Solubilization of Sterols by Bile Salt Micelles and Monolayer Behavior of Mixed Systems of a Bile Acid with Different Sterols

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Abstract

In order to study thermodynamically the so-called lowering effect of plasma cholesterol level caused by dietary intake of phytosterols (plant sterols) or phytostanols, solubilization was investigated for the respective single systems of cholesterol (Ch), cholestanol (Chsta) and stigmasterol (Stig) and for their 1:1 mixed systems with two kinds of free bile salts (BS), sodium cholate (NaC) and sodium deoxycholate (NaDC), in pH 10 Kolthoff buffer solution at 37 °C. Even for the sterol / stanol solubilizates examined in the present study, compared with NaC, NaDC is stronger in solubilizing power, Sp, which is defined as the derivative of the solubilized amount, W, in mM by the total BS concentration, C_t (dW / dC_t), as being expected in terms of hydrophobicity and aggregation number. The following findings were made i) the amount solubilized by both BS micelles follows the order: Ch > Chsta > Stig among the three single solubilizates, ii) Chsta is much stronger in the lowering ability than Stig in regard to Ch solubilization, and iii) from the 1 : 1 mixture of Ch with Chsta, Chsta is selectively solubilized (Ch is selectively excluded) by BS micelles.

Different physicochemical properties of Langmuir films (monolayers) composed of mixed systems of a bile acid, deoxycholic acid (DC) with Ch and various plant sterols (Chsta and Stig), and DC with 1 : 1 St mixtures; (Ch + Chsta), (Ch + Stig) on the substrate of 5 M aqueous NaCl solution (pH = 1.2) at 25 °C, were investigated in terms of mean surface area per molecule (A_m), surface excess Gibbs energy ($\Delta G_{(ex)}$); these were analyzed on the basis of the respective surface pressure (π) vs. A isotherms as a function of mole fraction of sterols (X_{st}). Notable findings are: (i) all the binary component systems did form patched film type monolayers (ii) $\Delta G_{(ex)}$ was found to be greatly dependent on (a) the combinations of DC with different sterol species and (b) to be markedly varied by a difference in mixing ratio of DC to sterols.

1. Introduction

The most important property of bile salts (BS) is their ability to solubilize and bind solute molecules that are insoluble or only marginally soluble in water or other bulk solvents[1-8]. That is, water-insoluble components in bile (such as cholesterol (Ch) and lecithin) and lipolytic products (monoglycerides and fatty acids) are solubilized by BS micelles in the small intestine. The amount of solute solubilized is generally more dependent on BS concentration than on common surfactants consisting of a hydrophobic chain with a simple hydrophilic head group, indicating that the aggregation mode is not so cooperative. The

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micellar structure as well as aggregation number depends not only on given conditions such as pH, ionic strength, and temperature but also on the BS species themselves. In fact, the structure of micelles formed is greatly changeable, in particular around their critical micellization concentration (CMC) even for a BS species[8]. It follows that the solubilization of various hydrophobic solutes by BS micelles is different in terms of interaction between host and guest molecules or the driving force causing it.

It has been established that several plant sterols can induce a decrease in the serum total and low-density lipoprotein cholesterol (Ch) level, which in turn cause suppression of atherogenesis[9]. The mechanisms for the decrease in Ch concentration accompanied by the coexistence of plant sterols, are not completely interpreted yet, while it is clearly demonstrated that the effect is based on the plant sterols reducing the rate of intestinal Ch absorption[10]. Plant sterols are absorbed from the intestine to a lower extent in comparison to Ch[11]. Sugano et al. showed first that phytostanols, derived from reduction of the double bonding of phytosterols, can more effectively lower the plasma Ch level than phytosterols by using rats[12]. Based on systematic studies on solubilization of Ch and phytosterols or phytostanols, Ikeda et al. have revealed that it is an essential condition that phytosterols inhibit Ch solubilization by bile salts in the intestine lumen; this implies that the suppression of Ch absorption results from the lowering of the relative amount of Ch solubilized by bile salt micelles when phytosterols or phytostanols coexist with Ch. In other words, the inhibition of Ch absorption takes place at the unstirred water layer (UWL) covering epithelium cells[13-15].

It is well-known that bile salts play an important role when some lipids migrate in living tissue and are absorbed through the intestinal wall[16-18]. The bile salts form mixed micelles with phospholipids and do solubilize the ingested fat, Ch and fat-soluble compounds. A comprehensive understanding of transport mechanism requires some knowledge of the physicochemical properties of lipids and bile salts regarding their behavior in an aqueous environment[19-21]. Many studies to clarify the biological activities of the bile salts have been presented from pharmacological and physicochemical points of view[22-26]. Nevertheless, the physicochemical mechanism in vivo has not been clarified as yet. One of the main reasons for this is attributable to the complexity of living systems.

In earlier works we have examined the relation of the solubilizing power to the free energy change on solubilization, and have developed thermodynamic equations for evaluating various thermodynamic parameters when mixed solubilizate systems are solubilized by bile salt micelles in buffer solution at pH 10 and 37℃[27]. In that solubilization study, the solubilizates studied were the respective single systems of Ch, stigmasterol (Stig) and cholestanol (Chsta) and their 1 : 1 mixed systems of Ch / Stig and Ch / Chsta, while the solubilizers were sodium cholate (NaC) and sodium deoxycholate (NaDC). From the study on mixed solubilizate systems, selective solubilization of sterols was highlighted. On the other hand, in the previous monolayers studies, we have reported that various combinations of binary mixed systems of Ch with a free deoxycholic acid (DC / Ch)[28] and Ch with different conjugated bile acids {glycochenodeoxycholic acid (GCDC) / Ch, glycoursodeoxycholic acid (GUDC) / Ch, taurochenodeoxycholic acid (TCDC) / Ch and tauroursodeoxycholic acid (TUDC) / Ch}[29] were found to show that Ch does not mix with bile acids but to form a "patched monolayer". This patched monolayer formation suggests that the lateral interaction between Ch and bile acids is extremely unfavorable, whereas bile acids themselves are well mixed with each other, and the lack of the lateral interaction interprets why bile acids are generally poor at solubilizing Ch. In this paper, the monolayer behavior of single systems and of binary (or ternary) component systems composed of DC with sterols; Ch, Stig, Chsta, being formed at the interface of air / water (at pH = 1.2 with addition of NaCl), was investigated in terms of surface pressure (π) and molecular occupation surface area (A) relation. A series of π-A curves at every 0.1 mole fraction of each sterol for mixed systems were obtained at 25.0℃ and various thermodynamic analyses were carried out. Since we have recently investigated the monolayer behavior of binary component systems of dipalmitoyl phosphatidyl choline (DPPC) with Ch and plant sterols and a stanol mentioned above (these are abbreviated as St or Sts in the text) in order to make clear the role of different Sts played in cell membranes[30]. The

results obtained in the present study will be discussed in comparison with the previous DPPC / Sts binary component systems.

2. Competitive solubilization of Sterols by Bile Salts

In Fig. 1 (A) and (B) are shown the relations of Solubilized amounts (W, in mM) vs. BS concentration (C_t) for the mixed systems of Ch / Stig in NaC (left) and NaDC (right) solutions and for those of Ch / Chsta in NaC (left) and NaDC (right) solutions, respectively. In all the frames, the results for the respective single systems (shown by open and closed circles) are included for comparison. As is seen, W decreases in the order: Ch > Chsta > Stig in NaC as well as NaDC solutions. The relations obtained in NaDC solution are very linear, while those in NaC solution the plots show curvature, suggesting that the state of a micelle, especially in terms of aggregation number and structure, changes with concentration, as has been previously reported[4, 8].

The W values of the respective components in the mixture are shown together with the total W values. Comparing (A) and (B), the total W value of Ch / Stig mixture (A) is greater than each single system over the whole BS concentration ranges. In contrast, the total W value is lower than both single systems in Fig. 1 (B). It is noteworthy that Ch is surprisingly reduced as if Ch was excluded by Chsta. In the ranges below 80 mM for NaC and below 40 mM for NaDC, Ch is completely kicked out of micelles by Chsta. Selective solubilization was observed for this combination. It should be noted here that for another combination, i.e. Stig / Chsta mixed system, the present HPLC method did not allow us to separately analyze the composition of solubilized amount except for the total amount; we should have employed a

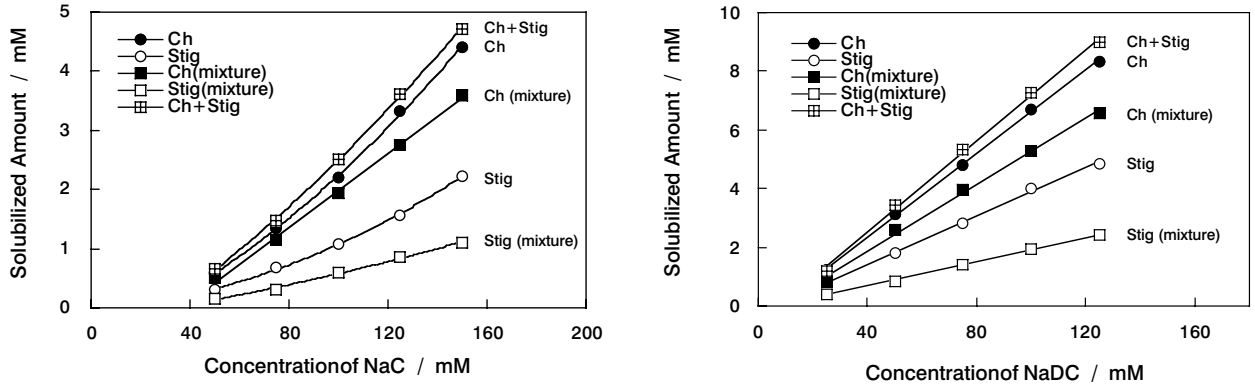

Fig. 1-A Solubilized amounts of Ch and Stig single systems and their mixed solubilizates in micellar solutions of NaC(left) and NaDC(right) for total and respective components of the mixed solubilizates.

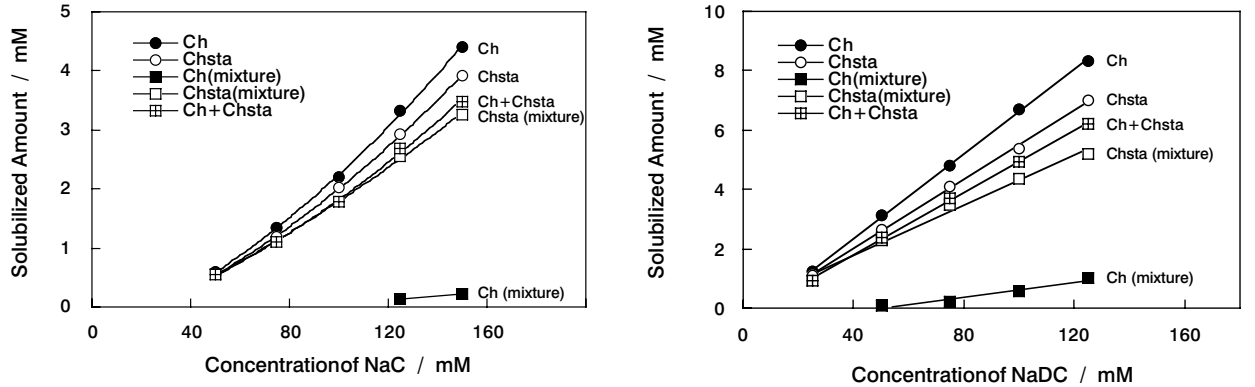

Fig. 1-B Solubilized amounts of Ch and Chsta single systems and their mixed solubilizates in micellar solutions of NaC(left) and NaDC(right) for total and respective components of the mixed solubilizates.

different column or not methanol but a different liquid as eluent. So we cannot report a set of analyzed data for Stig / Chsta at present.

To see the mixing effect more clearly, W values of the respective single systems and their 1:1 mixed systems are expressed by histograms for Ch / Stig and Ch / Chsta combinations in NaC and NaDC solutions.

From histograms for Ch / Stig mixed solubilizate systems (not shown here, but can be seen from Fig. 1 (A)) it was found that the mixing results in a small increase of total amount, but in regard to each component, W is reduced. Therefore, it may be said that W of Ch is a little decreased by coexistence of Stig. On the contrary, looking at Fig. 2 for Ch / Chsta mixed systems, the total W is lower than the respective singular systems. Again, attention should be paid to the fact that most or all Ch is excluded from micelles. The histogram clearly demonstrates that BS micelles can selectively solubilize a sterol from sterol / stanol mixtures.

For easy comparison Fig. 3 was constructed. The mole fraction in solubilized steroid / stanol mixture is plotted as a function of BS concentration. In the mixed system of Ch and Stig, Stig occupies nearly 20% with no visible BS concentration dependence. On the other hand, in the Ch / Chsta mixed systems, Chsta is selectively solubilized by BS micelles. At the higher concentration range, Ch becomes to be solubilized to some extent, but at physiological concentration range, that is 30 mM, Ch does not coexist with Chsta in BS micelles. Table 1 lists the reciprocals of solubilizing

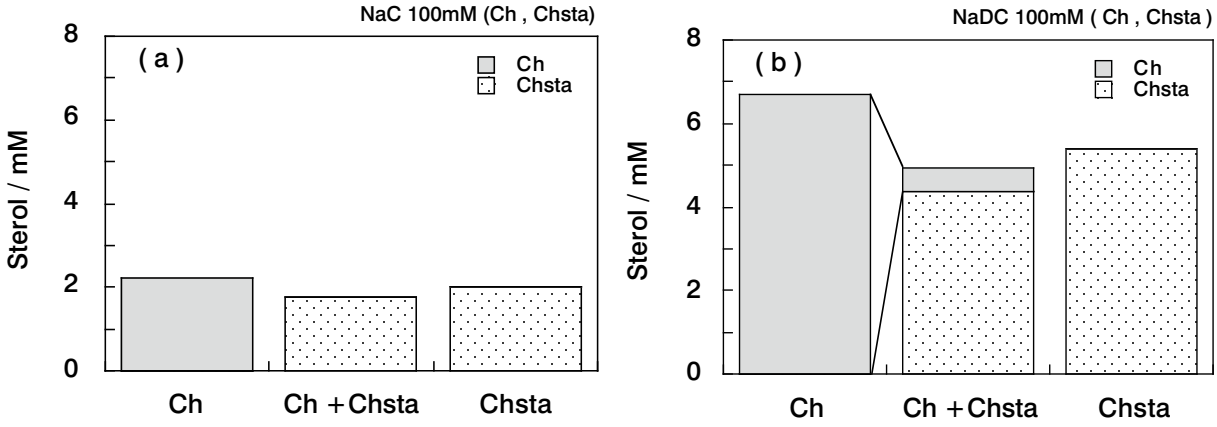

Fig. 2 Concentrations of the respective single and binary mixed sterols of Ch and Chsta solubilized by (a) NaC and (b) NaDC micelles in pH 10 Kolthoff buffer solution at 37 ℃.

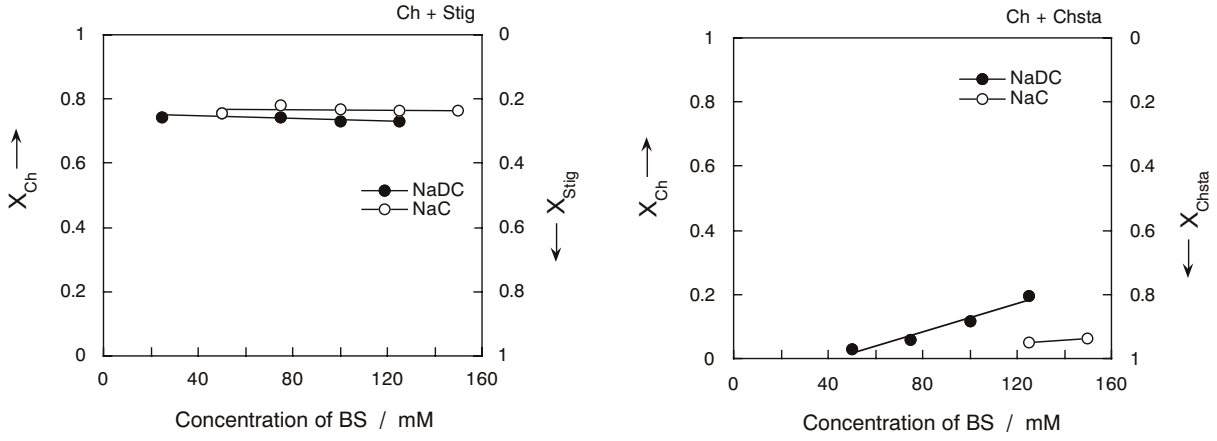

Fig. 3 Selectivity represented by mole fractions X_{Ch} and X_{Stig} or X_{Chsta} in the solubilizate mixtures within micelles as a function of BS concentration.

Table 1 The reciprocal of solubizing power (Sp^{-1}) corresponding to the molecularnumber required for solubilizing a sterol molecule.

	SP^{-1}				
	Ch	Stig	Chsta	Ch+Stig	Ch+Chsta
NaDC	14.0	24.3	17.3	13.4	19.0
NaC[a)]	27.6	60.0	31.4	31.4	35.2

a) Sp was determined at 100 mM of NaC.

power (Sp^{-1}) of Ch, Stig and Chsta in addition to their mixed systems solubilized in NaC and NaDC solutions. The Sp^{-1} corresponds to the molecular number required for solubilizing a solubilizate.

Looking at Table 1, one solubilizate molecule is likely to be solubilized by a BS micelle in average, while the distribution in solubilizate molecule number may mostly range from zero to three (It has been reported that the mean aggregation number values were ca. 10 for NaC and ca. 30 for NaDC[31].). Even if zero to three solubilizate molecules are solubilized by one BS micelle, the better affinity of solubilizate to BS micelles must be recognized by each other, when a restricted space inside a micelle is allowed for a solubilizate molecule to occupy it; this may lead to the selctivity between sterols and stanols.

Comparing Ch and Chsta which are of a sterol-stanol relation, Ch is superior somewhat to Chsta in terms of solubilized amount because Ch's double bond, which has a slight hydrophilicity as compared with the B-ring of Chsta, may lead to a favorable positioning or orientation toward the solubilizing site of BS micelles[27]. However, looking at total figure of the parts of B, C, D rings with the side chain, the difference at carbon 5, i.e. either double bonded or hydrogen-saturated, is likely to divide sterol / stanol mixture into types of more and less easily penetrating trend toward the given hydropholic space in micelles. In the case of Ch / Chsta mixed system, the steric structure of B-C-D-rings of Chsta seems to avoid steric hindrance more easily than that of Ch when inserting the hydrophobic part inside micelle. Although A/B rings are trans for Chsta in contrast to cis for BSs, more suitable space needed for hydrophobic interaction may be given to Chsta. This may allow Chsta molecules to occupy more fastly and more stably the solubilizing sites of micelles than Ch, and consequently Ch molecules cannot find any more room for solubilization. Sugano et al.[32] and Ikeda et al.[33] have shown an interesting comparison between sitosterol and sitostanol in regard to lowering effect of plasma Ch concentration by using four types of feeds containing (a) Ch only, (b) Ch + sitosterol, (c) Ch + sitostanol and (d) no Ch added. They found that (c) Ch + sitostanol system was the most effctive for rats and rabbits. This also indicates that stanol can more greatly reduce the micellar solubilization of Ch than sterol.

Examining the effect of difference in side chain length, Stig which has the same steroid skeleton as Ch shows the lowest solubilized amount as long as a comparison is made among single species. This suggests that the length of side chain, its bulkiness and flexibility (whether there exists a double bond or none) are involved in sterol / stanol solubilizing-micelle formation and, in addition, that the side chain attached to the steroid skeleton is more deeply inserted into the hydrophobic space and interacts directly with the inside of micelles. In this sense the bulky and non-flexible side chain of Stig can fit to somewhat less extent within the hydrophobic space, leading to a weaker interaction. However, when Ch and Stig coexist, their side chains seem to have a hydrophobic interaction rather favorable to penetration into the hydrophobic space, thus the total solubilized amount of the Ch / Stig mixed system is greater than that of the Ch / Chsta mixed system.

3. Monolayer Behavior of a Bile Acid with Sterols

The obtained π-A isotherms are shown in Fig. 4 for the some mixed systems, (a) DC + Stig and (b) DC + (Ch + Stig) as selected examples. Here it is noted that the experiment to determine surface pressure (π) as a function of mean surface area

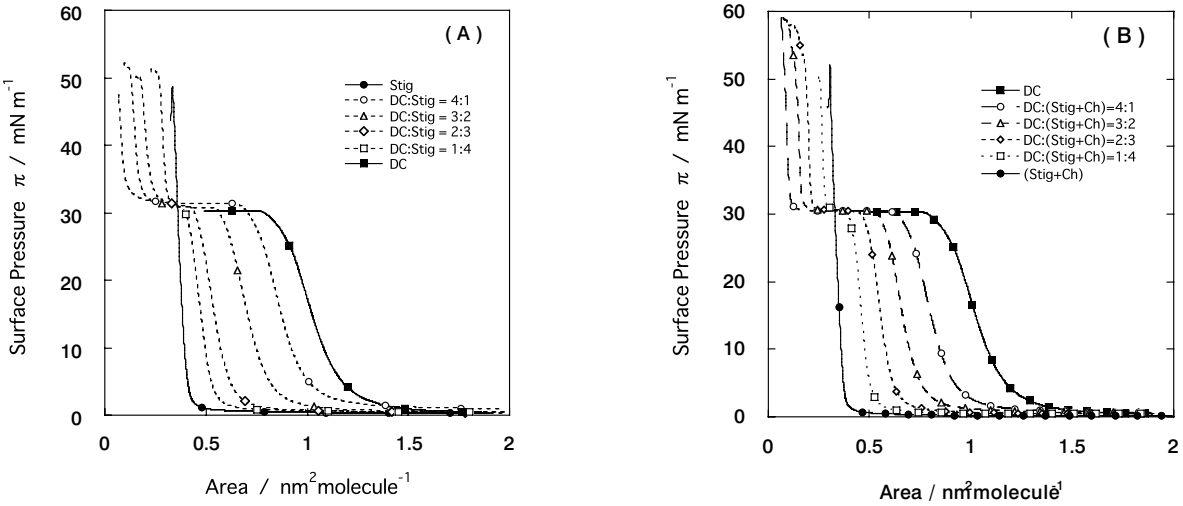

Fig. 4 The representative π-A isotherms at different mixing ratios for two component systems: (A) DC/Stig and for three component systems: (B) DC/(Stig+Ch)

per molecule (A) was started from A = ca. 2 nm^2 molecule^{-1} at a speed of 0.05 nm^2 min^{-1} molecule^{-1}. As will be discussed later (and the details will be reported elsewhere), it was found in the second trial for all the mixed systems that the different initiation of the π-A measurement (from initial surface area per molecule A = ca. 1 nm^2 molecule^{-1} at the same speed as above or at twice more rapid speed) did lead to π-A curves being different from those shown in Fig. 4. This condition dependence suggests that the π-A isotherms obtained are not of completely thermodynamical equilibrium and thus the various data values derived from analyses on the basis of π-A data are nothing other than apparent ones.

It can be seen from Fig. 4 that all the Sts show a rapid rise of π starting from the narrower limiting (or extrapolated) mean molecular surface area (A_0) compared to DC; this rapid rise of π-A curves of Sts implies the formation of liquid condensed (LC) film. In contrast, DC has a larger A_0 value and a slower rise up to 30 mN m^{-1}. DC does form liquid expanded (LE) film, but at the pressure of π-A curves' break point, DC demonstrated that a transition from LE to LC took place. This remarkable contrast suggests that the attitude of sterol molecules floating on the water surface is different from that of DC molecules; Sts having only one hydroxyl group at carbon number 3 as a hydrophilic head group do float vertically putting their hydrocarbon side chains toward the air and this attitude can easily lead to a strong hydrophobic interaction among the molecules while DC having two hydroxyl groups at positions 3 and 12 in addition to a carboxyl group does lay oneself down flat on the surface. This attitude of DC is difficult to make van der Waals attraction without coming tightly close to each other (in this situation the LE-LC transition takes place), and the magnitude of the interaction itself might be weaker than those among St molecules. The difficulty in van der Waals attraction may result in extremely poor miscibility of DC with St molecules in the two dimensional (2-D) phase.

Comparing A_0 values in nm^2 among the respective Sts, they increase in the order of Ch (0.39) < Stig (0.40) < Chsta (0.42). It is noted that the magnitude difference is not so marked, ranging between 0.39 and 0.42, but the order suggests that the difference in chemical structure of flexible side chain attached to the position of carbon number 17 of steroid skeleton, is clearly reflected. Interestingly, compared with Ch, Stig has the biggest side chain but the A_0 value is most close to that of Ch; this seems to come from the double bond of the side chain leading to more contact between side chains with a ethyl group at the position of carbon number 24 when the condensed liquid film is formed. Chsta has much wider A_0 value than Ch. The difference between the two lies only in absence or presence of the double bond of the steroid skeleton, apparently. However, the skeleton of Ch is cis and that of Chsta is trans structure of A/B

rings, being similar to the A/B ring structure of bile acids. This may have resulted in a greater distance between stanol molecules themselves as compared to the sterol molecules investigated in the present study.

When π-A curves of a given binary mixture are analyzed, it is essential to examine whether the relation of mean molecular surface areas (A_m) with mole fraction (X_{St}) satisfies the additivity rule or not, and if not so, which of negative and positive deviations is observed. Figure 5 shows the plots of A_m vs. X_{St} at discrete surface pressures for examining if the additivity rule of A_m holds or not for selected two combinations. The negative deviation from linearity corresponds to non-ideal mixing leading to an enhanced interaction between DC and St molecules.

As a powerful tool for evaluating the interaction among molecules in the formed monolayer comprising two or more components and its thermodynamic stability, the surface excess Gibbs energy, $\Delta G_{(ex)}$, can be used as the followings[34, 35]. The Gibbs energy change upon mixing of species 1 and 2, ΔG_{mix} for a real mixed system, is considered to be a sum of ideal and excess Gibbs energy changes as $\Delta G_{mix} = \Delta G_{(id)} + \Delta G_{(ex)}$. For ideal mixing the Gibbs energy change involves only the entropy term as $\Delta G_{(id)} = RT(X_1 \ln X_1 + X_2 \ln X_2)$, where X_i (i=1,2) denotes mole fraction in the mixture and RT is the gas constant times Kelvin temperature. Further, the excess Gibbs energy can be expressed as:

$$\Delta G_{(ex)} = \int_0^{\pi} \left[A_{12} - (X_1 A_1 + X_2 A_2) \right] d\pi \qquad (1)$$

where A_{12}, A_1 and A_2 represent the real area of mixed system and the respective areas of pure components 1 and 2, and π is the surface pressure. It is noted that if the monolayer is an ideally mixed one, $\Delta G_{(ex)}$ is zero because A_{12} should be equal to $(X_1A_1+X_2A_2)$ corresponding to the additivity rule[25, 30]. Fig. 6 shows the $\Delta G_{(ex)}$ as a function of mole fraction of the respective Sts at a few selected pressures (5, 15 and 30 mNm^{-1}). $\Delta G_{(ex)}$ was calculated on the basis of Eq. (1).

All the frames (A) to (F) simultaneously give us a vivid impression that shows remarkable changes in positive or negative value and clear dependence on species as well as composition. In its entirety it may be said that all the diagrams can be in common divided into two ranges above and below X_{st} = 0.5, i. e., St majority and DC majority regions. All over looking lets us have the following classification. Type I; $\Delta G_{(ex)}$ values are negative at almost all pressures: (B) DC / Chsta and (D) DC / (Ch+Chsta). Type II; Partially positive and partially negative depending on composition at all pressures: (C) DC / Stig, (E) DC / (Ch+Stig) and (F) DC / (Chsta+Stig). Type III; Positive at lower pressure but negative at higher

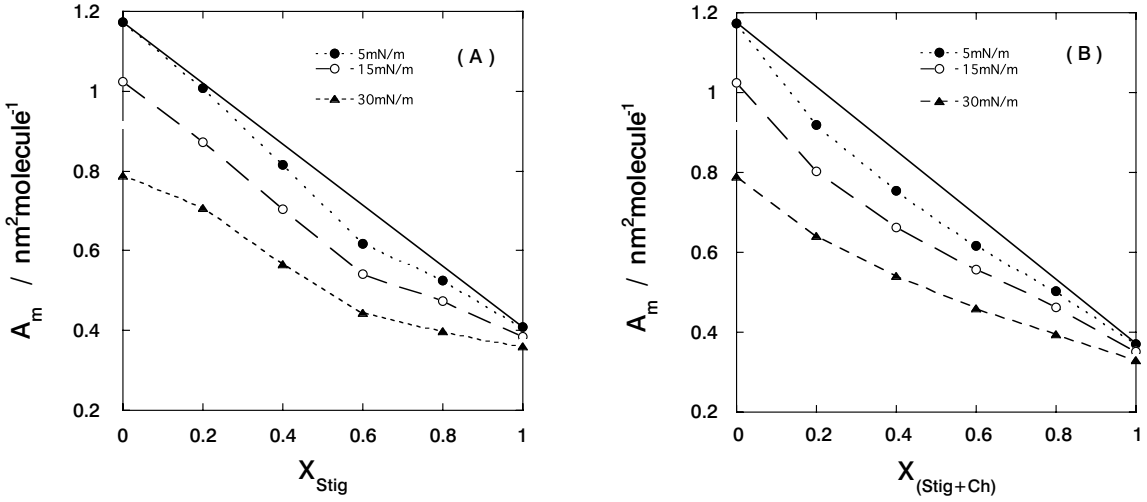

Fig. 5 The plots of mean molecular surface area (A_m) versus mole fraction of sterol(s) (X_{st}) at discrete surface pressures for examining if the additivity rule of A_m holds or not. The negative deviation from linearity (the solid line) corresponds to non-ideal mixing leading to an enhanced interaction between DC and St molecules. Selected systems of (A) DC/Stig and (B) DC/(Stig+Ch) are shown here.

pressures: (A) DC / Ch. It is clearly seen that this classification comes from the molecular structure difference.

More the detailed examination makes us know that the results are sorted in terms of extrema most of which appear at X_{st} = 0.2 and/or = 0.8, as follows. Comparing the $\Delta G_{(ex)}$ values at π = 30 mNm^{-1}, minima are found for: (A) DC / Ch remarkable at X_{st} = 0.2, (B) DC / Chsta remarkable at X_{st} = 0.2, (D) DC / (Ch+Chsta) remarkable at X_{st} = 0.2 and = 0.8, (E) DC /(Ch+Stig) remarkable at X_{st} = 0.2, (F) DC / (Chsta+Stig). These differences shown above are considered to be reflective of interrelations of DC and St molecules and among different St species.

Shifting our view point to the $\Delta G_{(ex)}$ value at X_{st} = 0.2, if the absolute value of negative $\Delta G_{(ex)}$ is put in order, we obtain the following relation: DC / (Ch+Chsta) > DC / Chsta > DC / (Ch+Stig) > DC / (Chsta+Stig). Interestingly, DC / Stig mixed system has a slightly positive value, indicating that a certain repulsive interaction between both molecules is caused by an unfavorable matching of DC with Stig molecules. Even in the solubilization of Ch, Chsta and Stig by bile salt micelles, the solubilized amount of Stig was the lowest; this corresponds to the present finding [unpublished data]. In addition, the DC / Stig mixed system, different from the other binary systems, did produce a minimum at X_{st} = 0.6 (DC : Stig = 2 : 3).

Paying attention to DC / (Ch+Chsta) ternary system the minimum of $\Delta G_{(ex)}$ appeared at the range of X_{st} = 0.2 to 0.3. When 1 : 1 mixture of Ch and Chsta was competitively solubilized by micelles of sodium deoxycholate (NaDC), Chsta was found to be a stranger in the competition as if Chsta completely occupied the space inside the NaDC micelles by excluding Ch molecules[27]. This means that the affinity between DC and Chsta is much stronger than that between DC and Ch. The affinity difference is seen to be reflected even in frames (A) and (B).

Regarding 1 : 1 mixture of Ch and Stig as solubilizate in competitive solubilization by 125 mM NaDC micellar solution, the respective single solubilizates were solubilizated as; Ch, 8.34 mM and Stig, 4.84 mM, while the total of solubilizated amount was synergistically enhanced as: Ch, 6.58 mM, Stig,

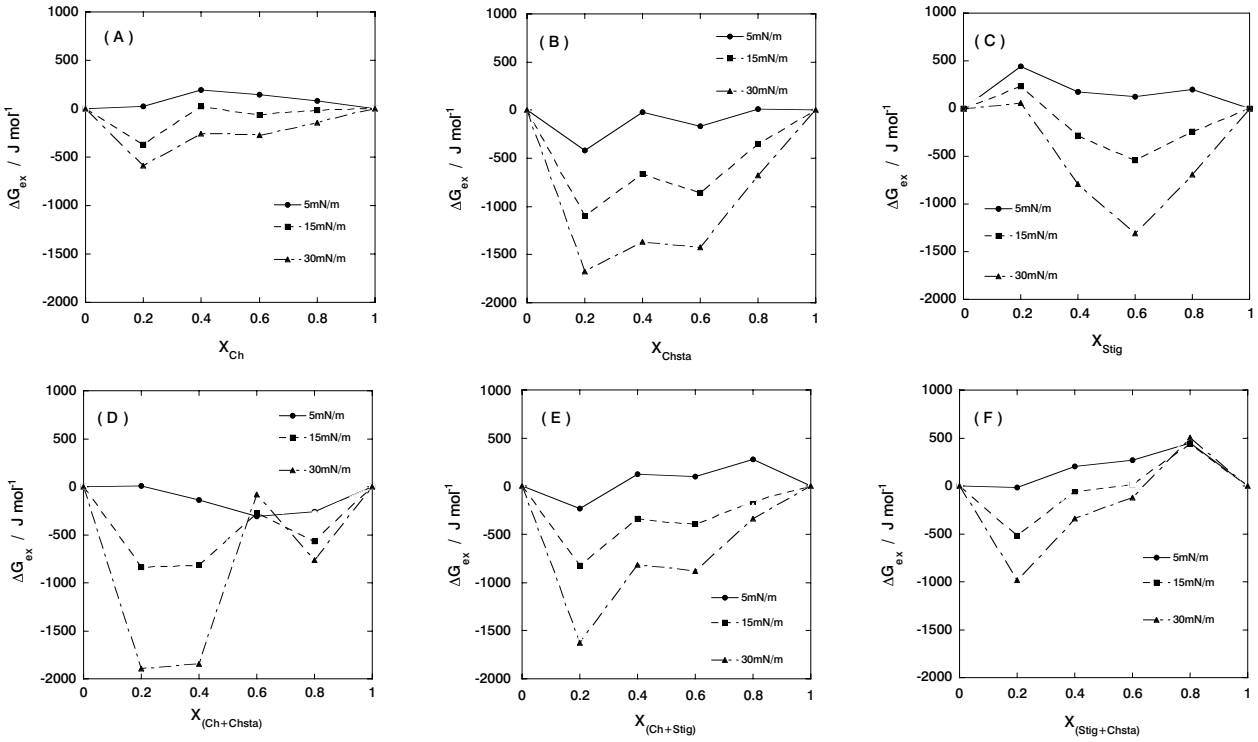

Fig. 6 The excess Gibbs energy change as a function of mole fraction of sterol(s) at discrete surface pressures, (A) DC/Ch, (B) DC/Chsta, (C) DC/Stig, (D) DC/(Ch+Chsta), (E) DC/(Ch+Stig) and (F) DC/(Stig+Chsta).

2.41 mM and total 9.0 mM. This phenomenon may be related to the results shown in (E) of Fig. 6.

Acknowledgments

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