Ultracentrifugal Study of Effect of Sodium Chloride on Micelle Size of Fusidate Sodium

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Abstract □ Apparent micellar molecular weights were determined with the antibiotic fusidate sodium by ultracentrifugation in varying counterion concentrations (Na^+). The effects of buffer salts, pH, sodium chloride concentration, and drug concentration were studied. The results strongly support the concept of the formation of primary micelles composed of five monomer units, followed by aggregation of the pentomers into larger micelles as salt concentration increases.

Keyphrases □ Fusidate sodium micelles—molecular weights determined by ultracentrifugation, effects of buffer salts, pH, and sodium chloride and drug concentrations □ Micelle size of fusidate sodium—determined by ultracentrifugation, effects of buffer salts, pH, and sodium chloride and drug concentrations □ Ultracentrifugation—determination of fusidate sodium micelle molecular weights in varying counterion concentrations □ Sodium chloride effect—micelle size of fusidate sodium

The counterion concentration has been shown to control the size of micelles in solutions of bile salts (1, 2) and also in solutions of the steroid-like antibiotic fusidate sodium[1] (I) (3–5). The critical micelle concentration (CMC) of I was found by surface tension measurements (3) to be 3–4 m*M* in solutions containing 0.01–0.6 *M* NaCl. Aggregation numbers of I micelles were not reported at counterion concentrations below 0.01 *M* nor above 0.6 *M* NaCl.

In view of the studies of Godtfredsen *et al.* (5) showing that I in humans is excreted through the bile and that rather large concentration changes occur during storage of the bile in the gallbladder, it was decided to study the apparent micelle molecular weights of I by ultracentrifugation through as wide a counterion concentration range as possible. In addition, the effect of I concentration and of rotor speed on micelle size was investigated. The results tend to confirm the postulate of Small (1) that primary micelles, perhaps pentomers, are formed through hydrophobic interactions of the steroid-like structure, followed at increasing ionic strength by the formation of secondary micelles through hydrogen bond-type interactions of the hydrophilic groups.

EXPERIMENTAL

No secondary spots were observed with I[2] by TLC (3, 4). All chemicals used in this work were analytical reagent grade. Molecular weights were determined with an analytical ultracentrifuge[3] equipped with schlieren optics and a temperature control. All determinations of molecular weight were made at 25°, with the camera lens focused at the two-thirds level of the cell, as is required (6) for aqueous solutions. Schlieren patterns were read on a two-dimensional microcomparator[4], followed by data analysis according to Method II of Van Holde and Baldwin (7).

The pH, when not controlled by buffers, varied between 8 and 9, depending on the I concentration. Compound I has a reported pKa of 5.35 (3). Tromethamine–hydrochloric acid buffer was made up at 0.1, 0.05, 0.025, and 0.0125 *M* tromethamine to pH 7.3. Sodium phosphate buffers also were made up at 0.1, 0.06, 0.03, and 0.015 *M* to pH 7.2. The partial specific volume of I was taken to be 0.774 ml/g, as reported previously (3).

RESULTS AND DISCUSSION

The effect of sodium chloride concentration on the apparent *z*-average molecular weight of I micelles is shown in Fig. 1, where $M_{z_{app}}$ is plotted against the molarity of sodium chloride for 18 m*M* I. Rotor speed effects were negligible in the 18 m*M* I solution; with the 0.6 *M* NaCl mixture, equilibrium was obtained at seven different speeds from 14,290 to 44,040 rpm with no resultant change in the calculated molecular weight. Overall, the data tend to fall on one curve with some scatter but extrapolate to the molecular weight of the I monomer of 538.

Also plotted in Fig. 1 are the results of Carey and Small (3) for 20 m*M* I at pH 10 in carbonate buffer, which extrapolate at zero added salt to about a molecular weight of 2500, *i.e.*, to the pentomer of I. Therefore, these investigators considered the pentomer as the size of primary micelles for I solutions.

Measurements of the molecular weight were also made on 18 m*M* I solutions in phosphate buffers and tromethamine buffers at pH 7.2 and 7.3, respectively. The plot shown in Fig. 1 for phosphate rises steeply and linearly from the monomer (538 daltons) at low phosphate molarity up to about 10,000 daltons when the total phosphate molarity reaches 0.1. The data end there, since increasing the phosphate content further resulted in slow gel formation up to 0.15 *M* phosphate and in rapid precipitation at any concentrations above 0.15 *M* phosphate. An attempt was made to study the effect on I micelles of sodium chloride added to 0.06 *M* phosphate buffer. However, independently of the order of mixing, I

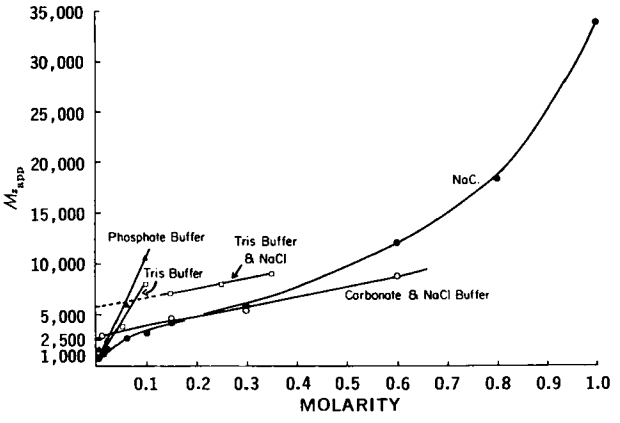

Figure 1—*Effect of various salt concentrations and pH on the apparent z-average micellar molecular weight of 18 m*M *I solutions. Key: ●, sodium chloride; ○, carbonate–bicarbonate buffer (0.01* M*) plus added sodium chloride, pH 10.0; ▲, phosphate buffer, pH 7.2; and □, tromethamine (tris) buffer alone and tromethamine (tris) buffer (0.05* M*) plus added sodium chloride, pH 7.3.*

[1] Sodium 3α,11α,16β-trihydroxy-29-nor-8α,9β,13α,14β-dammara-17(20),24-dien-21-oate 16-acetate.

[2] Lot 22151, supplied by Dr. W. O. Godtfredsen, Leo Pharmaceutical Products, Ballerup, Denmark.

[3] Model E, Beckman Instruments, Palo Alto, Calif.

[4] Nikon model 6, Nippon Kogaku, K. K. Japan.

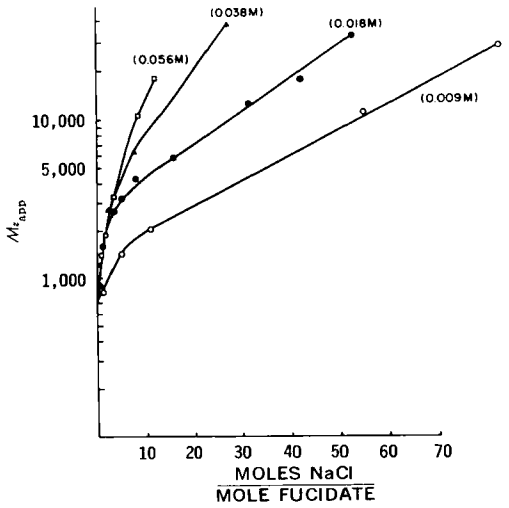

Figure 2—*Logarithm of the apparent z-average micellar molecular weight of I solutions as a function of the ratio of salt to drug for four different I concentrations.*

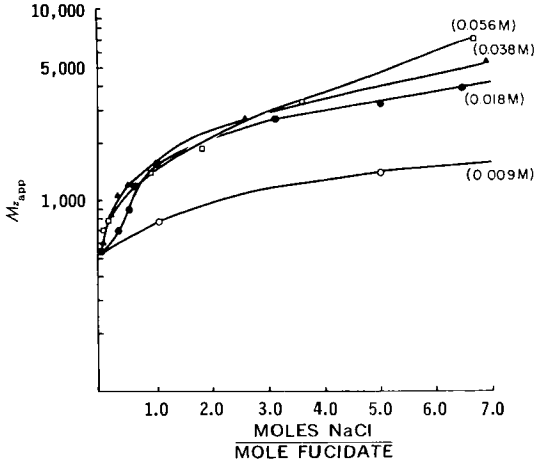

Figure 3—*Logarithm of the apparent z-average micellar molecular weight of I solutions as a function of the ratio of salt to drug in regions of low salt concentrations. Abscissa is expanded 10-fold over that of Fig. 2.*

would not dissolve in 0.06 *M* phosphate with added 0.1 *M* NaCl, and no data were obtained.

With increasing tromethamine buffer concentration at a constant pH of 7.3, a rapid linear increase in micelle size was seen, with slow gel formation again occurring at tromethamine concentrations above 0.1 *M*. However, addition of sodium chloride to the 0.05 *M* tromethamine buffer did not cause precipitation of I as it did with the phosphate system. A linear growth of micellar molecular weight was found that paralleled the data of Carey and Small (3) with carbonate–salt buffer at pH 10. Extrapolation to zero added salt, however, gave the decamer molecular weight rather than the pentomer obtained in carbonate buffer.

It has been stated (8, 9) that pH, counterion concentration, drug concentration, and temperature are important factors defining the size of micelles in solution. As shown by comparing the micelle molecular weight of I in phosphate and in tromethamine buffers at the same pH, however, care must be taken that interactions with the buffer salts themselves do not introduce additional micelle growth separate from any pH effect *per se*. Molecular models indicate that phosphate hydrogen bonds with the hydroxy groups of I. Thus, with phosphate bridging, I aggregates grow rapidly from the hydrophilic side (at low total buffer salt content) as well as from the hydrophobic side of the molecule. A similar argument, except for weaker hydrogen bond bridging than is present with phosphate, would explain the rapid growth of I micelles with the concentration of tromethamine buffer and also account for the continued solubility of I in the presence of added sodium chloride.

The sodium chloride curve in Fig. 1 suggested a logarithmic response of micellar weight to salt; therefore, various semilogarithmic graphs were attempted of the calculated molecular weights for four different I concentrations: *(a)* against molarity of sodium chloride, *(b)* against the square root of ionic strength, *(c)* against the mole fraction of sodium chloride, and *(d)* against the ratio of sodium chloride to I. Only the latter plots are shown in Fig. 2, because they contain linear portions that point out especially well the effect of sodium chloride concentration on micelle size. Excellent linearity was observed for all concentrations of I from 9 to 56 m*M* if the salt was present in 10-fold excess over the drug.

Unfortunately, the ultracentrifugal equipment available did not permit study at I concentrations in the vicinity of the CMC because of the limited instrument sensitivity with the standard 12-mm centrifuge cell depth.

To study the low salt region of Fig. 2 in more detail, the log $M_{z_{app}}$ was plotted against an abscissa expanded 10-fold (Fig. 3). It is clear from Fig. 3 that curves for 9, 18, and 38 m*M* I extrapolate to the monomer molecular weight at zero salt but that the curve for 56 m*M* I seems to extrapolate to a slightly higher value of M_z. Also, a rapid dissociation is indicated for the I micelles in 18 m*M* drug concentration as the salt to I ratio drops below unity. It is seen that an aggregation number of three (M_z = 1600) is not obtained for the 18, 38, and 56 m*M* I solutions until the salt to drug ratio reaches unity but that the curves seem to meet near that common point before diverging into higher molecular weight ranges.

To illustrate the effect of I concentrations on micellar size, the data from Figs. 2 and 3 were plotted in Fig. 4 as log $M_{z_{app}}$ against concentration of I at several different salt to drug ratios. Also shown is the micelle growth curve in the absence of a supporting electrolyte. Two effects are immediately obvious from Fig. 4. First, there exists a family of horizontal lines at salt to drug ratios from 0 to 2.5, where the apparent micellar weight is independent of I concentration after an initial rapid growth from the monomer to some constant molecular weight. The micellar weight does increase, however, with an increasing salt to drug ratio. It appears that no large micelles are formed until the salt to drug ratio is approximately three and until the extrapolated micelle molecular weight reaches 2600; that is, until pentomers are formed. Beyond that point, rapid logarithmic growth in micellar size occurs up to the occurrence of a phase change, often noted at molecular weights of 35,000 or greater as the separation of an oily layer from the solution.

Figure 4 strongly indicates that two mechanisms of aggregation are involved in the formation of micelles of I. First, with low salt to drug ratios, the salt reduces charge–charge repulsions of the car-

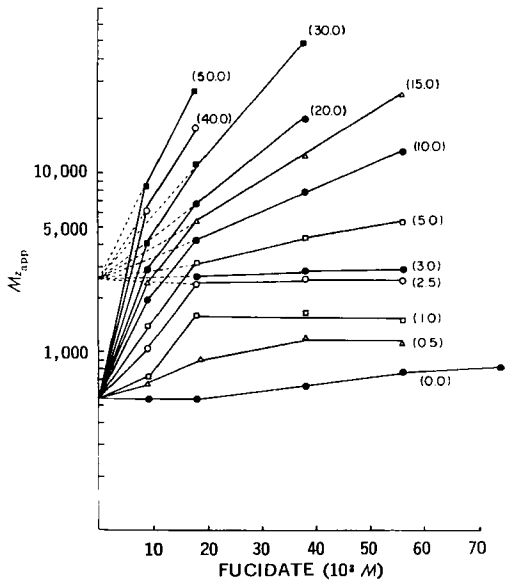

Figure 4—*Logarithm of apparent z-average micellar molecular weight of I solutions as a function of I molarity for several different sodium chloride to drug ratios. Notations on each curve indicate the salt to I molar ratio.*

boxyl group of I, thus making possible the approach and subsequent hydrophobic bonding of the steroid-like ring structure of neighboring molecules. This can continue until pentomers become the dominant species, after which increasing salt gives rise to secondary micelles. These are formed, as suggested by Small (1), by dehydration of the weaker nonionic groups and resultant hydrogen bond formation between primary micelles, analogous to a salting-out effect.

On a molar basis, the free energy of dimerization of I was calculated from the salt-free system. At the highest concentration studied, 74 m*M*, the molecular weight was 850. Thus, from both the expression for the *z*-average molecular weight (Eq. 1):

$$M_z = \frac{\sum_i m_i M_i^3}{\sum_i m_i M_i^2} = \frac{m_1 M_1^3 + m_2 M_2^3}{m_1 M_1^2 + m_2 M_2^2} \qquad \text{(Eq. 1)}$$

and the relationship between monomer and dimer concentrations (Eq. 2):

$$m_1 = m_{0_1} - 2m_2 \qquad \text{(Eq. 2)}$$

the concentration of monomer and dimer could be obtained, assuming ideal solution properties and the absence of larger aggregates. The equilibrium dimer concentration (m_2) was found to be 15 m*M*, and the monomer concentration (m_1) at equilibrium was 44 m*M*. The symbol used for initial monomer concentration was m_{0_1}. Then the equilibrium constant for the reaction:

$$2 \text{ fusidate} \rightleftharpoons \text{fusidate}_2$$

Scheme I

was given by:

$$K = \frac{[\text{fusidate}_2]}{[\text{fusidate}]^2} = 7.75 \qquad \text{(Eq. 3)}$$

and:

$$\Delta G^\circ_{298} = -RT \ln K = -1200 \text{ cal/mole} \qquad \text{(Eq. 4)}$$

On the other hand, with sodium chloride added to give a salt to I ratio of 0.5, dimerization was facilitated. A molecular weight of 900 was reached at a I concentration of only 18 m*M*, and repetition of the thermodynamic calculation for dimerization led to a value of 56 for *K* and of −2400 cal/mole for ΔG°_{298}. In this second computation, it was assumed, as shown by Carey and Small (3), that salt was not specifically or firmly bound to the dimer but acted only as a supporting electrolyte, that is, to mask charge at the anionic head of I. This value of ΔG°_{298} is in agreement with values calculated for dimerization of sodium lauryl sulfate (10) and in fair agreement with the −3400 cal/mole calculated for I from CMC data (3) at pH 10 in carbonate buffer, although in the latter work I dimers were not specified exclusively in the composition of the micelle. Work is continuing to interpret the multiple equilibria involved as the salt to I ratio leads to the formation of larger aggregates in solution.

REFERENCES

(1) D. M. Small, *Advan. Chem. Ser.*, **84**, 31(1968).

(2) J. P. Kratohvil and H. T. Delli Colli, *Can. J. Biochem.*, **46**, 945(1968).

(3) M. C. Carey and D. M. Small, *J. Lipid Res.*, **12**, 604(1971).

(4) P. Eneroth and J. Sjovall, in "The Bile Acids," P. P. Nair and D. Kritchevsky, Eds., Plenum, New York, N.Y., 1971.

(5) W. O. Godtfredsen, K. Roholt, and L. Tybring, *Lancet*, **1**, 928(1962).

(6) L. I. Epstein, P. Nixon, and A. J. Richard, *Can. J. Chem.*, **51**, 3309(1973).

(7) K. E. Van Holde and R. L. Baldwin, *J. Phys. Chem.*, **62**, 734(1958).

(8) T. C. Laurent and H. Persson, *Biochim. Biophys. Acta*, **106**, 616(1965).

(9) M. C. Carey and D. M. Small, *Arch. Intern. Med.*, **130**, 506(1972).

(10) P. Mukerjee, K. J. Mysels, and C. I. Dulin, *J. Phys. Chem.*, **62**, 1390(1958).

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Synthesis of Potential Adrenergic Blocking Agents: 2-Substituted Aminomethylnaphtho(2,3-*b*)-1,4-dioxans

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Abstract □ Eleven 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans were synthesized. The nucleophilic displacement of 2-tosyloxymethylnaphtho(2,3-*b*)-1,4-dioxan by appropriate amines was carried out using dimethyl sulfoxide as the solvent. Preliminary pharmacological evaluation revealed a potentiation of norepinephrine at low doses and a noncompetitive antagonism at high doses in the rat vas deferens and a dose-related hypotensive action of short duration in the anesthetized rat.

Keyphrases □ Aminomethylnaphtho(2,3-*b*)-1,4-dioxans, 2-substituted—synthesized and screened as potential adrenergic blocking agents □ Dioxans—synthesis and screening of 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans as potential adrenergic blocking agents □ Adrenergic blocking agents, potential—synthesis and screening of 2-substituted aminomethylnaphtho(2,3-*b*)-1,4-dioxans

Bovet and Simon (1) were the first investigators to demonstrate that 2-substituted aminomethyl-1,4-benzodioxans (I) possessed the ability to block certain actions of epinephrine. Piperoxan blocked norepinephrine by means of competitive antagonism in a study utilizing the rat vas deferens (2). The effects of