as a specific surface-active agent (Moilliet, Collie & Black, 1961a). Thus the reduction in surface tension is caused by some specific group being attracted to the air-water interface.

Similarly, it is difficult to visualize how chlorhexidine could form a micelle of the type formed by colloidal surfactants. It has been known for many years that water-soluble dyestuffs often exist in solution in an aggregated form (Vickerstaff, 1954). Aggregation of these dyes is not necessarily associated with adsorption at the air–water interface (Alexander & Stacey, 1952) and it has been suggested that aggregation is primarily due to some specific forces, in particular those due to hydrogen bonding, rather than amphipathy in the dyestuff molecule (Moilliet, Collie & Black, 1961b). We hypothesize that the aggregation of chlorhexidine to form micelles is more akin to the aggregates formed by dyestuffs

than by colloidal surfactants and that the force responsible may be hydrogen-bonding associated with the diguanido-groups. Figs 1 and 2 both show the effect of the counter-ion on the CMC of chlorhexidine. It is well known that the counter-ion can alter the CMC and this is usually associated with a change in micellar size.

Klevens (1950) gives several examples where the micelles of a more soluble salt have solubilized a less soluble salt. This is clearly shown by chlorhexidine digluconate and diacetate in Fig. 4. Two points emerge from these results. Firstly, the concentration at which solubilization commences (0·60–0·80% of chlorhexidine base) is slightly higher than the CMC of chlorhexidine digluconate as determined by surface tension and conductivity measurements (0·59%) and, secondly, below the CMC there is a slight, but definite, decrease of solubility of chlorhexidine diacetate compared with its solubility in water. This might be expected from solubility product considerations.

Chlorhexidine slowly hydrolyses in aqueous solution to give, among other products, *p*-chloroaniline (Goldman, J. & Goodall, R. R., unpublished observation). The formation of micelles might lead to some protection of the hydrolysable group and thus reduce the rate of hydrolysis, but for the conditions and concentrations studied, no change in hydrolysis rate above and below the CMC could be detected [cf. sodium lauryl sulphate (Nogami, Awazu & Kanakubo, 1963)].

Hugo & Longworth (1964) and Rye & Wiseman (1964) have pointed out similarities between the mode of action of chlorhexidine and the quaternary ammonium antibacterial compounds. The fact that these latter compounds and chlorhexidine lower the surface tension of water and form micelles is a further similarity. However, it must be remembered that although at the CMC the surface tension of cetrimide solutions is similar to that of chlorhexidine diacetate solutions, the concentration of the former is lower, on a molar basis, by about one order of magnitude.

INTERACTION WITH MACROMOLECULES

Fig. 5A shows that like many other antibacterial compounds, chlorhexidine interacts with polysorbate 80. The higher the concentration of the polysorbate 80, the greater is the interaction. Also, the ratio of free to total chlorhexidine is dependent at low chlorhexidine concentrations on the actual concentration of chlorhexidine present. This is shown more clearly in insert A, Fig. 5A. Table 3 compares the inactivation of

TABLE 3. COMPARISON OF THE INACTIVATION OF CHLORHEXIDINE DIACETATE BY POLYSORBATE 80 AS DETERMINED BY A DIALYSIS METHOD AND A BACTERICIDAL TECHNIQUE USING *Staph. aureus*

Concentration polysorbate 80	Method	% inactivation of 0·1% chlorhexidine diacetate
1·0	Dialysis at 25°	37·5
1·0	Bactericidal at 30°	61·0
3·0	Dialysis at 25°	70·0
3·3	Bactericidal at 30°	86·0

chlorhexidine diacetate by polysorbate 80 as determined in this present work with the inactivation as determined by an *in vitro* bactericidal technique using *Staphylococcus aureus* (Mr. R. Hall, unpublished observations).

There are a number of possible reasons for the agreement between the two methods not being closer. The two series of experiments were made at different temperatures, concentrations of polysorbate 80 were slightly different and replaced thermodynamic activities, and no allowance was made for possible interference from the Donnan effect. Closer examination shows that the effects of all these factors are likely to be small. A more likely reason for the difference is that whilst the physico-chemical technique only measures the interaction of the surfactant with chlorhexidine, the bactericidal test in addition measures any interference by the surfactant on the uptake of chlorhexidine by the bacteria (Wedderburn, 1964).

Fig. 5B shows the effect of various concentrations of acetate ion on the interaction between chlorhexidine and polysorbate 80. Acetate ion might be expected to decrease the solubility of chlorhexidine diacetate leading to increased interaction with the polysorbate 80. Acetate ion would also be expected to affect the polysorbate 80. Thus it might change the micellar size and lower the CMC. It is not possible to predict the magnitude of these changes (Elworthy & Macfarlane, 1965). However, the CMC of polysorbate 80 is so low (0·078% w/v) that reduction of this value could not increase the amount of micellar material sufficiently to account for the increase in interaction observed. The major effect leading to increased interaction between chlorhexidine diacetate and polysorbate 80 in the presence of excess acetate ion is, therefore, mainly due to the "salting-out" of the chlorhexidine.

The effects of ethanol on the aqueous solubility of chlorhexidine diacetate and the CMC of polysorbate 80 are shown in Table 2. There is a fall in interaction between chlorhexidine diacetate and polysorbate 80 with increasing concentrations of ethanol. The fall in interaction in the presence of 10 and 20% of ethanol is probably more a result of the increased solubility of chlorhexidine diacetate in the aqueous phase than of the slight reduction in micellar surfactant. That interaction still occurs between chlorhexidine diacetate and polysorbate 80 in the presence of 50 and 60% of ethanol when no micelles of surfactant are present must mean that chlorhexidine diacetate can interact with monomeric polysorbate 80 in the presence of aqueous ethanol. As with phenols (Mulley & Metcalf, 1956), this interaction may well be hydrogen-bonding between the diguanido-groups and the ether oxygens of the polyoxyethylene chains. Because the polysorbate 80 is in a non-micellized form in the presence of the two higher concentrations of ethanol, it will diffuse more rapidly through the dialysis membrane. This will result in some error associated with the interaction values at these concentrations.

There are essentially three areas in a micelle where a compound can be solubilized (Riegelman, Allawala & others, 1958). These are the central portion, the surface of the micelle and the intermediate "palisade" layer. Chlorhexidine is essentially insoluble in hydrocarbon solvents and we consider that, like dimethyl phthalate, it is probably adsorbed on the

surface of the micelle. A suggestion for the shape of the interaction curves in Fig. 5A can now be made. Fig. 5A shows relatively much greater interaction at low chlorhexidine concentrations, followed by a roughly linear relation between total and free chlorhexidine. This could be caused by the two different types of interaction, namely adsorption on the micellar surface and hydrogen-bonding with the polyoxyethylene chain, which it is postulated chlorhexidine diacetate and polysorbate 80 can undergo.

Both methylcellulose and polyvinylpyrrolidone with chlorhexidine diacetate gave equilibrium concentrations which were essentially equal on both sides of the membrane. From this it follows that chlorhexidine diacetate interacts with neither. Miyawaki, Patel & Kostenbauder (1959) have previously shown that methyl- and propyl-*p*-hydroxybenzoates interact with both methylcellulose and polyvinylpyrrolidone. However, neither of the quaternary ammonium compounds tested by Deluca & Kostenbauder (1960) interacted with polyvinylpyrrolidone although one, cetylpyridinium chloride, did interact with methylcellulose.

The experimental work reported here shows that in common with other antibacterial agents, chlorhexidine shows some inactivation by polysorbate 80. Although the amount of inactivation is not so large as with certain other antibacterials, nevertheless, for optimum activity the concentration of non-ionic surfactant should be kept as low as practicable.

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