Transport Behavior of Lipids Solubilized by Bile Salts across Porous Membranes

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ABSTRACT

In order to study how the bile salts (BSs) and lipids behave in the vicinity of microvillus, the membrane transport properties of (1) a sodium salt of deoxycholic acid (NaDC) and its mixture with monooleoylglycerol (MO) in 0.15 M NaCl saline solution at 37 ℃, (2) the mixture of NaDC with cholesterol (Ch), and in addition, (3) sodium salts of chenodeoxycholic acid (NaCDC), of ursodeoxycholic acid (NaUDC) and of cholic acid (NaC) and (4) their respective mixtures with Ch in tetraborate-carbonate buffer solution at pH 10.0 and 37 ℃.

It was demonstrated that the surfactant properties such as critical micellization concentration (CMC) and micellar size or diffusion coefficient (accordingly, hydrodynamic radius) were determinable from the flux or permeability measurements. The comparison among the respective pure systems of BSs led to a conclusion that the micellar size decreased in the order of NaDC > NaCDC > NaUDC > NaC and the CMC values determined were in agreement with those in literature. The hydrodynamic radius of MO-solubilizing micelles was estimated to be approximately 17-20 Å, and the radii of the Ch-solubilizing micelles are approximately 12 - 15 Å, and interestingly, smaller than those of the respective BS alone (single-component) micelles ranging from 14 to 22 Å.

MO molecules solubilized may probably enhance the interaction between MO and NaDC molecules by better contacting with the respective hydrophobic groups in a mixed micelle (the flexible structure of MO molecule enables it), and in this situation, the smaller micelles compared with those of pure NaDC must be more favorable. As for the mixed micelle formation of lipids and BSs or lipid solubilization by BSs, MO is considered to be solubilized by NaDC (and probably by the other BSs also) to form "solution type of mixed micelle", while Ch and other sterols such as cholestanol and stigmasterol are solubilized by BSs to form "adsorption type of mixed micelles".

1. INTRODUCTION

It is well-known that bile salts (BSs) play an important role when some lipids migrate in living tissue and are absorbed through the intestinal wall [1-4]. The BSs form a mixed micelle with phospholipids and solubilize the ingested fat, cholesterol (Ch) and fat-soluble compounds. A comprehensive understanding of transport mechanism requires some knowledge

of the physicochemical properties of lipids and BSs regarding their behavior in an aqueous environment [5-8]. Many studies to clarify the biological activities of the BSs have been presented from pharmacological and physicochemical points of view [9-14]. Nevertheless, the physicochemical mechanism in vivo has not been clarified as yet. One of the main reasons for this may be attributed to the complexity of the living system. According to electron microscopic observations, the surface of epithelial cells of the intestine was visualized and found to be covered by villus on which numerous microvillus grow thickly to enhance the absorption of the digested materials. Dimensions of 30-150 μm with 1-1.5 mm in depth for villi and of 0.01-0.1 μm with approximate 1 μm in depth for microvilli have been reported respectively [15, 16]. Furthermore, diffusion around the absorptive villi or microvilli constitutes a primary process to absorptive mechanisms. In this context, it is important to elucidate how the BSs behave in the vicinity of such microvilli [16].

In order to better understand the behavior of BSs, we have examined the transport properties of a typical free BS and its mixture with monoacylglycerol through artificial membranes. In this model system, sodium salt of deoxycholic acid (NaDC) was used as the BS and monooleoylglycerol (MO), one of the acylglycerols, was used as a lipid [17].

Continuously, to extend our understanding of the interrelation (or interaction mode) between BSs and lipids to other BS homologues and another type of lipid, a comparative study was carried out using four different BSs and cholesterol (Ch) paying attention to the interaction between BSs and Ch [18]. The artificial porous membranes, with pore sizes of 0.01 μm and 0.1 μm which correspond to the space between microvillus, were selected, because digestive absorption has been shown to take place predominantly in the vicinity of such microvilli. The results obtained from membrane transport studies [17, 18], such as the diffusion coefficients or the sizes of BS micelles, in the absence and presence of solubilized MO and Ch.

2. EXPERIMENTAL

2-1. Materials

Four bile salts (BSs) were used in this study ; sodium salts of deoxycholic acid (NaDC), of chenodeoxycholic acid (NaCDC), of ursodeoxycholic acid (NaUDC) and of cholic acid (NaC). All of these salts were obtained from Calbiochem. Co. (CA, USA) and used with no further purification. Monooleoylglycerol (MO) was kindly donated by Kao Co. (Tokyo, Japan) and was used as received. Cholesterol (Ch) was also used as received (from Sigma Co., USA). p-Aminobenzoic acid (PABA) and other inorganic salts were reagent grade materials (from Wako Pure Chem. Ind., Osaka, Japan). Triple-distilled water was used for all the solutions. In most cases, two types of porous nitrocellulose membranes (purchased from Sartorius Co.) were employed and their pore size (and thickness) were 0.01 μm (90 μm), and 0.1 μm (100 μm), respectively.

2-2. Apparatus and Measurements

The diffusion cell and its operation were the same as reported previously [19], with a schematic representation of the apparatus shown in Fig. 1.

In all experiments, the initial sample and buffer solution used as the medium with or without addition of a known amount of BS was placed in the left (Chamber L) and right (Chamber R) hand sides of the membrane, respectively (see Fig. 1). An aliquot was withdrawn from the solution in the Chamber R at different time intervals using a microsyringe (Hamilton Co.) and the increases of BSs and MO or Ch concentrations with time were monitored using of a spectrophotometer (JASCO

UVIDEC-320). The concentration of MO was determined by applying the acetylaceton method (Triglyceride-Test, Wako Pure Chem. Ind.) to each 100 μl of the sample solution [20, 21], while the Ch concentration was determined using an enzymatic assay (Determiner TC555, Kyowa Medex Co., Japan) for each 50 μl from Chamber R [22]. For each 10 μl of the sample solution taken out, the BSs' concentration change with time was measured according to the enzymatic assay (ENZABILE-2, Daiichi Pure Chem. Co., Itd., Japan) [23].

Prior to the above measurements, a diffusion measurement across the membrane was carried out using PABA as a standard diffusate [16].

In the case of PABA, the concentration change with time across the porous membrane was monitored by taking 10 μl of the sample solution out for each spectroscopic measurement (λ = 280 nm).

Preparation of mixed solutions for measurements : known amounts of (i) MO and NaDC and of (ii) Ch and BSs were dissolved (i) in 0.15 M NaCl saline solution and (ii) in tetraborate-carbonate buffer solution (0.05M $Na_2B_4O_7$ + 0.05M Na_2CO_3) at pH 10.0, respectively, in test tubes with a glass stopper, and the mixed solutions were shaken and incubated in a thermostated water bath at 37 ℃ for 24 hours. During the preparation of thses mixed solutions, air was completely removed by replacement with dry N_2 gas to avoid oxidation of MO and Ch.

3. RESULTS and DISCUSSION

3-1. Transport Behaviors of Bile Salt Systems in the Absence of Solubilizates

3-1-1. NaDC in 0.15 M NaCl Saline Solution

The diffusivity of NaDC across the porous membranes was initially examined for NaDC dissolved in saline solution at 37 ℃. Figure 2 shows typical results regarding the concentration changes in Chamber R against the lapsed time. At each starting concentration, good linearity holds over the NaDC concentration range examined. This suggests that the transport of the system is at a steady state. The flux of NaDC, J_{NaDC}, can be obtained by dividing the slope by the effective membrane area [24], and the results obtained for two

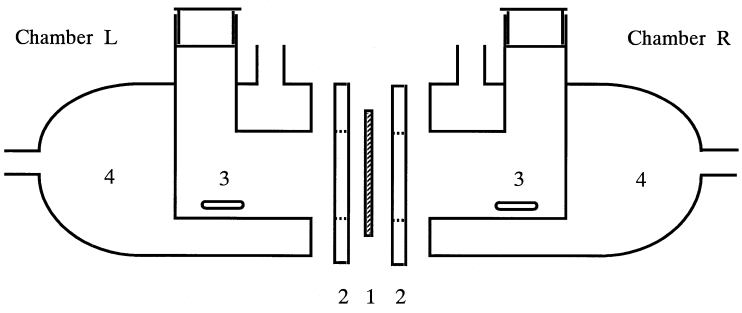

Fig. 1. Schematic representation of diffusion cell. 1, Porous membrane filter; 2, Silicon gasket; 3, Magnetic stirrer; 4, Double-jacketed glass cell. Each of compartment has the volume of 10 cm^3. The membrane is fixed between the circular face of two chambers. The effective surface area of the membrane is just π cm^2. Two silicon O rings at both sides of the membrane are supported to avoid the leak of solution. The structure of two chambers are double-jacketed to circulate the water thermostated at 37℃ and the solutions in compartments are stirred at speed of 400 rpm by magnetic stirrers. (From Ref. 18)

different membranes are plotted as a function of NaDC concentration in Fig. 3. A sharp kink point at a certain NaDC concentration is evident (Fig. 3). Irrespective of difference in pore size of the membranes, this inflection point occurs at the same NaDC concentration.

The break suggests that the flux change was caused by micelle formation of NaDC with the concentration giving the break point corresponding to the critical micelle concentration (CMC) of NaDC in agreement with literature values [6, 25].

3-1-2. Four Bile Salts in pH 10 Buffer Solution

Similarly, the diffusivity and permeability were examined for the respective BS systems, NaDC, NaCDC, NaUDC and NaC alone, in buffer solution at pH 10 and 37 ℃. Again, based upon the concentration changes of BSs in Chamber R versus the lapsed time, except for the initial stage, a satisfactory linearity was confirmed to hold over all the BS concentration range, suggesting a stationary transport for all of these systems. The flux of BS across the membrane, J_{BS}, was plotted as a function of each BS concentration, similarly to the results as shown in Fig. 3. Again, a sharp kink point was observed at a certain BS concentration for all of the BSs investigated (not shown here). The BS concentration giving the break was found to correspond to the literature CMC values of these specific bile salts [6, 25, 26].

These results indicate that either in the saline or buffer solution each value of the flux may be, straightforwardly, converted into permeability. Generally, the flux, J_M of a solute M across the membrane is described simply as a function of solute concentration, C_M,

$$J_M = P_M \Delta C_M \quad (1)$$

where P_M is a proportional constant called the permeability coefficient of M, and ΔC_M, the concentration difference of M between Chambers L and R. As seen in Fig. 3, two straight lines intersect with each other in the respective BS systems. Thus, from Eq. 1, the slopes below and above the intersection concentration (CMC) correspond to the permeability coefficients peculiar to the monomer and micellar states, respectively. In general, P_i has physical meaning in terms of the diffusion

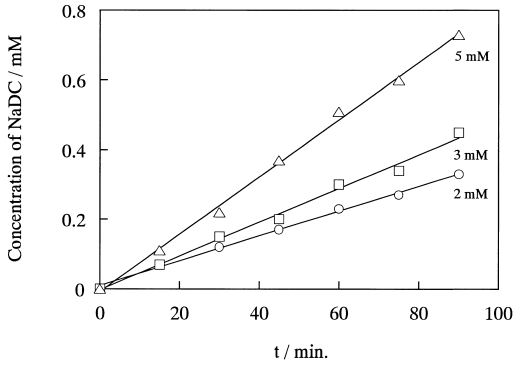

Fig. 2. Concentration change with time in Chamber R as a function of initial NaDC concentration in Chamber L. Numerical values indicate the concentrations of NaDC. Flux (J) is determined from the slope (in 0.15 M NaCl solution at 37℃). (From Ref. 17)

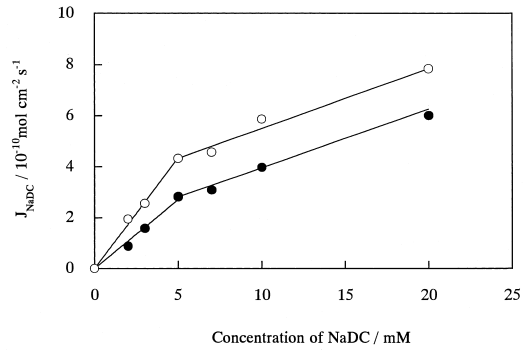

Fig. 3. Flux (J) changes of NaDC alone systems with concentration through the membranes of pore sizes 0.01 μm (●) and 0.1 μm (○) (in 0.15 M NaCl solution at 37℃). (From Ref. 17)

coefficient, tortuosity and porosity factors and membrane thickness as follows : $P_i = fD_i / h$, where D_i is the diffusion coefficient, f is the frictional coefficient of membrane and h is the membrane thickness. Moreover, $f = \varepsilon / \tau$, where the f value reflects porosity factors (τ) and tortuosity (ε) of the membrane [27]. If these factors, except for the diffusion coefficient, are assumed to be a constant characteristic of the membrane employed (the membrane coefficient), then the permeability coefficient would be attributed only to the diffusion coefficient of solute through the pores in the membrane. Note that if a molecule, such as p-aminobenzoic acid, is sufficiently smaller than pore sizes of the membrane, then the diffusion coefficient of PABA in aqueous solution can be used as a reference value for that porous membrane. Thus, the following relation may be applied to estimate of the bile salt diffusion coefficients, both below and above CMC.

$$\frac{P_{PABA}}{D_{PABA}} = \frac{P_{BS}}{P_{BS}} \qquad (2)$$

Further, this expression can even be applied at different temperatures by assuming that the temperature dependence of the membrane coefficient is negligible.

Since the P_i values are all measurable and literature value of D_{PABA} in solution at 25 ℃ is available ($D_{PABA} = 8.4 \times 10^{-6}$ cm^2 s^{-1} at 25 ℃ [16, 28]), the unknown diffusion coefficients of BS in their monomeric and micellar states can be determined. The P values of PABA were preliminarily determined at 25 ℃ (and 37 ℃) for 0.01 μm and 0.1 μm of membranes as 1.1×10^{-4} cm s^{-1} (25 ℃) and 1.2×10^{-4} cm s^{-1} (25 ℃), respectively in 0.15 M NaCl solution. According to the treatment described above, P_{NaDC} and D_{NaDC} values for two membranes different in pore size were obtained and are summarized in Table 1. As expected, the diffusion coefficients through different membranes gave different values depending on the concentration ranges above and below CMC of NaDC, but almost the same P values for the respective membranes. Even in the buffer solution, a P_{PABA} value was determined to be 1.2×10^{-4} cm s^{-1} at 25 ℃ (0.1 μm in pore size). P_{BS} and D_{BS} data are included in Table 1 and clearly indicate that monomeric BS molecules can migrate through the membrane faster than their aggregates.

Table 1. The Data of Permeabilities (P), Diffusion Coefficiants (D) and Hydrodynamic Radii (r) Estimated from the Stokes-Einstein Equation for Four Bile Salts at Monomeric (mon). and Micellar (mic.) States in Saline (0.15M NaCl) and Buffer Solution (pH = 10) at 37℃, through the Membrane of Pore Size 0.1 μm. The Data in Parenthesis, ( ), Were Obtained through the Membrance of 0.1 μm.

	in Saline Solution		in Buffer Solution							
	NaDC		NaDC		NaCDC		NaUDC		NaC	
	mon.	mic.	mon.	mic.	mon.	mic.	mon.	mic.	mon.	mic.
P / 10^{-5} cm s^{-1}	8.7 (5.4)	2.3 (2.2)	10	2.5	11	3.3	11	3.4	9.8	4.0
D / 10^{-6} cm^2 s^{-1}	6.4 (4.8)	1.7 (1.9)	6.0	1.5	6.6	2.0	6.6	2.1	5.9	2.4
r / Å	5.1 (6.8)	20 (17)	5.4	22	5.0	16	5.0	16	5.5	14

3-2. Dispersion or Solubilization of Monooleoylglycerol and Cholesterol in Bile Salts

3-2-1. Dispersion of Monooleoylglycerol / NaDC Mixtures in 0.15 M NaCl solution

First, it is important to comment on the mixing ratio of NaDC to MO in the mixtures employed for this study. Figure 4 shows the plot of transmittance of the NaDC-MO mixture in saline solution against mole fraction of NaDC, X_{NaDC} = [NaDC] / {[NaDC]+[MO]}. The solid circles are obtained for the mixed systems containing NaDC, the concentration of which ranges below 5 mM , indicating that NaDC can appreciably aid the dispersion of MO even in the concentration range below CMC. Open circles are for the mixtures of MO and NaDC whose concentration is above CMC. Obviously the NaDC-MO mixtures are divided into three categories (E, M and S). At X_{NaDC} = ca. 0.3 and ca. 0.6; the mixtures below X_{NaDC} = 0.6 were turbid while those above 0.6 were transparent. The regions partitioned by broken and solid lines in terms of transmittance are denoted by E, M, and S as shown in Fig. 4. The region E may be assigned to an emulsion and the region S, micellar solution solubilizing MO. Thus, Fig. 4 indicates that in the mole fraction region above X_{NaDC} > 0.6, MO is completely solubilized by NaDC. At X_{NaDC} = 0.6, the mixing ratio of MO vs. NaDC equals to 2 : 3, that is, at least three NaDC molecules can solubilize two MO molecules.

Thermodynamically stable mixed micelles of NaDC with MO are formed only if there exists 1.5 times more NaDC than MO in molar terms.

It is interesting to compare this MO behavior with Ch solubilization by NaDC; although NaDC as well as sodium salt of chenodeoxycholic acid (NaCDC) is the best solubilizer among the bile salts examined, 15 NaDC molecules are needed to solubilize one Ch molecule [6, 12, 21], as will be described in detail later.

The region M between about X_{NaDC} = 0.3 and 0.6 may be assigned to an intermediate state between emulsion and micellar solution.

However, this region will not be considered further, because the analysis is beyond the scope of the present work. In the present study, we are concerned only with the mixed systems within the micellar solution range.

3-2-2. Cholesterol / Bile Salt Systems in pH 10 Buffer Solution

In the plots of solubilized amount of Ch against the respective BS concentration, the amount of solubilized Ch increases linearly with an increase in the BS concentration over the wide concentration range (not shown here) [18]. The linearity may suggest that the size and shape of BS micelles solubilizing Ch are almost constant irrespective of the BS concentration. In order to analyze the results quantitatively, we have used solubilizing power (capacity) S_p, i.e., the slope (dC_{Ch}/dC_{BS}) of the curve [6, 29]. The reciprocal of

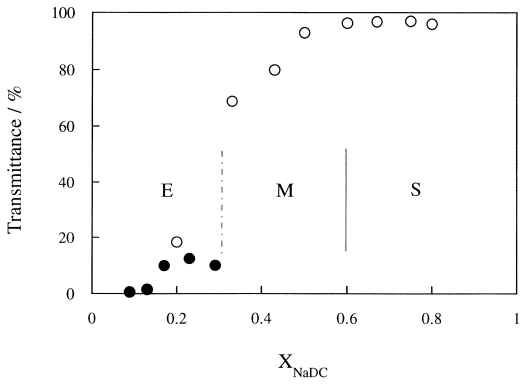

Fig. 4. The plots of transmittance of MO-NaDC mixture in 0.15 M NaCl solution against mole fraction of NaDC in MO-NaDC mixture at 37℃. NaDC concentration: (●) below 5 mM (CMC), (○) above 5 mM. E, M and S denote regions of emulsion, intermediate state and solubilizing micellar solution, respectively. (From Ref. 17)

solubilizing power (S_p^{-1}) indicates the number of BS molecules per molecule of solubilized Ch.

The results are summarized together with two kinds of hydrophobic indices (HIs) in Table 2. The first HI has been defined by Miyajima et al. on the basis of the geometrical evaluation of hydrophobic surface area [30] (called the geometrical HI [31-33]), while the other HI was introduced by Heuman on the basis of the logarithms of BS capacity factors determined using reverse-phase high performance liquid chromatography (HPLC) [34] (called the empirical HI [31-33]). With both of these HIs, the larger the value, the greater the hydrophobicity. Table 2 shows that the magnitude of solubilizing power for Ch decreases in the following order, NaDC > NaCDC > NaC > NaUDC. It should be noted that this is the same order as the empirical HI. Comparison of NaDC (3α, 12α) and NaCDC (3α, 7α), which are both dihydroxy BSs, in terms of Sp^{-1} and each HI, reveals very little differences, other than the location of hydroxyl group. On the other hand, a comparison of NaUDC (3α 7β) with NaC (3α, 7α, 12α) which is trihydroxy BS, indicates that a β-oriented hydroxyl group results in greater effective hydrophilicity than two hydroxyl groups of 7α and 12α. The correlation of Ch solubilization with hydrophobicity of each BS species has been discussed in detail elsewhere [31-33].

3-3. Transport Behavior of Monooleoylglycerol / NaDC and Cholesterol / Bile Salt Solubilization Systems

3-3-1. Monooleoylglycerol / NaDC Mixed System in 0.15 M NaCl Solution

In the case of the mixed solution of NaDC with MO, the situation is not as simple as that of the NaDC alone solution system. As seen in Fig. 4, depending on the mixing ratio, either solubilization or emulsification takes place.

Figure 5 shows the NaDC and MO fluxes across the membrane (0.01 μm) as a function of NaDC concentration at a fixed MO concentration of 10 mM. As seen in the figure, the fluxes increased linearly with an increase in NaDC concentration, but deviated from linearity at NaDC concentrations lower than 20 mM.

The NaDC concentration in the linear region is above X_{NaDC} = 0.6 (see Fig. 4). The measured points at the lowest concentration (10 mM of NaDC) are for the mixed system of X_{NaDC} = 0.5, and the deviation from the linearity is interpreted by the fact that the measured point is assigned to the intermediate

Table 2. Comparison of Solubilization Data with Hydrophobic Indices

	Sp^{-1} (Solubilizing Power)	Miyajima's HI	Heuman's HI
NaC	52	6.91	+ 0.13
NaDC	15	7.25	+ 0.72
NaCDC	20	7.27	+ 0.59
NaUDC	250 *	5.48	- 0.31

* a reference value [28]

region, M, between emulsion and solubilization, as is seen in Fig. 4. In this region, somewhat larger particles (like micelles swelled with supersaturated MO) in between emulsion and micelle may pass through the membrane pore.

In Fig. 6, the flux of MO-NaDC micellar solution in which the MO concentration is fixed at 10 mM, is plotted against NaDC concentration for the membranes of 0.01 μm (a) and 0.1 μm (b). Although the MO concentration is constant, a slight increase in amount of transported MO was observed with increase in NaDC concentration. Since for all the data shown in Fig. 6 X_{NaDC} ranged between 0.67 and 0.80, the samples examined are all micellar solutions. The slightly lower value of flux at lower NaDC concentration is attributed to the smaller concentration of micellar species because the concentration is closer to CMC and singly dispersed species of NaDC can scarcely carry the MO molecule.

Figure 7 shows the flux measured as a function of solubilizate concentration in the micellar solution systems in which the NaDC concentration was kept at 40 mM for both membranes.

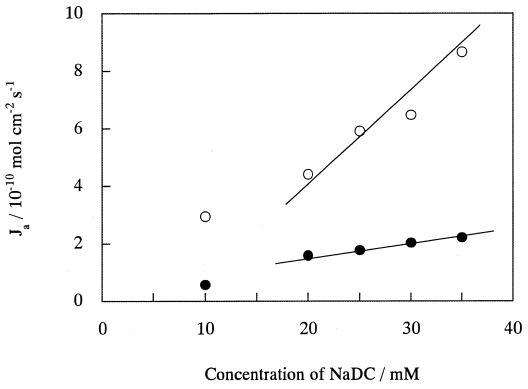

Fig. 5. The fluxes (J_a) of MO (●) and NaDC (○) as a function of concentration of NaDC in mM (in 0.15 M NaDC solution at 37℃). (From Ref. 17)

The flux (open squares) increases very slowly with an increase in MO concentration. Comparing the slopes of the flux vs. concentration curves of the micellar solutions (open squares in Figs. 6 and 7), the permeability of MO-solubilizing micelles is more effectively enhanced by increasing NaDC concentration.

Interestingly, the curve of NaDC (open circles) has a negative slope (Fig. 7). This negative slope does not mean that the permeability of NaDC is negative. This apparent decrease corresponds to the relative decrease in NaDC content in the MO-NaDC mixed system.

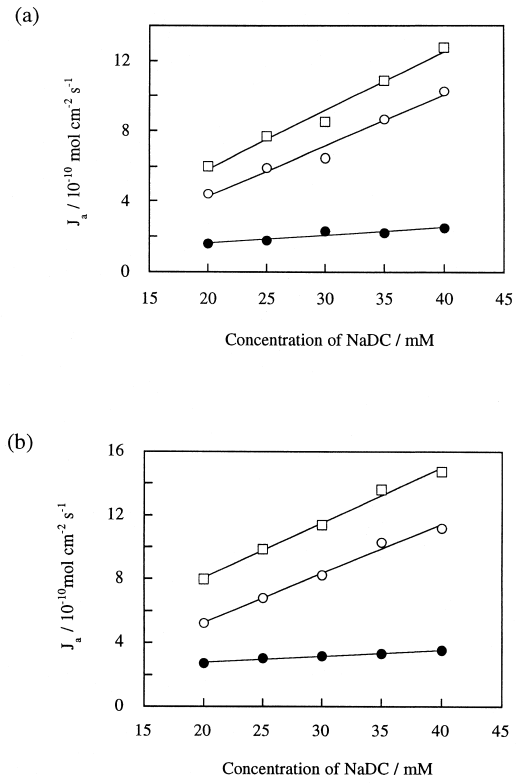

Fig. 6. The plots of fluxes (J_a) of MO (●), NaDC (○) and the total (□) against NaDC concentration for the systems in which MO concentration is fixed at 10 mM (in 0.15 M NaCl solution at 37℃). Membrane pore size: (a) 0.01 μm, (b) 0.1 μm. (From Ref. 17)

3-3-2. Cholesterol / Bile Salt Mixed Systems in Buffer Solution

The fluxes of Ch, J_{Ch} in Ch-BSs mixed micellar solution are also plotted against solubilized Ch concentration (Fig. 8). The plots of J_{Ch} vs. solubilized Ch concentration exhibit very good linearity, with a high regression (greater than 0.99 for the respective Ch-BSs systems). The linear relations shown in Fig. 7 were obtained for the MO-NaDC mixed system by keeping the NaDC concentration at 40 mM. The linearity of J_{Ch} vs. Ch concentration plot in Fig. 8 was obtained from the Ch-BSs mixtures in which not only the Ch concentration but also the BS concentration was simultaneously varied.

According to nonequilibrium thermodynamics, the phenomenological equations for the mixed miceller solution can be subject to two kinds of driving forces, so that J_{Ch} may be described by the following expression:

$$J_{Ch} = P_{11}\,\Delta C_{Ch} + P_{12}\,\Delta C_{BS} \qquad (3)$$

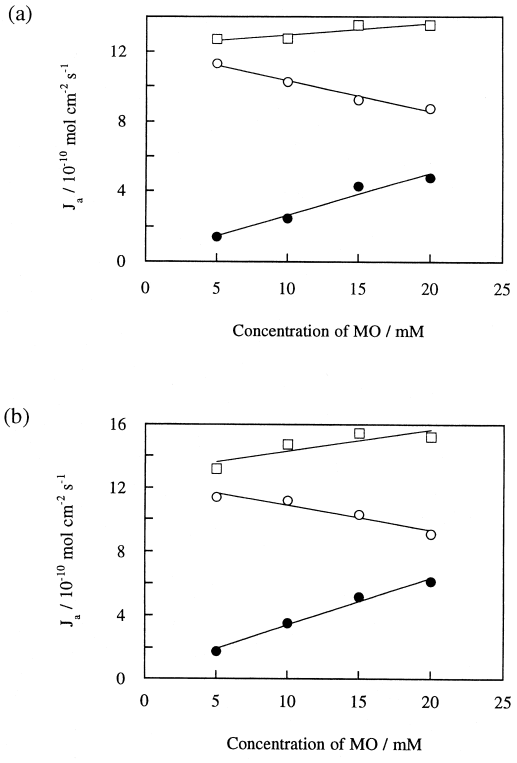

Fig. 7. The plots of fluxes (J_a) of MO (●), NaDC (○) and the total (□) against MO concentration for the systems in which NaDC concentration is fixed at 40 mM (in 0.15 M NaCl solution at 37 ℃). Membrane pore size: (a) 0.01 μm, (b) 0.1 μm. (From Ref. 17)

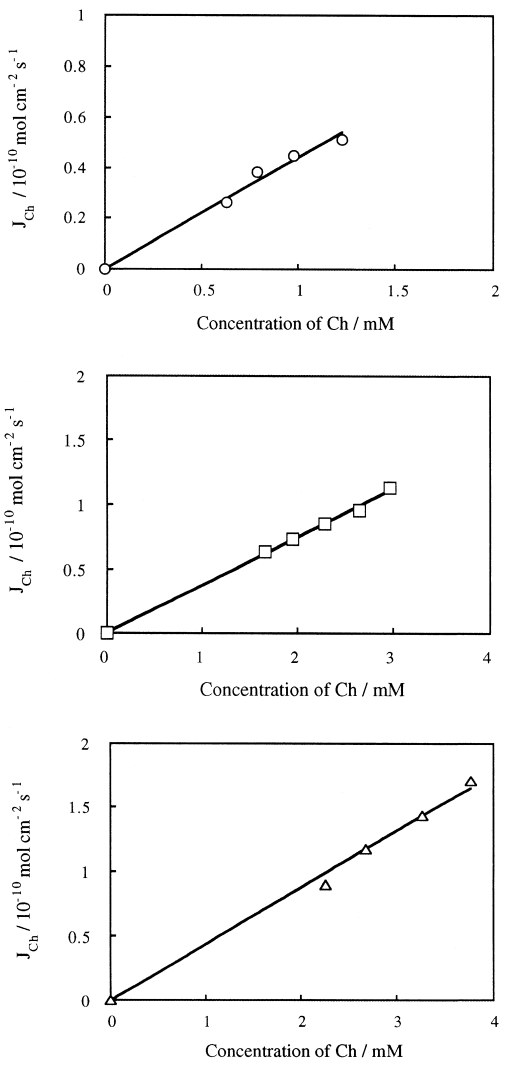

Fig. 8. The plots of fluxes (J_{Ch}) of cholesterol against concentration of solubilized cholesterol for three mixed systems with NaC (○), NaDC (□) and NaCDC (△). (From Ref. 18)

where P_{11} is the major proportional constant and P_{12} is the minor proportional constant for J_{Ch}. Provided that the mixed solution consists of only BS micelles and the mixed micelles with Ch, ΔC_{Ch} and ΔC_{BS} correspond to the respective concentration differences of the mixed micelles with Ch and of the BS micelles without Ch. Here, P_{11} is defined as the proportional coefficients between the flux and the corresponding Ch concentration difference, and P_{12} represents the physical quantities concerning the interaction between the BS alone micelle and the mixed micelle with Ch in the mixed solution. Interestingly, J_{Ch} increases linearly with an increase in Ch concentration for each BS-Ch mixed system, whereas no linear relationship was found between J_{Ch} and BS concentration (Fig. 8). This suggests that the value of P_{12} may be negligibly small compared with that of P_{11} and that the concentration of coexisting BS alone micelles contributes little to the Ch transport. The curves of J_{Ch} in Fig 8 show that J_{Ch} corresponds just to the flux of Ch-BSs mixed micelles themselves, and the linearity of J_{Ch} against Ch concentration change suggests that P_{Ch} is a constant independent of Ch concentration change.

Using the slopes from the flux vs. Ch concentration curves, the permeability coefficient of Ch-solubilizing mixed micelles was calculated (Table 3).

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Table 3. Hydorodynamic Data of the Respective Bile Salt Micelles Solubilized Cholesterol

	P / 10^{-5} cm s^{-1}	D / 10^{-6} cm^2 s^{-1}	r / Å
NaC-Ch	4.4	2.7	12
NaDC-Ch	3.7	2.2	15
NaCDC-Ch	4.4	2.7	12
NaUDC-Ch	—	—	—

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