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Surface and Solution Properties of Steroid Antibiotics: 3-Acetoxylfusidic Acid, Cephalosporin P_1 and Helvolic Acid†

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ABSTRACT: The colloid/chemical properties of the fusidane antibiotics, 3-acetoxylfusidic acid, cephalosporin P_1, and helvolic acid, and their sodium salts, were investigated. The sodium salts of 3-acetoxylfusidic acid and cephalosporin P_1 were found to be detergent-like molecules with micellar properties comparable to the parent compound sodium fusidate and the bile salt sodium cholate. Critical micellar temperatures (cmt) were less than 0°C except for sodium helvolate which being sparingly soluble did not form micelles between 0 and 50°C. Potentiometric titrations of dilute solutions gave apparent pK values (5.2–6.5) in the range expected for carboxylated steroid detergents. The apparent pK values increased significantly once the detergent concentration exceeded the critical micellar concentration (cmc). Micellar properties were determined by surface tension, titration with a water-soluble dye (Rhodamine 6G), light scattering, and solubilization of lecithin and cholesterol. Cmc's, in the range of 1.5 to 5.6 mM, were found which varied slightly depending on the method employed and in all cases fell slightly in the presence of added NaCl. The number of monomers per micelle (aggregation number) in concentrations well above the cmc was extrapolated from Debye light scattering plots in 0.15 M NaCl. The values varied from 6 for fusidate to 14 for 3-acetoxylfusidate with sodium cephalosporin P_1 having an intermediate value. Each detergent readily solubilized the phospholipid lecithin. The maximum solubility of cholesterol in lecithin–detergent mixed micelles varied from 6 to 10 mol %. The counterion binding of micelles in water and in 0.15 M Na^+ was calculated from the log cmc–log Na^+ concentration curves and from Debye light scattering plots. The percent counterions bound to fusidane micelles was similar to that bound to sodium cholate micelles, but was significantly less than that found with typical straight-chain detergent micelles. Pressure–area isotherms were determined on a Langmuir-Pockels surface balance for each compound on an aqueous subphase containing 5 M NaCl at pH 2. Each of the isotherms was distinctive and the area of each antibiotic at its collapse point varied from 95 to 124 $Å^2$ per molecule, all significantly larger than cholic acid (90 $Å^2$ per molecule). These areas correlated well with estimates of the areas of the salts from surface tension measurements (107–115 $Å^2$) and with areas calculated from Stuart-Briegleb molecular models of the salts lying flat (103–118 $Å^2$). It is suggested that the unique physical chemical characteristics of these amphiphilic antibiotics may be important in their antibiotic activity and in their ability to mimic many of the physiological properties of the bile salts. Owing to their close chemical and biophysical similarity to bile salts, these drugs may serve as model compounds for detergent replacement in bile salt deficiency syndromes.

A wide variety of drugs are surface active and aggregate to form micelles in aqueous solution (Florence, 1968). This is of considerable importance in drug action because the structural requirements for surface activity and micelle formation are often similar to those for interaction of a drug with receptor sites, serum proteins, or membrane components (Tanford, 1973). Steroids of the fusidane family represent a unique class of soluble amphiphiles in that they structurally resemble the bile salts, the alimentary biodetergents of vertebrates (Godtfredsen, 1967; Florence, 1968; Carey and Small, 1971). The aggregation properties of sodium fusidate and some of its derivatives including their glycine and taurine conjugates were previously studied, and a marked similarity with the micellar properties of the common vertebrate bile salts was found (Carey and Small, 1971, 1973). One of these derivatives, taurodihydrofusidate, was actively secreted into bile of primates without appreciable metabolism and significantly influenced the biliary secretion of bile salts, phospholipids, and cholesterol (Beaudoin et al., 1973).

In this paper we evaluate the colloidal properties of 3-acetoxylfusidic acid (Godtfredsen, 1967), shown previously

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to influence biliary lipid secretion in the rat (Montet et al., 1973) and two other analogous fusidane antibiotics, cephalosporin P_1 and helvolic acid, which have chemical and antimicrobial properties similar to fusidic acid (Godtfredsen, 1967) and which are secreted in high concentrations in bile when administered to mice (Chain et al., 1943; Ritchie et al., 1951). We have explored the micellar, surface, potentiometric, and solubilizing properties of these compounds and their sodium salts in water and NaCl solutions and compared them with the parent compound sodium fusidate and with a bile salt sodium cholate in an attempt to determine in detail the effect of the chemical structure of the fusidane antibiotics on their surface and colloid behavior and to assess whether these drugs could be developed as potential bile salt substitutes for bile salt deficiency states.

Experimental Procedure

Materials. Sodium fusidate (sodium salt of 3α,11α-dihydroxy-16β-acetoxyfusida-17(20)-[16,21-*cis*],24-dien-21-oic acid, Figure 1) and sodium 3-acetoxyfusidate (sodium salt of 3α,16β-diacetoxy-11α-hydroxyfusida-17(20)-[16,21-*cis*],24-dien-21-oic acid, Figure 1), fermentation products of *Fusidium coccineum* (K. Tubaki), were gifts from Dr. W. O. Godtfredsen (Leo Pharmaceutical Products, Ballerup, Denmark). Cephalosporin P_1 (3α,7β-dihydroxy-6α,16β-diacetoxyfusida-17(20)-[16,21-*cis*],24-dien-21-oic acid, Figure 1) from the culture fluid of a strain of Cephalosporium (G. Brotzu) was supplied by Dr. W. E. J. Cuthbertson (Glaxo Research Ltd., Stoke Poges, Bucks, U.K.) and helvolic acid(7α,16β-diacetoxyfusida-1-en-3,6-dione-17(20)-[16,21-*cis*],24-dien-21-oic acid, Figure 1), from the culture filtrates of an *Aspergillus fumigatus* (mut. helvola yuill), was supplied by Dr. S. Okuda (Institute of Applied Microbiology, The University of Tokyo, Tokyo, Japan). Sodium cholate (sodium salt of 3α,7α,12α-trihydroxy-5β-cholanoic acid) was obtained from the Maybridge Chemical Co. (Tintagel, Cornwall, U.K.). Egg yolk lecithin (Grade 1) was purchased from Lipid Products (South Nutfield, Surrey, U.K.) and cholesterol was obtained from the Hormel Institute (Austin, Minn.) and recrystallized twice from hot ethanol. All lipids except cephalosporin P_1 were judged to be at least 99% pure by thin-layer chromatography (TLC) (200-μg spots) on silica gel G developed with isooctane–ethyl acetate–acetic acid, 40:40:12 (v/v/v) (acidic steroids) and chloroform–methanol–H_2O, 65:35:4 (v/v/v) (neutral lipids), sprayed with ferric chloride–H_2SO_4 followed by heating to 200°C. The cephalosporin P_1 sample consisted of 90% cephalosporin P_1, the remainder being composed of isocephalosporin P_1 (~9%) which has a 6α-hydroxy-7β-acetoxy structure and helvolic acid (~1%). The material was recrystallized in the cold (4°C) from hot ethanol (50°C) 2–4 times until TLC homogeneity was achieved. Rhodamine 6G (Allied Chemical Company, Morristown, N.J.) used for critical micelle concentration (cmc) determinations was spectrophotometrically pure. All common chemicals were reagent grade. NaCl was roasted in a muffle furnace at 600°C for 6 hr to oxidize and remove organic impurities. All glassware was Pyrex brand and alkali-acid washed by steeping overnight in tanks of EtOH–2 *N* KOH (50:50, v/v) and 1 *N* HNO_3 followed by thorough rinsing with water and drying in an air oven at 110°C. Water was tripley distilled, the second distillation being from an automatic distillation apparatus (Corning Glass Works, Corning, N.Y.) followed by distillation in a seasoned all-Pyrex laboratory distillation assembly over $KMnO_4$.

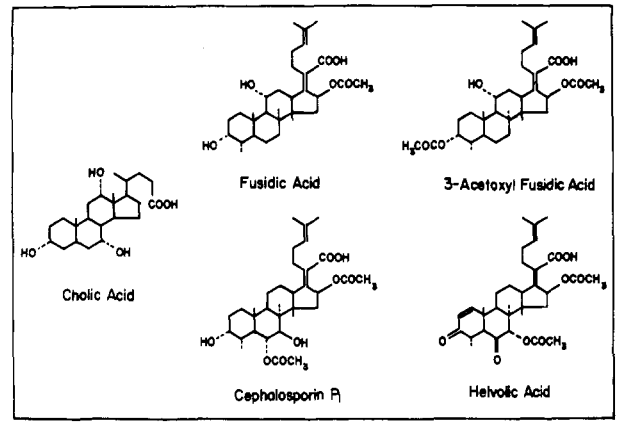

FIGURE 1: Conventional chemical configuration of cholic acid (3α,7α,12α-trihydroxy-5β-cholanoic acid) and the four fusidane antibiotics studied in this work.

Methods. (a) Solutions. The sodium salts of cephalosporin P_1 and helvolic acid were prepared by dissolving a weighed amount of the acids in MeOH, adding a stoichiometric quantity of 2 *N* NaOH, and evaporating the MeOH–H_2O solvent with a vacuum pump in a glass desiccator over P_2O_5. Aqueous or carbonate–bicarbonate buffered solutions of the salts were prepared gravimetrically in 5–50 ml volumetric flasks. Critical micellar temperatures (cmt) of 1% (w (g)/v, approximately 17 m*M*) solutions were determined by the clearing point method (Small and Admirand, 1969) and solubilities of 2.5% (w/v, approximately 43 m*M*) solutions in NaCl of varying molarity were carried out as previously described (Carey and Small, 1973). For Rhodamine 6G determinations of the cmc, the stock solutions were prepared in cuvettes with the addition of a known volume of the solvent. Concentrations (w/v) of solutions were checked in triplicate by the dry weight method after correcting for the weight of the added electrolyte. The pH of all solutions was either buffered to pH 10.0 (0.01 *M* carbonate–bicarbonate buffer) or adjusted to pH 10 ± 0.2 by the addition of 2 *N* NaOH. Solutions of cephalosporin P_1 at pH 10 in the cold were partly transformed into the isomeric compound isocephalosporin P_1 (Godtfredsen, 1967). By TLC about 40% of cephalosporin P_1 and 60% of isocephalosporin P_1 were present at equilibrium (~24 hr). When these solutions were heated or left for longer than 5 days at room temperature significant amounts of deacetylcephalosporin P_1 formed which precipitated from solution. Most physical-chemical measurements were therefore carried out on these solutions within 2–3 hr of their constitution; however, light scattering measurements on cephalosporin P_1 took up to 24 hr. The solution temperature for all studies was 25 ± 0.2°C unless otherwise specified.

(b) Potentiometric Titration. Titrations were carried out with 0.6 or 0.95 *N* HCl after prior adjustment of the pH of the solution to about 12.0 with 2 *N* NaOH. A 17–20 m*M* (1%, w/v) detergent solution in 5 ml was placed in the glass cup of a manual titration assembly through which N_2 saturated with H_2O was circulated. After the addition of small aliquots of HCl, the equilibrium pH was measured with a H^+ sensitive glass electrode (Corning Glass Works, Corning, N.Y.) fitted to an expanded scale pH meter (Radiometer, Copenhagen, Denmark). In the case of 3-acetoxylfusidate and cephalosporin P_1, a systematic series of titrations was carried out at concentrations (approximately 0.25–20 m*M*) which ranged from well below to well above their crit-

ical micellar concentrations (cmc's). The procedures for measuring equilibrium and precipitation pH's identifying supersaturation, calculating the solubilities of the acids and apparent pK values (pK_a) have been described elsewhere (Small, 1971).

(c) Surface Tension. Equilibrium surface tensions were measured with a Fisher Tensiometer employing a platinum Wilhelmy blade (2 × 1 cm). The procedures for preparing the solutions, marking and sweeping the solution surface, and timing the aging of the surface have been documented previously (Carey and Small, 1971). The solution concentrations ranged from 0.125 to 20 mM in 0.01 M carbonate–bicarbonate buffer to which 0.14 M NaCl had been added. The pH of all solutions was 10 ± 0.2. The cmc of each detergent was estimated from the break points in the surface tension vs. log detergent concentration plots. As the solutions contained a swamping excess of neutral electrolyte, the interfacial areas at surface saturation were calculated from the simplified form of the Gibbs adsorption isotherm equation (Pethica, 1954).

(d) Dye Titration. Critical micelle concentrations were determined by titration of a stock solution of each detergent with buffered (pH 10.0 ± 0.2) Rhodamine 6G (2.5×10^{-6} M) solutions at 25 ± 0.2°C. In order to study the effect of increasing counterion concentration on the cmc's a systematic series of titrations was carried out in 0.01, 0.05, 0.15, 0.3, and 1.0 M NaCl/carbonate–bicarbonate buffer. The experimental details for estimating the cmc's from each set of spectrophotometric curves and the validation of the method have been described (Corrin et al., 1946; Tori and Nakagawa, 1963; Carey and Small, 1969, 1971).

(e) Surface Balance. A 2 × 1 cm platinum Wilhelmy blade was suspended from a Cahn electromicrobalance in order to measure surface tension in a Teflon-coated aluminum Langmuir-Pockels trough (40 cm × 14 cm) filled to the brim with 5 M NaCl (in triply distilled water) adjusted to pH 2.0 with HCl. Using calibrated acid–alkali washed pipets specific amounts of fusidic acid, 3-acetoxylfusidic acid, cephalosporin P_1, and helvolic acid, dissolved in a double distilled organic solvent mixture (hexane–ethanol, 9:1, v/v), were spread on the aqueous surface. After allowing the solvents to evaporate (3–5 min), the trough was totally enclosed in a large plastic box and automatic compression of the surface was carried out by a piston to which a Teflon barrier extending across the aqueous surface was attached. The rate of compression was adjusted to 2.5 cm/min as this rate gave reproducible results. Continuous pressure–area isotherms were recorded on an x–y recorder and carried out in triplicate. The solution surface was cleaned before each run by sweeping the surface marked with a few grains of pure talc with a manually operated Teflon barrier followed by suction with a vacuum line and an air-jet. The Wilhelmy blade was washed thoroughly in distilled water and heated to incandescence before and after each run. All experiments were carried out at 23 ± 1°C in water-saturated air at atmospheric pressure.

(f) Light Scattering. A Brice-Phoenix light scattering apparatus (Model 2000) with a light wavelength of 546 nm was employed. A range of detergent solutions, above and below the cmc, was prepared with 0.14 M NaCl in 0.01 M carbonate–bicarbonate buffer (pH 10 ± 0.2) and the solutions were aged for 24 hr. It was confirmed by TLC that an isomeric mixture containing 60% isocephalosporin P_1 and 40% cephalosporin P_1 but no deacetylcephalosporin P_1 was present at equilibrium. These were then filtered directly

Table I: Micellar and Molecular Data of Steroid Detergents (25°C, pH 10.0).

	Mol Wt (Anhydrous)	Mp[a] (°C)	Cmc (mM) in 0.15 M Na^+ Buffer: Dye Titration	Cmc (mM) in 0.15 M Na^+ Buffer: Surface Tension	Cmc (mM) in 0.15 M Na^+ Buffer: Light Scattering	Cmt (°C) 1% Soln. in H_2O	Cholesterol Solubility (mol %) (Detergent–Lecithin Molar Ratio 7/3 in H_2O)[b]	App pK_a (17–20 mM Solution in Water)	Solubility in NaCl Solutions (2.5% w/v)	% of Na^+ Ions Bound to Micelles in Water[c]	Molecular Area (Å^2): Surface Balance	Molecular Area (Å^2): Surface Tension	Molecular Area (Å^2): Molecular Model	Aggregation No. in 0.15 M Na^+	Apparent Partial Specific Vol $(\bar{v})$ (4%, w/v)
Sodium 3-acetoxylfusidate	580.74	209–210 (acid)	1.65	3.30	2.07	<0	10	6.45	Sol. 0.5 M Insol. 0.75 M	16	124	115	118	14 (L.S.)[d]	0.794
Sodium[e] fusidate	538.70	192–193 (acid)	2.46	3.70	5.57	<0	8	5.35	Sol. 1 M Insol. 2 M	4	107	107	107	6 (L.S.) 8 (U.C.)[f]	0.777
Sodium cholate	430.60	198 (acid)	2.25			<0	8	5.35	Sol. 1 M Insol. 2 M	14	90[g]	88[g]	93[g]	5 (U.C.)[g]	0.754
Cephalosporin P_1	596.74	146–147 (acid)	1.68	3.1	1.84	<0	6	5.58	Sol. 1 M Insol. 2 M	23	95[h]	73	103	9 (L.S.)	0.785

[a] By hot stage polarizing microscopy. [b] By coprecipitation from $CHCl_3$–MeOH. [c] Calculated from the slopes of log cmc – log NaCl curves. [d] L.S. = light scattering (Debye plots) at 23°C. [e] Data from Carey and Small (1971) except for light scattering results. [f] U.C. = ultracentrifugation by equilibrium or sedimentation and diffusion methods. [g] From Small (1971). [h] Area at second-order surface phase transition.

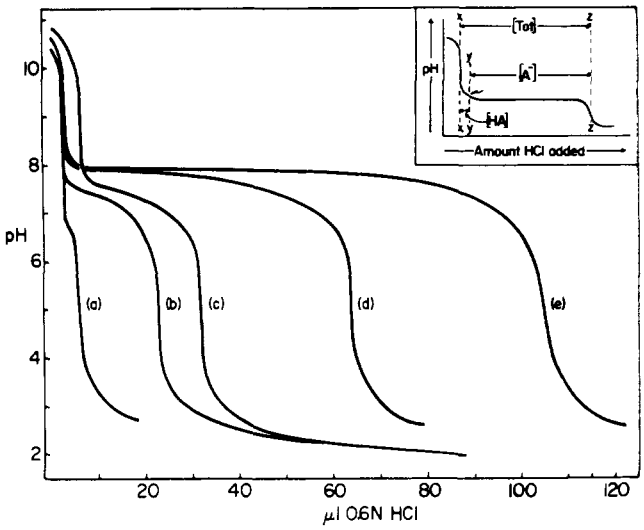

FIGURE 2: Concentration dependence of proton titration curves for sodium 3-acetoxylfusidate (25°C): (a) 0.5, (b) 3, (c) 4, (d) 10, and (e) 17 m*M*. The inset shows a hypothetical titration curve for solutions of steroid detergents with ionizable carboxyl groups: x = first equivalence point where titration of the steroid detergent with hydrochloric acid commences; y = Tyndall effect noted in this region of titration curves; this is the last point where the steroid detergent solution is in thermodynamic equilibrium as a single aqueous phase; z = second equivalence point where titration of the steroid detergent with hydrochloric acid is complete; Tot = total amount of acid required to complete the titration; HA = the amount of acid added from the first equivalence point (x) to point y which represents the maximum solubility of the protonated acid form of the detergent in the ionized detergent solution (A^-). For further explanation of the titration curves see the detailed description in the text.

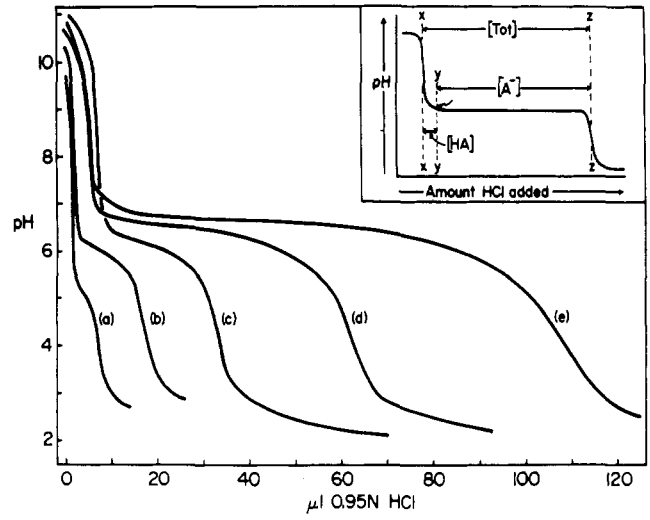

FIGURE 3: Concentration dependence of proton titration curves for sodium cephalosporin P_1 (25°C): (a) 0.9, (b) 2.9, (c) 5.0, (d) 10.3, and (e) 19.2 m*M*. The inset shows a hypothetical titration curve and the explanation of the symbols is identical with that given in Figure 2 (legend).

through pre-wetted Sartorius membranes (pore diameter 0.20 μm) under low nitrogen pressure into square cells (30 × 30 mm) of 30-ml capacity. The scattered light intensity was measured at angles of 0 and 90° at 23 ± 1°C. Refractive index increments (dn/dc) were determined with a Brice-Phoenix differential refractometer (Model BP 2000 V) at 23 ± 0.1°C at a wavelength of 546 nm. The apparatus was calibrated with a series of aqueous sucrose reference solutions.

(g) Mixed Micellar Solutions. Mixed micelle formation with each antibiotic and lecithin and cholesterol as 10% (w/v) solutions was investigated by composing a series of mixtures in which 7 parts of the sodium salt of each steroid and 3 parts of egg lecithin on a molar basis with varying amounts of cholesterol were dissolved in $CHCl_3$–MeOH (1:1, v/v) in 5-ml volumetric flasks. The organic solvent was removed under reduced pressure over P_2O_5 until constant weight was achieved. Distilled water was then added and the mixtures vortexed for 3 min. The pH was adjusted to pH 10 ± 0.2 by the addition of a few microliters of 2 *N* NaOH. Each mixed micellar solution was gassed with dry N_2 for 1 min, sealed, and allowed to equilibrate with gentle agitation at 25 ± 0.2°C for 3–5 days. The maximum solubility of cholesterol in sodium cephalosporin P_1–lecithin mixtures was determined by light scattering at 24–48 hr. The volumetric flask with the highest relative cholesterol concentration as a clear mixed micellar solution at the end of the equilibration periods (optical density (OD) less than 0.020 at 750 nm/cm^2 cuvettes) was taken as the limit of cholesterol solubility in the system (Carey and Small, 1973).

(h) Apparent Partial Specific Volumes. Densities of 4% (w/v) solutions (actual concentrations known to four significant figures) of each detergent in water (pH 10.0) were measured in an Anton Paar Precision Density Meter (DTC 2) at 23.58 ± 0.01°C. Apparent partial specific volumes were calculated according to the formula (Sakura and Reithel, 1972):

$$\bar{v} = \frac{1}{d_0} - \frac{1}{x}\left(\frac{d - d_0}{d_0}\right)$$

where $\bar{v}$ is the apparent partial specific volume in milliliters per gram, d_0 is the density of the solvent, d is the density of solution, and x is the concentration of solute in grams/milliliter (w/v).

Results

The cmt's of 1% w/v solutions of 3-acetoxylfusidate and sodium cephalosporin P_1 in water (pH 10.0) were less than 0°C; however, due to the freezing of water, values could not be determined below 0°C (Table I). Sodium helvolate was sparingly soluble in water (pH 10.0) at 25°C and decomposed upon heating to 50°C in an attempt to measure its cmt.

Representative equilibrium proton titration curves for sodium 3-acetoxylfusidate and sodium cephalosporin P_1 are summarized in Figures 2 and 3, respectively. A hypothetical titration curve is shown in the insets in Figures 2 and 3 and corresponding parts of the actual titration curves are referred to according to these symbols. The initial part of each curve at high pH (to the left of x) represents the titration of a small added excess of NaOH with HCl. At the first inflection point (x), the convex slope of each curve becomes concave, indicating the first equivalence point where the following chemical reaction commences:

$$Na^+A^- + HCl \rightarrow HA + Na^+Cl^-$$

The pH falls steeply until a sharp break point appears in the curves. In the case of cephalosporin P_1 (Figure 3) the precipitation of the insoluble protonated acid at y is detected by the appearance of a Tyndall cone between x and the break point. In the case of 3-acetoxylfusidate (Figure 2) the precipitation pH lies on the other side of the break points. These pH values are plotted against the detergent concen-

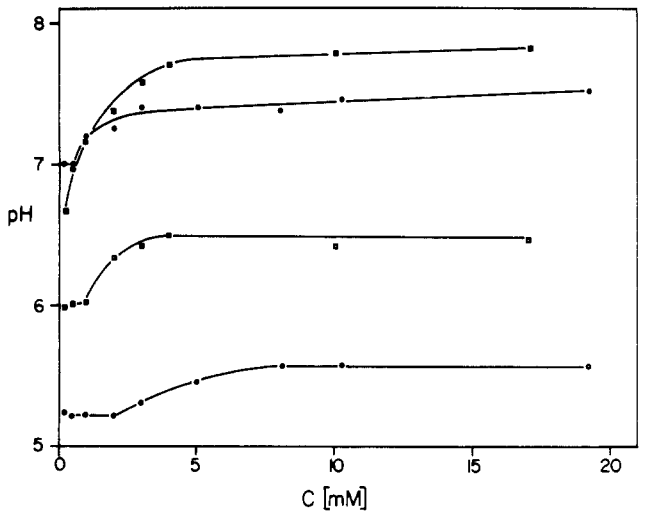

FIGURE 4: The apparent pH of precipitation and pK_a of 3-acetoxylfusidate (■,□) and sodium cephalosporin P_1 (●,○), respectively, as a function of the detergent concentration in water at 25°C. In both cases the pH of precipitation increases significantly with increases in concentration levelling off at about 5 mM. The apparent pK values are relatively constant at low concentrations, and increase sharply over a narrow concentration range to plateau again at concentrations greater than 3–7 mM. Significant differences in pH of precipitation and pK_a of both detergents are apparent.

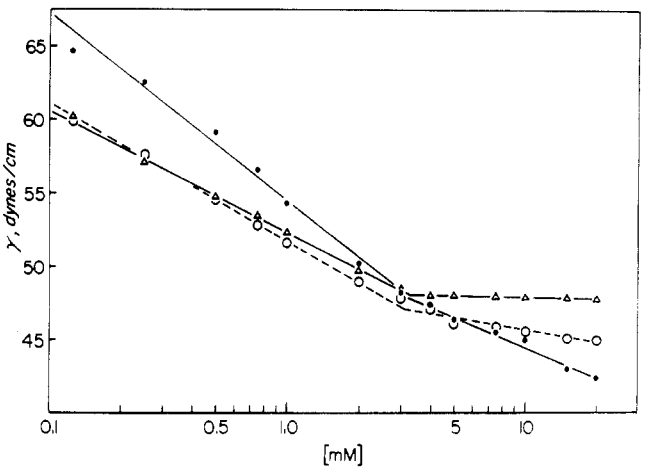

FIGURE 5: Surface tension–log concentration dependence of solutions of sodium cephalosporin P_1 (●), sodium 3-acetoxylfusidate (△), and sodium fusidate (○) from Carey and Small (1971) at 25°C (0.15 M Na^+, pH 10.0). The interfacial molecular areas of the molecules were calculated from the steep portions of the curves using the simplified form of the Gibbs adsorption isotherm equation. The cmc's correspond to the detergent concentration at the intersection of the two linear parts of each curve.

trations in Figure 4. With each successive addition of HCl, an equivalent amount of detergent is precipitated which appears to act as a buffer producing a levelling off or a plateau in the curves. With low concentrations of detergent this buffering effect is much less and the curve falls once the break point is passed. Toward the end of each titration this "plateau" portion changes at a second break point to a convex slope and when the above reaction is complete the curve shows a second inflection point (z) which is the final equivalence point. The quantity of HCl added from the first equivalence point to the pH of precipitation is equivalent to the solubility of the acid form of the detergent (HA) in the ionized detergent solution (A^-) (Figures 2 and 3, insets). The solubilities of the acid forms of cephalosporin P_1 and 3-acetoxylfusidic acid in concentrations below the cmc were 0.03 and 0.094 mM, respectively. The ratios of A^- to HA for concentrations well above the cmc (17–19 mM) were 60:1 (cephalosporin P_1) and 34:1 (3-acetoxylfusidate). No significant supersaturation of the solutions was observed. The apparent pK values calculated from the simplified version (Small, 1971) of the formula proposed by Back and Steenberg (1950) are plotted with the precipitation pH values as a function of the concentration of both detergents in Figure 4. The precipitation pH values increase significantly at very low concentrations and gradually level off between 3 and 5 mM. The precipitation pH values of 3-acetoxylfusidate in concentrations above 5 mM are about 0.4 of a pH unit higher than that of sodium cephalosporin P_1. The apparent pK values of 3-acetoxylfusidate are also consistently higher than the values for cephalosporin P_1. In both cases the apparent pK values are essentially constant up to a concentration of 1–2 mM but increase over a range of 2–4 mM (3-acetoxylfusidate) and 2–8 mM (sodium cephalosporin P_1) levelling off at values which are nearly 1 pH unit higher (Figure 4). The apparent pK values for the other detergents as 17–20 mM solutions are listed in Table I.

The dependence of the equilibrium surface tension values (dynes/centimeter) on increasing sodium fusidate, 3-acetoxylfusidate, and sodium cephalosporin P_1 concentration (mM) in 0.15 M Na^+ (pH 10.0 ± 0.2) is plotted semilogarithmically in Figure 5. The surface tension–log concentration curves form two straight lines which intersect at a concentration customarily taken as the cmc of the detergents (Elworthy and Mysels, 1966; Mukerjee and Mysels, 1971). The cmc's and interfacial molecular areas derived from these curves are summarized in Table I. Both above and below the cmc, the sodium cephalosporin P_1 curve falls steeply in comparison to sodium fusidate and 3-acetoxylfusidate.

The cmc's estimated by dye titration with Rhodamine 6G in increasing Na^+ (M) concentrations (25°C, pH 10.0) are plotted logarithmically in Figure 6 and the values in 0.15 M Na^+ are listed in Table I for comparison with the cmc's by other methods. The cmc's of the detergents in water decrease in the order Na^+ cephalosporin P_1 > Na cholate > Na fusidate > Na 3-acetoxylfusidate. All curves are linear and exhibit variable but slight decreases in the cmc's as the counterion concentration is increased up to the solubility limits (Table I). The percentage of Na^+ ions bound to the micelles in water (Table I) is equivalent to the slopes of these curves × 100 (Mukerjee, 1967). These values decrease in the order Na cephalosporin P_1 > Na 3-acetoxylfusidate > Na cholate > Na fusidate.

Pressure (π)–area isotherms for fusidic acid, 3-acetoxylfusidic acid, cephalosporin P_1, and helvolic acid are shown in Figure 7. The isotherm for fusidic acid (Figure 7a) begins at about 210 Å^2, at a pressure of 0–2 dyn/cm, and increases gradually at first but then more steeply to collapse at a pressure of 23 dyn/cm and 107–108 Å^2 per molecule. Upon further compression, the pressure remains constant at 23 dyn/cm until at approximately 28–30 Å^2 the pressure increases abruptly with further compression. The film became solid at 30 Å^2. Since 30 Å^2/molecule area is approximately one-third of the area at collapse, the film probably exists as a crystalline trilayer above 23 dyn/cm. 3-Acetoxylfusidic acid (Figure 7b) exhibits a moderately steep isotherm beginning at ~0 dyn/cm at 200 Å^2 per molecule and

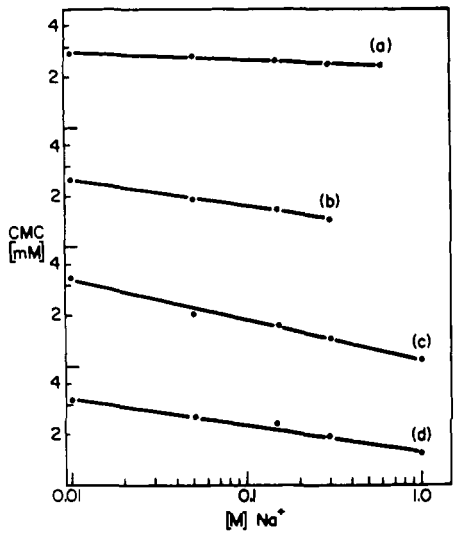

FIGURE 6: Dependence of cmc (mM) on the logarithm of the added electrolyte (NaCl) concentration for (a) sodium fusidate, (b) sodium 3-acetoxylfusidate, (c) sodium cephalosporin P_1, and (d) sodium cholate. All cmc's were estimated by the Rhodamine 6G titration method at 25°C (pH 10.0). The slopes of the curves correspond to the fraction of counterions bound to the micelles of each detergent in water (Table I).

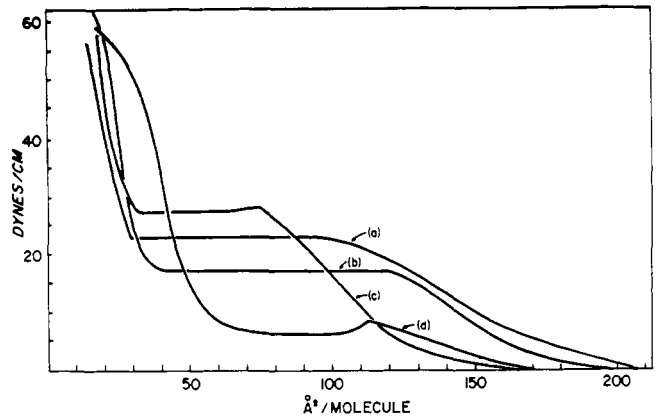

FIGURE 7: π-area isotherms (23 ± 1°C) of fusidane antibiotics on a subphase of 5 M NaCl (pH 2.0) with the use of an automated recording Langmuir-Pockels surface balance: (a) fusidic acid; (b) 3-acetoxylfusidic acid; (c) cephalosporin P_1; (d) helvolic acid. The film compression rate in all cases was 2.5 cm/min and the surface viscosity was qualitatively ascertained by observing the motion of a few grains of purified talc sprinkled on the film's surface.

rises steeply to collapse at a pressure of approximately 17 dyn/cm and an area of 124 Å^2/molecule. On further compression beyond the collapse point the pressure remains constant and the film remains fluid until about 40 Å^2 per molecule at which point the film becomes solid and the pressure increases very steeply. This change in viscosity from liquid to solid which coincides with the steep increase in pressure above a pressure of 17 dyn/cm indicates that the surface monolayer has collapsed to form a crystalline trilayer.

Cephalosporin P_1 (Figure 7c) exhibits a similarly shaped isotherm with several important differences from that of fusidic acid and 3-acetoxylfusidic acid. The isotherm begins at 170 Å^2 per molecule and collapses at a pressure of 29 dyn/cm and an area of 76–77 Å^2. This curve shows a slight but a distinctive change in slope at 18.5 dyn/cm and approximately 95 Å^2 which is consistent with the presence of a second-order phase transition in the surface at this point. With further compression, the pressure is constant at 27–28 dyn/cm and the film remains fluid until approximately 32 Å^2 when the film becomes solid and the pressure increases steeply. Above 40 dyn/cm and 25 Å^2 per molecule the film is solid by the talc test. As in the isotherm of 3-acetoxylfusidic acid, the change in viscosity and the steep increase in pressure at about 32 Å^2 indicate that at this area per molecule the surface also exists as a crystalline trilayer as this area is about one-third of the area at the second-order phase transition. The isotherm of helvolic acid (Figure 7d) differs significantly from the other three. The surface pressure starts to rise at 170 Å^2 per molecule but the slope of this part of the isotherm is much less steep than that of fusidic acid, 3-acetoxylfusidic acid, or cephalosporin P_1, indicating a readily compressible film. The isotherm peaks at 8–9 dyn/cm with an area per molecule of 114 Å^2 and the extrapolated collapse pressure is 6–7 dyn/cm with 127–128 Å^2 per molecule. This initial peak in the isotherm is due to the formation of an unstable monolayer, for continued compression of the film leads to a fall in pressure of 2–3 dyn/cm. On further compression the pressure remains nearly constant until a pressure rise commences at approximately 70 Å^2 per molecule increasing gradually at first to 55 Å^2 per molecule at 23–24 dyn/cm and then more steeply upon continued compression. The viscosity change noted in the monolayer at areas less than 70 Å^2 indicates increasing rigidity of the film and below 50 Å^2 the film was solid. The steep increase in pressure below 49–50 Å^2 per molecule indicates that the surface exists as a trilayer in this region also. The areas per molecule at the collapse pressures are compared to the areas of the salts derived from the surface tension measurements and Stuart-Briegleb molecular models in Table I.

In order to measure the cmc of the detergents by light scattering, the turbidity (τ) of a range of concentrations in the vicinity of the cmc for each detergent is plotted against concentration (milligrams/milliliter) in Figure 8. Contrary to the usual practice where τ, the total turbidity, is plotted, we have plotted the solute turbidity (τ') vs. concentration. This is the difference between the apparent turbidity of the solution (τ) and the apparent turbidity of the solvent (τ_{sol}). The absolute turbidity τ_{abs} was calculated from the equation:

$$\tau_{abs} = 1.27n^2AFi_{90}/i_0$$

where n is the refractive index of the solution, A is a constant relating the working standard to an opal glass reference standard, F is the filter factor, and i_{90}/i_0 is the scattering ratio of intensities at 90 and 0° for the wavelength 546 nm. An estimate of the cmc's (in 0.15 M Na^+) is given by the concentration at which τ begins to increase abruptly. These correspond to the concentrations at the intersection of the gradual and steep straight lines of each curve in Figure 8. The values are compared with other estimates of cmc's in Table I. The refractive index increments (dn/dc), obtained from the slopes of curves plotting $n - n_0$ against c where n is the refractive index of the solution, n_0 is the refractive index of the solvent, and c is the concentration in 10^3 g/ml, are listed in Table II.

The weight average micellar mass and micellar charge were estimated from the light scattering measurements by plotting the results according to the method originated by Debye (1949a,b) in Figure 9. In this method $H(c - c_0)/(\tau - \tau_0)$ is plotted against $c - c_0$, where c is the solution con-

Table II: Light Scattering Results.

Steroid	dn/dc^a (ml/g)	$H^b \times 10^6$ (cm^2/g^2)	$I^c \times 10^4$ (mol/g)	$S^d \times 10^4$ (mol ml/g^2)	Micellar Wt $\times 10^{-3}$ (g/mol)	Aggregation No.^{e} (Rounded Off)
Na Fusidate	0.200	4.372	3.13	−9.0	3.20	6 (5.9)
Na 3-Acetoxyl-fusidate	0.189	3.905	1.21	0	8.26	14 (14.2)
Na cephalo-sporin P_1	0.191	3.988	1.98	0	5.05	9 (8.5)

[a] Refractive index increment. [b] Optic constant defined in text. [c] Intercept of Debye plots (Figure 9). [d] Slope of Debye plots (Figure 9). [e] Number of monomers per micelle. Actual values given in parentheses.

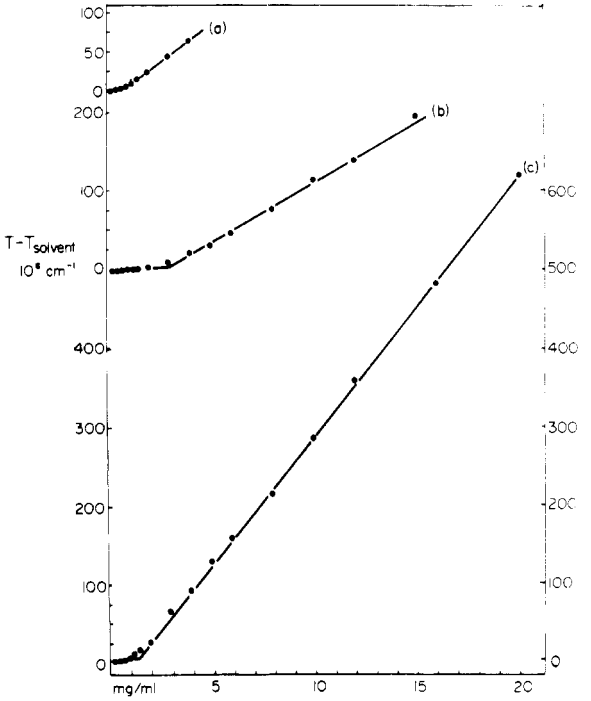

FIGURE 8: Reduced turbidity ($\tau-\tau$ solvent) as a function of concentration (mg/ml) for (a) sodium cephalosporin P_1, (b) sodium fusidate, and (c) sodium 3-acetoxylfusidate (23°C, 0.15 *M* Na^+, pH 10.0). The concentration of the detergent of the intersection of the two straight lines in each curve corresponds to the cmc. The turbidity-concentration relationships at high concentrations were utilized for calculating the Debye plots in Figure 9.

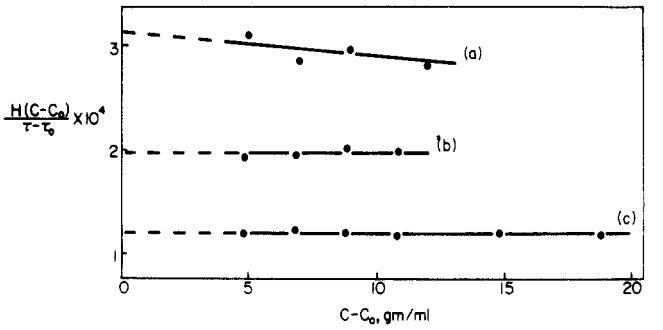

FIGURE 9: Debye plots of light scattering data for (a) sodium fusidate, (b) sodium cephalosporin P_1, and (c) 3-acetoxylfusidate (23°C, 0.15 *M* Na^+, pH 10.0). The reciprocal of the extrapolation of each curve to the ordinate gives the weight average micellar mass of each detergent. The zero slopes of lines b and c were employed to estimate the effective micellar charge in 0.15 *M* Na^+. The negative slope of a suggests that the micelles are polydisperse and that aggregation number increases with concentration. All measurements were done in 0.15 *M* Na^+ at 23°C (pH 10).

centration in grams per milliliter, c_0 is the critical micellar concentration in grams per milliliter, τ is turbidity at concentration c, and τ_0 is the turbidity at concentration c_0. H (values listed in Table II) is an optical constant:

$$H = 32\pi^3 n^2 \left(\frac{n - n_0}{c}\right)^2 \Big/ 3\lambda N^4$$

where n_0 is the refractive index of the solvent, n is the refractive index of the solution, λ is the wavelength of light (546 nm), and N is Avogadro's number. The expression of Prins and Hermans (1956a,b) which describes the Debye plots can be written in the form (Kratohvil and DelliColli, 1968): $H(c - c_0)/(\tau - \tau_0) = I[1 + S(c - c_0)]$, where I is the intercept and S is the limiting slope of the plots presented in Figure 9. As a close approximation, the extrapolated intercepts (I, Table II) of these curves are equal to the reciprocal of the weight average micellar mass ($1/M$) and the slope, S (Table II), is a function of both the micellar charge and the aggregation number (agg. no.) (number of monomer ions per micelle). Aggregation numbers are obtained by simply dividing the average micellar mass by the anhydrous molecular weight of the salt. These values and aggregation numbers rounded off to the nearest whole number are summarized in Table II.

Clear mixed micellar solutions were formed when all steroid detergents except helvolate were mixed in a molar ratio of 7:3 with egg lecithin in H_2O (10%, w/v). Using the same molar ratio of solubilizing lipids, it was found that cholesterol was readily solubilized as a complex four-component micellar system similar to vertebrate bile (Table I). A 3–5-day equilibration period was normally satisfactory to equilibrate these systems; however, in the case of mixed micelles containing sodium cephalosporin P_1, it was necessary to make the final readings at 48 hr for upon standing for 3 days or longer the detergent underwent alkaline decomposition. The cholesterol solubilities expressed in moles per 100 mol of total lipids (Table I) decreased in the order sodium 3-acetoxylfusidate (10 mol %) > sodium fusidate = sodium cholate (8 mol %) > sodium cephalosporin P_1–isocephalosporin P_1 equilibrium mixture (6 mol %).

The apparent partial specific volumes ($\bar{v}$) (Table I) of all sodium salts are similar and show a slight decrease in the order 3-acetoxylfusidate > sodium cephalosporin P_1 > sodium fusidate > sodium cholate. The values for sodium fusidate and sodium cholate are similar to the values obtained by pycnometry in previous studies (Small, 1971; Carey and Small, 1971).

Discussion

The results of the present investigation confirm our previous findings on fusidic acid and extend these studies to

three other fusidane derivatives all of which exhibit important colloid chemical differences from each other and from the parent compound. 3-Acetoxylfusidate, fusidate, and sodium cephalosporin P_1 form micelles of similar size to sodium cholate and solubilize lecithin and cholesterol as efficiently as bile salts. The micellar properties of sodium fusidate were shown previously to be similar to taurine conjugated bile salts with respect to cmc, micellar charge, and micellar mass (Carey and Small, 1971). However, the response of the cmc's of 3-acetoxylfusidate and sodium cephalosporin P_1 to added NaCl appears to resemble that of the unconjugated bile salt sodium cholate (Figure 6). The titration curves of 3-acetoxylfusidate and cephalosporin P_1 (Figures 2 and 3) are comparable to the titration curves of unconjugated bile salts (Small, 1971); however, the solubilities of the protonated fusidane acids in their respective micelles are much less than the solubility of the bile acids in bile salt micelles. The differences in apparent pK values between 3-acetoxylfusidate and sodium cephalosporin P_1 both below and above the cmc are significant. These detergents differ from each other by the second acetoxyl group at the 3 position of 3-acetoxylfusidate and the 7β-OH group of cephalosporin P_1. These differences only marginally influence the cmc's but are reflected in the significantly larger micellar mass of 3-acetoxylfusidate. It is evident, therefore, that the greater charge density on the surface of the larger 3-acetoxylfusidate micelles will increase their ability to bind protons over that of sodium cephalosporin P_1 micelles. However, an acetoxyl group on the C-3 of steroids is known to exhibit significant long-range effects as shown by the kinetics of 17β-oxidation in a series of estratrienes (Egorova et al., 1973). Long-range effects might also be expected to augment electronic delocalization at the carboxyl group in fusidane derivatives via the highly strained boat configuration of the B ring (Cooper, 1966; Godtfredsen, 1967) and this would lead to the weaker acidic properties of 3-acetoxylfusidate and explain its higher pK_a both above and below the cmc. The cmc's of each steroid are similar to each other and to those of both free and conjugated bile salts as might be expected from the remarkable structural similarity between the molecules. The Rhodamine 6G method gives slightly lower estimates of the cmc's of steroid detergents but higher estimates of cmc of straight-chain detergents as we have noted previously (Carey and Small, 1971; M. C. Carey and D. M. Small, manuscript in preparation). The cmc's decrease slightly in relation to the strength of their nonionic polar groups indicating that substituent acetoxyl groups are slightly less polar than hydroxyl groups on these steroids. The slight fall in cmc with added NaCl (Figure 6) is typical of all steroid detergents including bile salts, but is much less than what is observed with typical anionic or cationic detergents (Shinoda et al., 1963; Mukerjee, 1967; Mukerjee and Mysels, 1971). The major factor in the resistance of these detergents to the cmc lowering effects of Na^+ is related to the small aggregation numbers, the unusual packing of the monomers in micelles which necessitates widely separated charged groups, and the pleurality of nonionic polar groups which confer partial nonionic properties to the molecules (Carey and Small, 1969).

The interfacial area of sodium cephalosporin P_1 from the surface tension curve (73 Å^2) is slightly too small for these steroid molecules lying flat as the interfacial areas of sodium fusidate and 3-acetoxylfusidate are 107 and 115 Å^2 under the same conditions. The reason for the anomaly in the cephalosporin P_1 area by this method is not readily explained by the π–Å^2 isotherm of cephalosporin P_1 which also gives an area of 76–77 Å^2 at the collapse point. However, in the isotherm of cephalosporin P_1 (Figure 7) there is a definite second-order phase transition in the surface at 95 Å^2. This area probably corresponds to the true area of the molecule lying flat as this break point can only indicate that the two-dimensional liquid film reaches its limit of compressibility at this area. Instead of the molecules collapsing out of the surface, they probably begin to tilt on their edges. The absence of an 11α-OH group and the presence of a 7β-OH group in the cephalosporin P_1 molecule allow each molecule to tilt on its equatorial axis while being firmly anchored in the surface by the 6α-acetoxy and 7β- and 3α-OH groups. At the true collapse pressure (27–28 dyn/cm) the molecules are probably all stacked on their sides, and the area now occupied by cephalosporin P_1 (76–77 Å^2) is consistent with this type of packing from consideration of molecular models. A similar type of second-order phase transition occurs in the isotherm of the bile salt lithocholate on a subphase of 3 M NaCl at pH 10.0 (Small, 1971). Three acetoxylfusidic acid molecules occupy the largest surface areas at the collapse point (124 Å^2) in agreement with the Gibbs adsorption isotherm area of the salt (115 Å^2) and the area calculated from a Stuart-Briegleb molecular model lying flat (118 Å^2). This is significantly bigger than the area of a fusidic acid molecule (107 Å^2) and is consistent with the 3-acetoxyl group expanding the area of the molecule at the interface. Helvolic acid possesses two carbonyl groups and these not only decrease the overall polarity of the molecule due to their equatorial orientation but are not very strong polar groups in addition. Therefore, helvolic acid molecules collapse out of the surface at very low pressures.

The dissymmetry ratios of the fusidane micellar solutions by light scattering were close to unity and the $\bar{v}$ values (Table I) which are similar to the value for sodium cholate suggest that the shape of the fusidane micelles is spherical and that their micellar packing is analogous to that of bile salt micelles. If the structure is indeed spherical, it is easy to form a sphere with up to nine space-filling models of these molecules by apposition of the hydrophobic backs with the polar groups projecting toward the outside (Figure 10). However, it is not possible to pack 14 3-acetoxylfusidate molecules into a sphere using this type of configuration alone. If one assumes that the first eight or nine molecules of the 3-acetoxylfusidate micelles pack hydrophobically, it is possible to hydrogen bond two–three pairs of hydrophobically associated dimers or the outside of this primary micelle to form a secondary micelle (Figure 10). This type of packing is consistent with the three-dimensional packing of the trilayer above the film collapse pressure, where the first two layers of molecules are hydrophobically "bonded" while the third layer is hydrogen bonded to the second. A similar type of secondary aggregation has been suggested for bile salts micelles at high counterion concentrations (Small, 1971). It should be noted that in the approach employed here, it is assumed that aggregation into monodisperse micelles occurs. This is an oversimplication especially when aggregation numbers are small and when monomers are rigid molecules (Mukerjee, 1974). Cmc and aggregation number data should therefore be regarded as average micellar properties at the cmc.

An estimate of the effective charge on each micelle in 0.15 M Na^+ can be derived from the Debye plots employing the procedure of Emerson and Holtzer (1967). The ef-

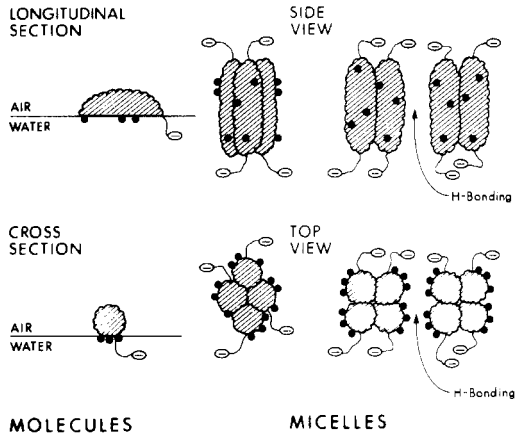

FIGURE 10: Suggested micellar structure of primary and secondary micelles formed by the fusidane derivatives. Each molecule is shown as it would lie at an air–water interphase in both longitudinal and cross-section. The primary micelles are formed by hydrophobic interactions between the broad hydrocarbon backs of the fusidane molecules. In this way up to nine molecules can be accommodated into a micelle without leaving an empty center. The secondary micelle is the suggested structure when the aggregation number of the micelles is greater than 10. These are composed of clusters of small primary micelles hydrogen bonded to each other by their exposed nonionic polar groups. In both situations, the micellar shape is spherical and experimentally this is confirmed by the disymmetry ratio from the light scattering studies which was close to one for all three detergents.

fective charge on sodium fusidate micelles in 0.15 *M* Na^+, however, cannot be estimated from the Debye plot as the negative slope (Figure 9a) suggests that the micelles are polydisperse and that their aggregation number increases with concentration (Mukerjee, 1972). The effective degrees of dissociation (the fraction of free charges) of the micelles of 3-acetoxylfusidate and sodium cephalosporin P_1 in 0.15 *M* Na^+ by this method are 0.54 and 0.48, respectively. The fractional micellar charges in water (Table I) from log cmc–log counterion plots give degrees of dissociation of 0.96, 0.84, and 0.77 for sodium fusidate, 3-acetoxylfusidate, and sodium cephalosporin P_1, respectively. These calculations indicate that sodium fusidate micelles are highly charged in water and are similar to taurine conjugated trihydroxy bile salt micelles (Carey and Small, 1969). On the other hand, both 3-acetoxylfusidate and sodium cephalosporin P_1 micelles bind a small number of Na^+ ions in water—about two Na^+ per micelle but in the presence of 0.15 *M* Na^+ about half the charges on each micelle are tightly bound. Similar calculations from light scattering data on sodium taurodeoxycholate and glycodeoxycholate solutions (Kratohvil and DelliColli, 1968; Carey and Small, 1969) indicate that slightly more Na^+ ions are bound by bile salt micelles than are bound by these fusidane-type detergents in water and in 0.15 *M* Na^+. In contrast typical long-chain anionic and cationic detergents have about 50% of their counterions bound in water and much more in 0.15 *M* Na^+ (Shinoda et al., 1963; Mukerjee, 1967).

If micelle formation is considered a mass action equilibrium, and assuming as a first approximation that the system is monodisperse, an estimate of the standard free-energy change (ΔF_m) in kilocalories per mole in 0.15 *M* Na^+ can be derived from (Mukerjee, 1967):

$$\Delta F_m = 2.303RT \log \text{cmc}$$

where *R* is the molar gas content, *T* is the absolute temperature, and cmc is the mean of the cmc's in 0.15 *M* Na^+ by all three methods (Table I). The values for ΔF_m are −3.63, −3.61, −3.59, and −3.29 kcal/mol for sodium cephalosporin P_1, 3-acetoxylfusidate, sodium cholate, and sodium fusidate, respectively. The values which are slightly larger than that of the parent compound sodium fusidate indicate a more favorable free energy of micelle formation. These values compare reasonably well with previously published estimates of this thermodynamic constant for taurine conjugated cholate and sodium fusidate (−3.53 and −3.37 kcal/mol in water at 30 and 20°C, respectively) (Carey and Small, 1971; Vitello, 1973).

Eight chemically related fusidane-type antibiotics of defined structure have now been isolated from natural sources (Godtfredsen, 1967). Fusidic acid has been shown to be a specific inhibitor of the translocation step of ribosomal protein chain elongation (Tanaka et al., 1968; Pestka, 1968; Haenni and Lucas-Lenard, 1968) by preventing the dissociation of a ribosome–translocation factor–guanosine diphosphate complex (Bodley et al., 1970; Brot et al., 1971). Furthermore, fusidic acid and related compounds possess the cyclopentanoperhydrophenanthrene ring system in common with the bile acids, and their sodium salts like the bile salts are detergents with comparable physical–chemical properties. In particular, they solubilize membrane lipids such as phospholipids and cholesterol as efficiently as bile salts. Furthermore, these steroidal antibiotics are secreted into bile when administered to animals. The free compounds are extensively metabolized by the liver (Godtfredsen and Vangedal, 1966; Montet et al., 1973); however, we have shown that 24,25-dihydrofusidic acid, conjugated in peptide linkage with taurine, is actively secreted in bile of Rhesus monkeys unchanged and significantly influences the secretion rates of the other biliary lipids (Beaudoin et al., 1973). The findings in this study suggest that the surface properties of these detergents are distinctive and their micellar properties are similar to sodium fusidate and unconjugated bile salts. The elucidation of the surface and solution properties of these antibiotics may shed new light on their structure–functional relationships in molecular biology. Furthermore, the close colloid chemical and physiological identity of these antibiotics with the bile salts suggests that these compounds and their derivatives are worth further exploration as models for detergent replacement in bile salt deficiency syndromes.

Acknowledgments

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Comments on the Proposed Rigidity of Staphylococcal Protease†

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ABSTRACT: It is proposed that the unusual low-field resonance observed in proton magnetic resonance spectra of staphylococcal protease (Markley, J. L., Finkenstadt, W. R., Dugas, H., Leduc, P., Drapeau, G. R. (1975), *Biochemistry 14,* 998) could be reassigned to an impurity of formic acid. This would reduce significantly evidence for the special rigidity of this protein.

In a recent publication (Markley et al., 1975) an unusual resonance was observed in the proton magnetic resonance (^{1}H NMR) spectrum of staphylococcal protease. This resonance (labeled X4), at ca. 8.2 ppm from an external standard of 5% Me_4Si[1] in CCl_4, was said to arise from solvent exchangeable N–H protons. A tentative assignment to nine buried amide N–H_2 groups was made. In order to explain the unusually narrow line width, some regularity in the bonding interactions of the buried amide groups was postulated. Mainly because of the behavior of this resonance, which did not completely exchange with solvent over the pH range 3 to 10 or on heating the protease solutions in D_2O to 80°C, several conclusions about the protease structure and its rigidity toward "breathing motions" were drawn. In this paper evidence will be presented for an alternative explanation for the behavior of peak X4, based on its assignment to an impurity in the sample.

Results and Discussion

The resonance X4 has a chemical shift of 8.16 ppm from external Me_4Si at pH values above 6. It was found experimentally in this work that at 270 MHz and 30°C this external Me_4Si standard resonates at 0.28 ppm downfield[2] from

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[1] Abbreviations used are: Me_4Si, tetramethylsilane; DSS, sodium 4,4-dimethyl-4-silapentane-5-sulfonate.

[2] This value is both temperature and frequency dependent, and will depend on the exact conditions used. There is therefore some uncertainty in the correction factor.