Permeability Alterations in Unilamellar Liposomes Due to Betaine-Type Zwitterionic and Anionic Surfactant Mixed Systems

A. de la Maza* and **J.L. Parra**
Instituto de Technología Química y Textil, Consejo Superior de Investigaciones Cientificas (C.S.I.C.), 08034 Barcelona, Spain

The alterations caused by betaine-type zwitterionic and anionic surfactant mixed systems in the permeability of unilamellar liposomes have been investigated. The partition coefficient of these systems, at different molar fractions, between the aqueous phase and the lipid bilayer of liposomes has been determined. These surfactant mixed systems were formed by *N*-dodecyl-*N,N*-dimethylbetaine (C_{12}-Bet) and sodium dodecyl sulfate (SDS) in the presence of 20 mM PIPES buffer and 110 mM Na_2SO_4, at pH 7.21. Unilamellar liposomes were prepared from egg phosphatidylcholine and phosphatidic acid (9:1 molar ratio). The release of the fluorescent agent 5-(6)carboxyfluorescein induced by the systems has been studied at sub-solubilizing concentrations. When the molar fraction of C_{12}-Bet/SDS is about 0.4, the critical micelle concentration values of these systems exhibit a minimum, whereas their partition coefficient between the aqueous phase and lipid bilayer of lipid bilayers shows a maximum. There is a consistent correlation between the partition coefficient and the ability of the different systems of surfactants to modify the permeability of liposomes.

KEY WORDS: Carboxyfluorescein release, interaction liposome-(dodecyl betaine/sodium dodecyl sulphate) mixed systems, partition coefficients, permeability changes, unilamellar liposomes.

Zwitterionic surfactants display strong interaction or complex formation with anionic surfactants in aqueous solutions (1). The effect of the micellar solution phase of these surfactant mixtures in avoiding or at least reducing the level of anionic/protein interaction has been suggested by several workers as a way of slowing down the irritation potential of anionic surfactants (2,3). Indeed, reduction in skin irritation by anionics has been reported in the presence of different amphoteric surfactants (4). Moreover, zwitterionic surfactants have been used as boosters of several anionic surfactants in industrial applications, and their mixed properties have been reported (5,6).

Liposomes are aqueous lipid dispersions organized as bilayers of lipid molecules and are widely used as simplified models of biological membranes (7–9). Knowledge of the physicochemical process involved in the interaction between surfactants and phospholipidic vesicles is of great interest because it can provide useful information for improving our understanding of the complex interactions between human skin and surfactants. Liposome-surfactant interaction leads to rupture of liposomic structures and to solubilization of the phospholipid components *via* mixed surfactant-phospholipid micelle formation (10). At sub-solubilizing concentrations, surfactants are incorporated into the phospholipid bilayers, where they bring about changes in physical properties (11,12), *e.g.*, bilayer permeability. At such concentrations, generally an equilibrium partition of surfactants between the bilayer and the aqueous medium governs the incorporation of surfactant into the bilayer (13). This partition coefficient is defined as the relationship between the surfactant concentration in the lipid bilayer per mol of lipid and in the surrounding aqueous medium (11,13).

In a recent paper, we reported (14) the partition coefficients of different surfactants, including *N*-dodecyl-*N,N*-dimethylbetaine (C_{12}-Bet) and sodium dodecyl sulfate (SDS), when each surfactant interacted individually with unilamellar liposomes. The influence of alkyl chainlength of alkyl betaines on this parameter has also been reported (15). Both investigations were based on the measurement of the release of half of the 5-(6)carboxyfluorescein dye from the interior of liposome vesicles. In the present study, we extend this investigation to mixed-system solutions of both surfactants to study the capacity of these systems to alter liposome permeability. This knowledge could be useful in improving our understanding of the synergism in these binary systems, and in establishing a criterion for the evaluation of their activity on phospholipid vesicles.

EXPERIMENTAL PROCEDURES

Materials. Phosphatidylcholine (PC) was purified from egg lecithin (Merck, Darmstadt, Germany) according to the method of Singleton *et al.* (16) and was shown to be pure by thin-layer chromatography. Phosphatidic acid (PA) from egg yolk lecithin was purchased from Sigma Chemical Co. (St. Louis, MO). Both lipids were stored in chloroform under nitrogen at −20°C until use. C_{12}-Bet was specially prepared by Albright and Wilson, Ltd. (Warley, West Midlands, United Kingdom); the active matter was 30% in aqueous solution, and the amino free content was 0.20%. SDS was obtained from Merck and further purified by a column chromatographic method (17). Piperazine-1,4-*bis*(2-ethanesulfonic acid) (PIPES buffer), obtained from Merck, was prepared as 20 mM PIPES adjusted to pH 7.2 with NaOH, containing 110 mM Na_2SO_4. Polycarbonate membranes and membrane holders were purchased from Nucleopore (Pleasanton, CA). 5-(6)Carboxyfluorescein (CF) was obtained from Eastman Kodak (Rochester, NY) and further purified by a column chromatographic method (18).

Preparation of unilamellar liposomes. Unilamellar vesicles of defined size (100 nm) were prepared by the extrusion of large unilamellar vesicles obtained by the reverse-phase evaporation method (19,20) based on protocol described earlier by Szoka and Papahadjopoulos (21). A lipidic film was formed by removing the organic solvent by rotatory evaporation from a chloroform solution of egg PC and PA (9:1 molar ratio).

The lipids were then redissolved in diethyl ether, after which the PIPES buffer containing 10 mM CF was added to the etheral solution of phospholipids. Gentle sonication led to the formation of a water-in-oil (W/O)-type emulsion. After evaporating the diethyl ether under reduced

*To whom correspondence should be addressed at Instituto de Tecnología Química y Textil (C.S.I.C.), C/ Jorge Girona 18-26, 08034 Barcelona, Spain.

pressure, a viscous gel was formed. Elimination of the final traces of organic solvent transformed the gel into a liposome suspension.

Unilamellar vesicles (of a uniform size distribution) were obtained by successive extrusion of vesicle suspensions through 800, 400, 200 and 100 nm polycarbonate membranes (22). Vesicles were freed of unencapsulated material by separation through Sephadex G-50 (Pharmacia, Uppsala, Sweden) by column chromatography. All liposomes were allowed to equilibrate for at least 1 h at room temperature. The range of phospholipid concentration in the liposome suspensions studied was 0.1–1.0 mM.

Phosphorus estimation. The phospholipid concentration of the liposome vesicles was determined by the ascorbic acid spectrophotometric method for total phosphorus estimation (23).

Surface tension measurements. Surface tension values were measured by the plate method (24) with a Krüss tensiometer (processor tensiometer K-12), which directly determines the real surface tension values at equilibrium. The critical micelle concentrations (CMC) of C_{12}-Bet/SDS mixed systems in PIPES buffer were determined by plotting surface tension values *vs.* the logarithm of surfactant concentrations.

Quasi-elastic light scattering. The mean vesicle size and polydispersity of unilamellar liposome preparations were determined by a photon correlator spectrometer (Malvern Autosizer IIc, Malvern, England). Samples were adjusted to an adequate concentration range with PIPES buffer. The measurements were made at 25°C and at a scattering angle of 90°.

Monitoring the release of CF from liposomes. Liposomes containing concentrated CF in the interior of the vesicles hardly fluoresce, but fluorescence strongly increases when CF is released from the interior into the bulk aqueous phase (25). Therefore, permeability changes in liposomes induced by surfactants can be determined by monitoring the increase in the fluorescence intensity of the liposome preparations due to the CF liberated (26,27). Fluorescence measurements were run on a Shimadzu RF-540 spectrofluorophotometer equipped with a thermoregulated cell compartment (Kyoto, Japan) at an excitation wavelength of 495 nm and emission of 515.4 nm.

The general procedure to assess the effect of surfactants on the release of liposomal content consists of treating aliquots of liposomes in buffered medium (loaded with CF) with identical volumes of buffered solution containing different concentrations of C_{12}-Bet/SDS mixed systems. Afterwards, the proportion of the fluorescent dye released is measured. The amount of released CF at the emission wavelength of 515.4 nm is calculated by means of the following equation (14):

$$\% \text{ CF release} = \frac{I_t - I_o}{I_\infty - I_o} \times 100 \qquad [1]$$

where I_o is the initial fluorescence intensity of the CF-loaded liposome suspension in the absence of surfactant, and I_∞ is the fluorescence intensity after destroying the liposomes by addition of Triton X-100 (60 μL of 10% vol/vol aqueous Triton X-100 solution to 2.0 mL of liposome suspension). I_t corresponds to the fluorescence intensity at the same wavelength measured 40 min after adding the surfactant solution to the liposome suspension.

Partition coefficients. To compare results from different mixed surfactant systems, a simple parameter is required. Throughout this study, we used a partition coefficient of surfactant between lipid bilayer and aqueous medium, defined as (11,13):

$$K = \frac{S_B/PL}{S_W} \qquad [2]$$

where S_W and S_B are concentrations of surfactant in the aqueous medium and bilayer, respectively, for a system containing PL (mM phospholipids). Defining the effective ratio of surfactant to PL, R_{eff}, as the ratio between surfactant concentration into bilayers (S_B) and the total PL concentration of liposomes (PL), it follows that:

$$K = \frac{R_{eff}}{S_W} \qquad [3]$$

Partition coefficients were determined experimentally by plotting surfactant concentrations resulting in a half-maximal value of CF release *vs.* liposome PL concentration. A linear relationship is established, which can be described by the following equation:

$$S_T = S_W + R_{eff} \times (PL) \qquad [4]$$

where the effective surfactant-to-PL molar ratio R_{eff} and the aqueous concentration of surfactant S_W are, respectively, the slope and the ordinate at the origin (zero PL concentration). The S_T parameter is the total surfactant concentration (mM).

RESULTS AND DISCUSSION

CMC. Figure 1 shows the surface tension as a function of total surfactant concentration for C_{12}-Bet/SDS mixed systems. The surface tension values for various molar fractions decrease with increasing total surfactant concentration, but these systems show a minimum in the vicinity of the CMC. Such a minimum has also been reported (5,28) for zwitterionic-anionic mixed systems. The surface tension at the CMC (γ_{CMC}) can be used as one of the criteria of surface activity (29), where the lower the γ_{CMC}, the higher the surface activity of the system. In the mixed surfactant system studied, the γ_{CMC} value for a 0.4 molar fraction of zwitterionic surfactant is 25.4 mN m^{-1}. Furthermore, the reduction in surface tension is larger for the mixed systems than for the single surfactant.

When plotting CMC values against the molar fraction of zwitterionic surfactant (Fig. 2), the CMC values of mixed systems decrease with increasing $X_{zwitter}$ and then show a minimum at $X_{zwitter} = 0.4$. The CMC values of each mixed system are given in Table 1.

If the mixed micelle were ideal, the CMC values would fall on the line predicted by the relationship (30):

$$\frac{1}{C_{12}} = \frac{X}{C_1} + \frac{1 - X}{C_2} \qquad [5]$$

where C_{12} is the CMC for the mixed micelle system of surfactants 1 (C_{12}-Bet) and 2 (SDS); C_1 is the CMC of

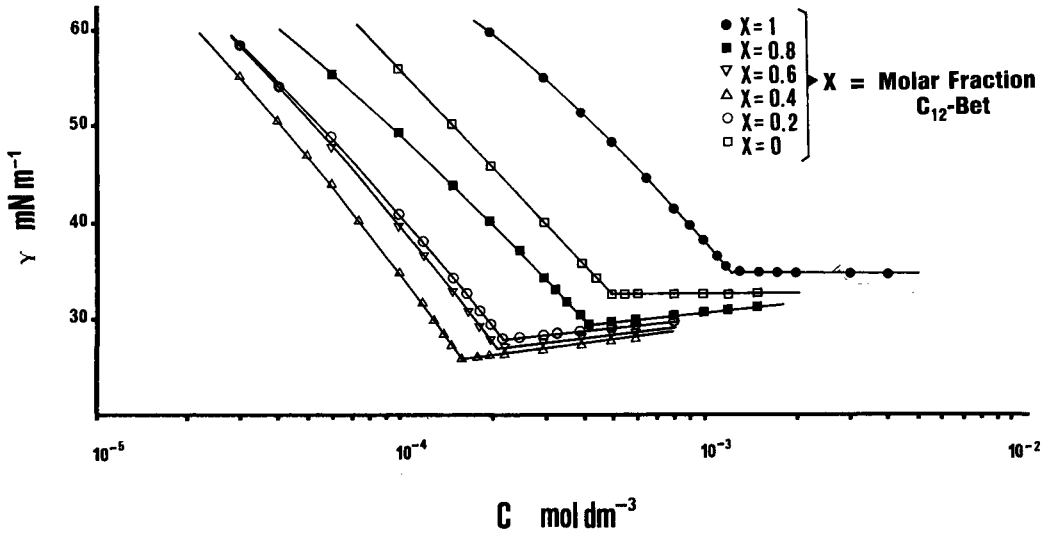

FIG. 1. Plots of surface tension against total surfactant concentration for different molar fractions of zwitterionic surfactant in *N*-dodecyl-*N,N*-dimethylbetaine/sodium dodecyl sulfate critical micelle concentration (C_{12}-Bet/SDS) mixed systems.

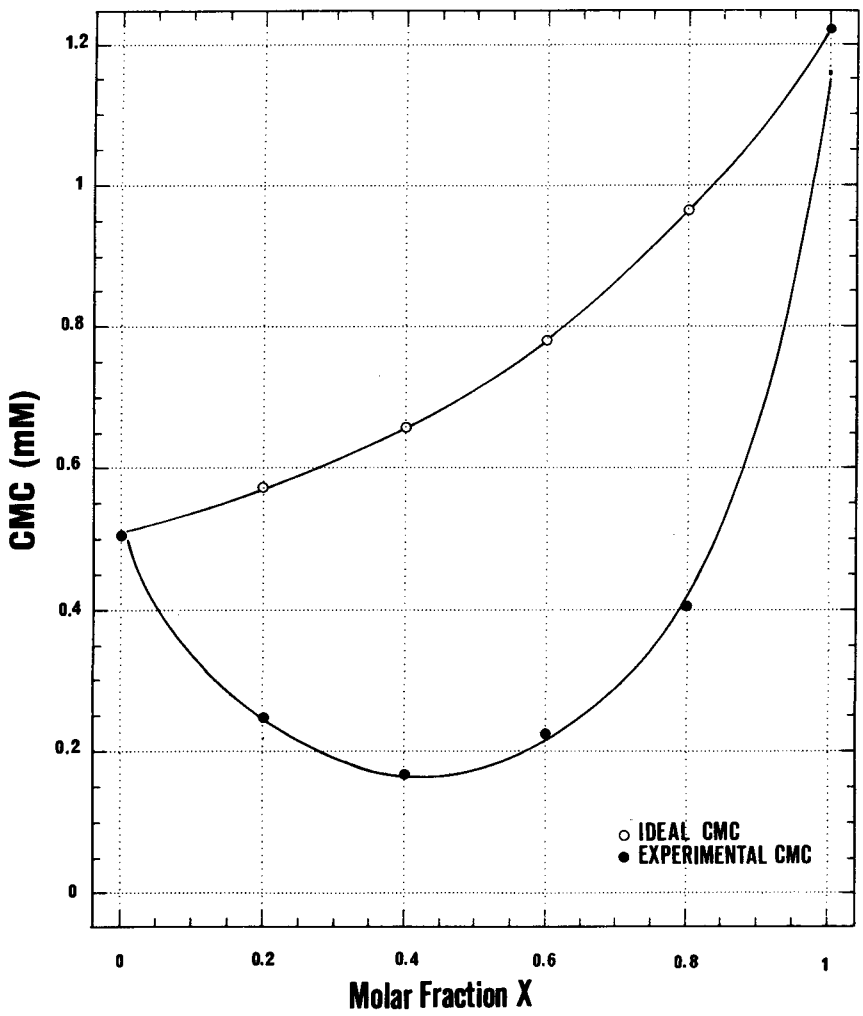

FIG. 2. Relationship between the critical micelle concentration (CMC) values (mM) and different molar fractions of the zwitterionic surfactant in *N*-dodecyl-*N,N*-dimethylbetaine/sodium dodecyl sulfate mixed systems.

TABLE 1

CMC Values at 25°C in Piperazine-1,4-*bis*(2 ethane sulfonic acid)

Zwitterionic surfactant molar fraction X	CMC (mM)	S_W (mM)	R_{eff} (mole/mole)	K (mM^{-1})
0	0.500	0.0890	0.2530	2.84
0.2	0.220	0.0389	0.1611	4.14
0.4	0.160	0.0261	0.1189	4.55
0.6	0.210	0.0267	0.1133	4.24
0.8	0.410	0.0444	0.1356	3.05
1.0	1.250	0.4200	0.4840	1.15

[a]S_W, surfactant concentration in aqueous medium; R_{eff}, effective surfactant to lipid ratio; K, partition coefficient values resulting in 50% carboxyfluorescein release for different molar fractions in *N*-dodecyl-*N,N*-dimethylbetaine/sodium dodecyl sulfate mixed systems; CMC, critical micelle concentration.

surfactant 1; C_2 is the CMC of surfactant 2 and X is the molar fraction of surfactant 1.

The theoretical CMC values for each molar ratio thus calculated also have been indicated in Figure 2. The difference between the experimental and theoretical CMC values shows that the binary system C_{12}-Bet/SDS has a negative derivation with respect to ideal behavior. A mixture of two surfactants usually forms mixed micelles in aqueous solution. It has been reported (1,31) that the mixed CMC for two oppositely charged surfactants becomes notably smaller than that of the individual surfactants due to association of the surfactants induced by electrostatic attraction. The present results can also be explained by assuming that association of the surfactants occurs easily by electrostatic attraction between the cationic portion of the betaine and the dodecyl sulfate ion in the mixed systems.

Particle size distribution of liposome preparations. The particle size of liposome vesicles in the range of starting PL concentrations from 0.1 mM to 1.0 mM varied little and was around 100 nm in all cases. In addition, the polydispersity index values were lower than 0.1, indicating that the size distribution was narrow.

Permeability studies. It is known that in lipid/surfactant systems reaching complete equilibrium may take several hours (32,33). However, a substantial part of the surface effect takes place within approximately 30 min after its addition to the liposomes (28).

To determine the time needed for a constant rate of CF release for liposomes in the range of the PL concentration investigated (0.165 mM and 0.990 mM), a kinetic study on the interaction of liposomes with C_{12}-Bet/SDS mixed surfactant systems was carried out. Unilamellar liposomes (0.165 mM phospholipid concentration) were treated with mixed systems of surfactants at different molar fractions (total surfactant concentration 0.05 mM). The subsequent changes in permeability were studied as a function of time. Results are shown in a three-dimensional picture in Figure 3. It shows that the permeability

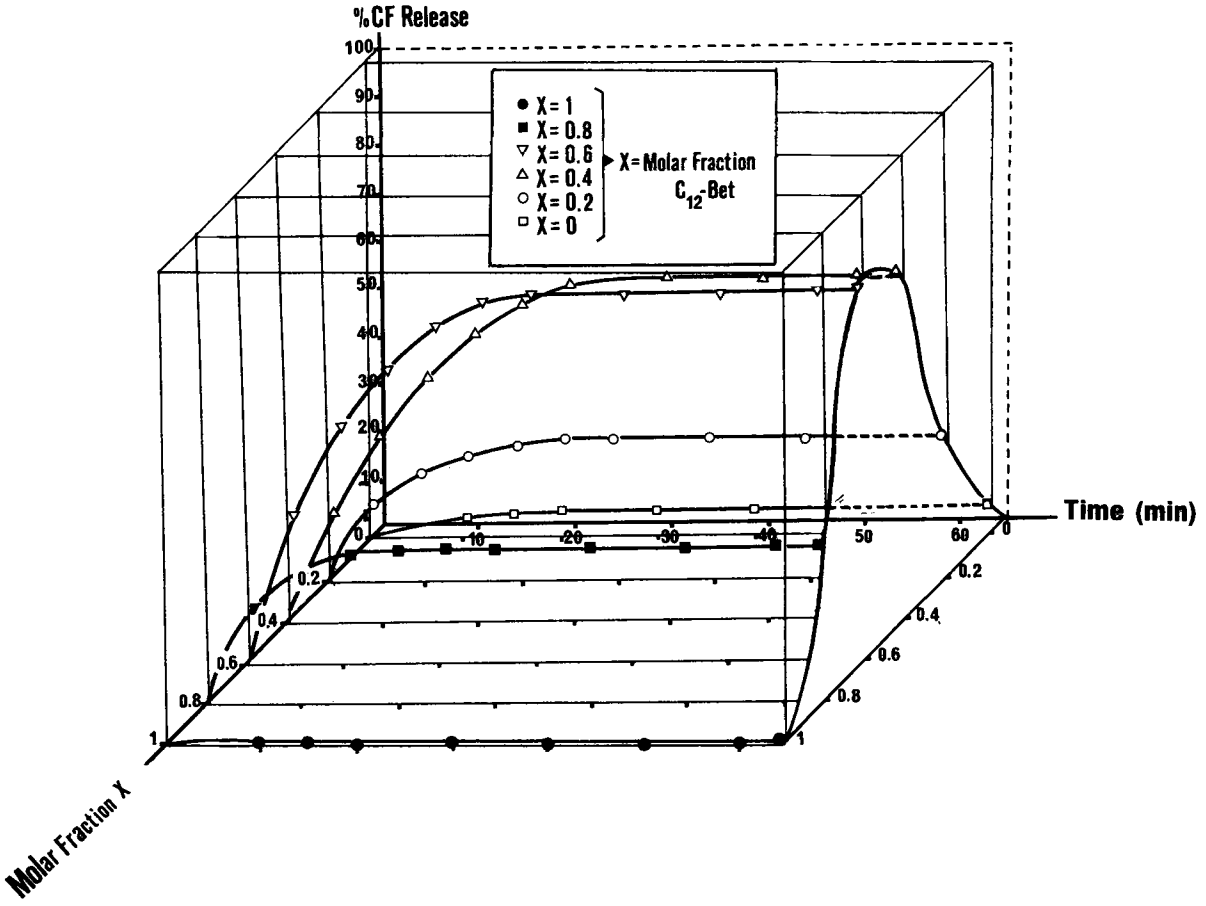

FIG. 3. Time curve of the release of carboxyfluorescein (CF) trapped in unilamellar liposomes (phospholipid concentration 0.165 mM), caused by different molar fractions of zwitterionic surfactant in *N*-dodecyl-*N,N*-dimethyl betaine/sodium dodecyl sulfate (C_{12}-Bet/SDS) mixed systems (total surfactant concentration 0.05 mM).

kinetics are similar for each system tested: About 40 min is needed to achieve a CF release equilibrium. Also, the increase in CF release is larger for the mixed systems than for a single surfactant. Thus, the highest value is achieved in the range between 0.4 and 0.6 for the molar fraction of zwitterionic surfactant ($X_{zwitter}$) with the lowest values for $X_{zwitter} = 1.0$ (100% C_{12}-Bet) and $X_{zwitter} = 0$ (100% SDS). As a consequence, changes in permeability were studied 40 min after addition of surfactants to the liposomes at 25°C. The CF release of liposome suspensions in the absence of surfactants 40 min after preparation was negligible.

To determine the partition coefficient of surfactants between aqueous media and lipid bilayers, a systematic investigation of liposome permeability changes caused by the addition of surfactant was carried out. To this end, changes in CF released from liposomes (lipid concentration from 0.165 mM to 0.990 mM) *vs.* surfactant concentration were determined 40 min after surfactant addition. The surfactant concentrations resulting in a half-maximal value of CF release were determined from these data and are presented as function of PL concentrations in Figure 4. A linear relationship was established in each case. These graphs are in agreement with Equation 4. For these straight lines, the effective surfactant-to-PL molar ratio, R_{eff}, and the aqueous concentration of surfactant, S_W, are, respectively, the slope and the ordinate at the origin (zero PL concentration). These results are given together with the CMC values in Table 1.

The surfactant concentrations in the aqueous medium (X_W) were always smaller than the corresponding CMC values in buffered medium. These results suggest that mixed-system surfactant-liposome interactions are determined mainly by the action of surfactant monomers on the lipid bilayers, unlike the behavior of the surfactants in solubilization processes (10,17), where micelle formation plays an important role. Likewise, both S_W and R_{eff} values decrease as the molar fraction of zwitterionic surfactant increases and show a minimum at $X_{zwitter} = 0.4$. When the R_{eff} values are related to the corresponding S_W values, partition coefficient K for each mixed surfactant system can be obtained (Table 1). These data show that the highest value is also obtained for a zwitterionic molar ratio of 0.4. Lower values are obtained for single surfactants. If the partition coefficient K for each mixed system is plotted as a function of its molar fraction, the graph shown in Figure 5 is obtained. The equation and the regression coefficient of the curve are:

$$Y = 2{,}865 + 8.129X - 9.776\ X^2$$
$$r^2 = 0.990$$

The curves of Figures 2 and 5 exhibit contrary tendencies, showing minimum CMC and maximum partition coefficient values for the same molar fraction of zwitterionic surfactant ($X_{zwitter} = 0.4$). Likewise, the results of Figure 3 and the partition coefficient values for each $X_{zwitter}$ establish a positive association between K values and the ability of each mixed system to modify the permeability of liposomes.

In conclusion, changes in the physicochemical properties (γ_{CMC} and CMC) of the mixed surfactant systems formed by C_{12}-Bet/SDS, which affect their surface activity, determine their capacity to alter the permeability of phospholipid vesicles. The partition coefficient of the mixed systems between the lipid bilayer and the aqueous

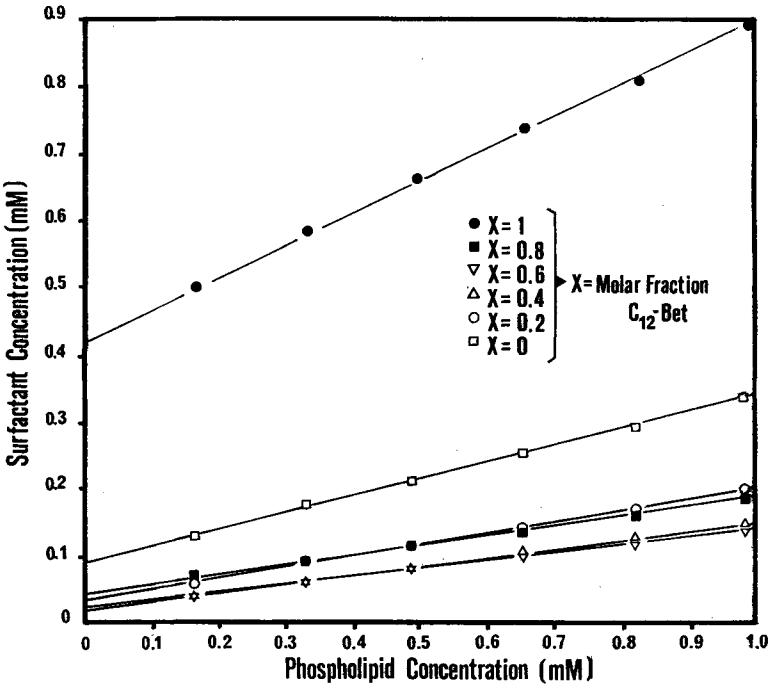

FIG. 4. Surfactant concentrations resulting in a half-maximal value of carboxyfluorescein release for different molar fractions of zwitterionic surfactant in *N*-dodecyl-*N,N*-dimethylbetaine/sodium dodecyl sulfate (C_{12}-Bet/SDS) mixed systems *vs.* phospholipid concentration.

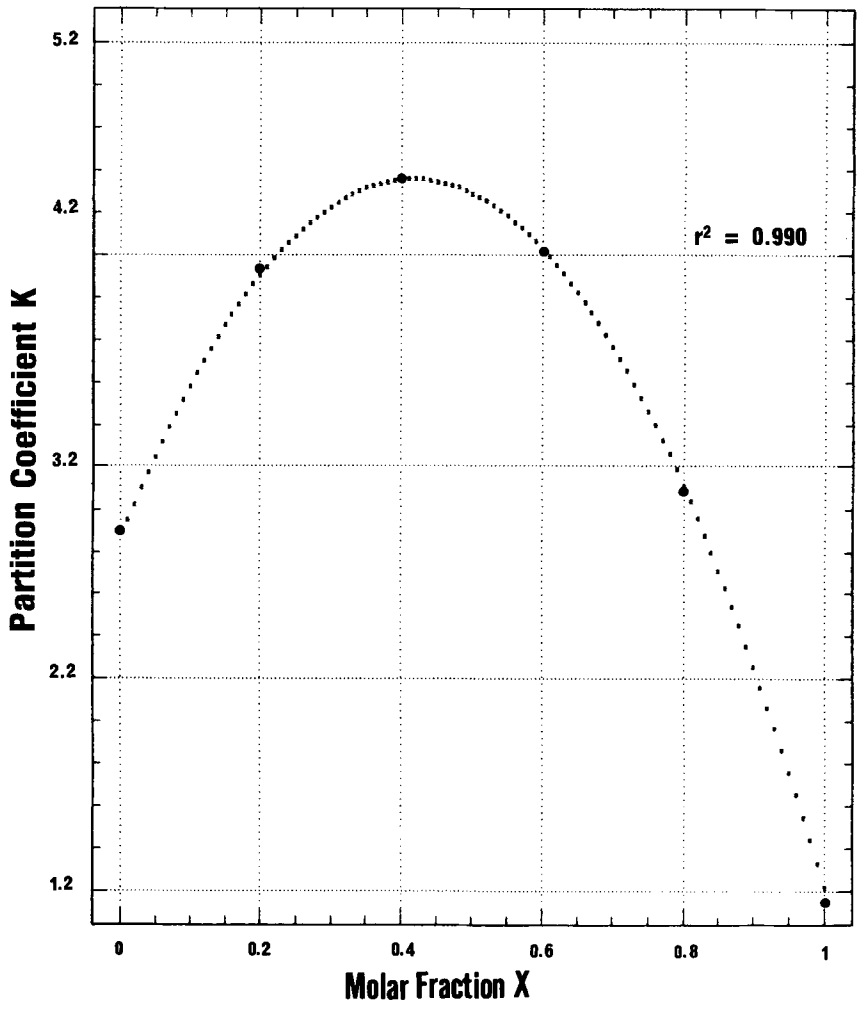

FIG. 5. Relationship between partition coefficients (mM^{-1}) and the molar fractions for different *N*-dodecyl-*N,N*-dimethylbetaine/sodium dodecyl sulfate mixed systems.

phase is also affected by these physicochemical changes and exhibits a maximum when the molar fraction of zwitterionic surfactant is 0.4.

ACKNOWLEDGMENT

We acknowledge the expert technical assistance of G. von Knorring.

REFERENCES

1. Iwasaki, T., M. Ogawa, K. Esumi and K. Meguro, *Langmuir 7*:30 (1991).
2. Cooper, E.R., and B. Berner, in *Surfactants in Cosmetics*, Surfactant Science Series, Vol. 16, edited by M.M. Rieger, Marcel Dekker Inc., New York, 1985, pp. 195–211.
3. García Dominguez, J., F. Balaguer, J.L. Parra and C.M. Pelejero, *Int J. Cosm. Sci. 3*:57 (1981).
4. Faucher, J.A., and E.D. Goddard, *J. Soc. Cosm. Chem. 29*:323 (1978).
5. Abe, M., K. Kato and K. Ogino, *J. Colloid Interface Sci. 127*:328 (1989).
6. Jansson, M., and R. Rymden, *Ibid. 119*:185 (1987).
7. Urbaneja, M.A., A. Alonso, J.M. Gonzalez Mañas, F.M. Goñi, M.A. Partearroyo, M. Tribout and S. Paredes, *Biochem. J. 270*:305 (1990).
8. Thewalt, J.L., and R.J. Cushley, *Biochim. Biophys. Acta 905*:329 (1987).
9. Abraham, W., and D.T. Downing, in *Factors Affecting the Formation Morfology and Permeability of Stratum Corneum Lipid Bilayers* in Vitro, edited by R.C. Scott, R.H. Guy and J. Hadgraft, IBC Technical Services Ltd, Great Britain, 1990, pp. 110–180.
10. Lichtenberg, D., J. Robson and E.A. Dennis, *Biochim. Biophys. Acta. 737*:285 (1983).
11. Jackson, M.L., D. Schmidt, D. Lichtenberg, A. Litman and A.D. Albert, *Biochemistry 21*:4576 (1982).
12. Schubert, R., K. Beyer, H. Wolburg and K.H. Schmidt, *Ibid. 25*:5263 (1986).
13. Lichtenberg, D., *Biochim. Biophys. Acta 821*:470 (1985).
14. De la Maza, A., J. Sanchez Leal, J.L. Parra, M.T. Garcia and I. Ribosa, *J. Am. Oil Chem. Soc. 68*:315 (1991).
15. De la Maza, A., J.L. Parra, M.T. García, I. Ribosa and J. Sanchez Leal, *Colloids and Surfaces 61*:281 (1991).
16. Singleton, W.S., M.S. Gray, M.L. Brown and J.L. White, *J. Am. Oil Chem. Soc. 42*:53 (1965).
17. Rosen, M.J., *J. Colloid Interface Sci. 79*:587 (1981).
18. Ralston, E., L.M. Hjelmeland, R.D. Klausner, J.N. Weinstein and R. Blumenthal, *Biochim. Biophys. Acta 649*:133 (1981).

19. Rigaud, J.L., A. Bluzat and S. Buschlen, in *Physical Chemistry of Transmembrane Ion Motion*, edited by G. Spach, Elsevier, Amsterdam, 1983, pp. 457-464.
20. Paternostre, M.T., M. Roux and J.L. Rigaud, *Biochemistry 27*:2668 (1988).
21. Szoka, F., and D. Papahadjopoulos, in *Liposomes: Preparation and Characterization*, edited by C.G. Knight, Elsevier, Amsterdam, 1981, Chapter 3.
22. Szoka, F., F. Olson, T. Heath, W. Vail, E. Mayer and D. Papahadjopoulos, *Biochim. Biophys. Acta 601*:559 (1980).
23. Rand, M.C., A.E. Greenberg and M.J. Taras, in *Standard Methods for the Examination of Water and Wastewater*, American Public Health Association, Washington, 1976, pp. 466-484.
24. Lunkenheimer, K., and D. Wantke, *Colloid and Polymer. Sci. 259*:354 (1981).
25. Parker, C.A., in *Photoluminiscence of Solutions*, Elsevier, New York, 1968, pp. 303-396.
26. Weinstein, J.N., E. Ralston, L.D. Leserman, R.D. Klausner, P. Dragsten, P. Henkart and R. Blumenthal, in *Liposome Technology*, Vol. III, edited by G. Gregoriadis, CRC Press, Boca Raton, 1986, pp. 183-205.
27. Ruiz, J., F.M. Goñi and A. Alonso, *Biochim. Biophys. Acta 937*:127 (1988).
28. Rosen, M.J., and B.Y. Zhu, *J. Colloid Interface Sci. 99*:427 (1984).
29. Rosen, M.J., *J. Am. Oil Chem. Soc. 51*:461 (1974).
30. Cox, M.F., N.F. Borys and T.P. Matson, *Ibid. 62*:1139. (1985)
31. Ogino, K., M. Abe, K. Kato and R. Sakama, *J. Jpn. Oil Chem. Soc. 36*:129 (1987).
32. Lichtenberg, D., Y. Zilberman, P. Greenzaid and S. Zamir, *Biochemistry 18*:3517 (1979).
33. Alonso, A., M.A. Urbaneja, F.G. Carmona, F.G. Cánovas, J.C. Gomez-Fernandez and F.M. Goñi, *Biochim. Biophys. Acta 902*:237 (1987).

[Received June 15, 1992; accepted March 23, 1993]