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Solubilization of Vitamin K_1 by Bile Salts and Phosphatidylcholine-Bile Salts Mixed Micelles

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Abstract—The solubilization of vitamin K_1 by bile salts (sodium deoxycholate, sodium cholate and their corresponding glycine conjugates) and phosphatidylcholine (egg)-bile salt mixed micelles has been investigated. The solubilization curves were not always linear with increasing bile salts, but the vitamin was appreciably solubilized in the region below their CMCs. In the bile salt solutions (20 mM, phosphate buffered saline, pH 7·5, ions strength 0·2), the solubilized vitamin ranged from 0·3 to 0·9 mM. With increasing phosphatidylcholine, the amount of vitamin solubilized was dramatically increased; at the molar ratio of 1 : 1 (both 20 mM), the amount of vitamin solubilized was about 25–30 times more than by the corresponding bile salts alone. There is a possibility that exogeneous phospholipid given orally as liposomal forms assists the solubilization of vitamin K_1, in the intestine. This characteristic is suggested as being responsible, in part, for the enhanced recovery of blood coagulation after oral administration of liposomal vitamin K_1 to warfarin-treated rabbits.

Gall bladder bile contains relatively high concentrations of bile salts (Scientific Tables 1970; Coleman et al 1979) which form mixed micelles with phosphatidylcholines in the normal physiological condition, thereby contributing to solubilization of cholesterol and fat in the intestine (Carey & Small 1978; Barrowman 1984).

We reported earlier (Nagata et al 1984) that liposomally associated vitamin K_1, administered orally, enhanced the recovery of blood coagulation in rabbits with warfarin-induced hypoprothrombinaemia. However, when administered orally, liposomes encounter changes of pH, digestive enzymes and bile salts, which affect the physical integrity of the vesicles (Lichtenberg et al 1979; Rowland & Woodley 1980; Yotsuyanagi et al 1983) and as a result their drug carrier functions. This could apply to the fate of vitamin K_1 so we therefore examined its solubilization by different kinds of bile salts and by phospholipid-bile salt mixed micelles with the aim of achieving effective absorption of the liposomal vitamin in the intestinal tract. The solubilization study was conducted using egg phosphatidylcholine (PC) and several free and conjugated bile salts as model bile constituents.

Materials and Methods

Materials

Vitamin K_1 (purest grade product in 1 g ampoule Lot No. DCK4780, Wako Pure Chem. Ind., Osaka) was used as received. Sodium cholate (SC), sodium deoxycholate (SDOC), sodium taurodeoxycholate (STDOC), sodium glycocholate (SGC) and sodium glycodeoxycholate (SGDOC), (Sigma, St Louis, MO) were used without further purification. Phosphatidylcholine (PC) was extracted from egg yolk and purified by column chromatography on silicic acid (Mallinkrodt, St Louis, MO) (Brandt & Lands 1967). All other chemicals used were of reagent grade.

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Measurement of vitamin K_1 solubilized by bile salts or mixed micelles

Vitamin K_1 and SDOC were dissolved in ethanol and the other bile salts in methanol as stock solutions while PC was in chloroform. The mixtures of each stock solution with different molar ratios of the components were evaporated to dryness under reduced pressure for 3 h and the residue was dispersed in 5 mL of distilled water or in 35 mM phosphate buffer (pH 7·5, ionic strength μ 0·2 with NaCl PBS) by vortexing (15 s, twice) and sonication (1 min, bath-type, Model B-12, Branson) at room temperature (25 °C). The resulting dispersed system was placed in a water-bath thermostatted at 25 °C and stood overnight for equilibrium. The amount of vitamin K_1 to be introduced in the solubilizing system was determined by preliminary tests so that the excess would be as small as possible above that solubilized by each surfactant-specified system.

To remove unsolubilized vitamin K_1 which exists as oil droplets, 1 mL of the emulsion was filtered through a membrane filter (pore size 0·1 μm, cellulose nitrate, Toyo Roshi Co. Ltd, Tokyo). The vitamin content in the filtrate was assayed at 330 nm after appropriate dilution with isopropanol–water (1 : 1, v/v) using a Shimadzu UV-260 spectrophotometer equipped with a constant temperature cell holder (25 °C). The PC content was assayed as phosphorus (Mrsny et al 1986). No vitamin was detected in the filtrate even immediately after dispersal by sonication.

Measurement of PC solubilized by bile salts

The mixture of PC and bile salt stock solutions was evaporated to dryness (Lichtenberg et al 1979). After the addition of 5 mL of water or phosphate buffer, the lipid mixture was fully hydrated until a uniform solution or suspension was obtained on a vortex mixer and by brief sonication. It was subsequently incubated at 25 °C overnight for equilibrium. The turbidity was measured at 660 nm and 800 nm using a 1 cm quartz cell.

Results and Discussion

Solubilization of vitamin K_1 by bile salts

Bile salts play an important role in the intestinal absorption of fat where fat droplets are emulsified for enzyme attack (Borgström et al 1979). Vitamin K_1 is fat-soluble and forms oil-droplets in water and may be similarly emulsified in the intestine, but the mechanism of its absorption differs from that of ordinary fats in that it is lipolysed before absorption. Bile salts are involved in the absorption of fat-soluble vitamins such as vitamin D (Schachter et al 1964) and α-tocopherol (MacMahon & Thompson 1970) and it is generally considered that these vitamins' absorption is due to the result of solubilization by bile salts or their mixed micelles with phosphatidylcholine. This is also probably applicable to the solubilization for vitamin K_1.

Fig. 1 shows the concentration changes of vitamin K_1 solubilized by SDOC as a function of the added vitamin K_1 where the total vitamin in the system is expressed in mM and the amount of bile salt is fixed. The turbidity changes of the dispersed system are also given. Our study was conducted to see whether the bile salt molecules were used in the emulsification of excess vitamin existing as droplets because the vitamin was likely to exist both in the micellar-solubilized and emulsified state. If a bile salt is appreciably transferred to the droplets, the amount of micellar-solubilized vitamin will decrease as the total vitamin in the system increases since the amount of the bile salt is kept constant. However, the concentration of vitamin K_1 solubilized reached a plateau, even when the SDOC concentration was 2 mM, indicating that the bile salt transferred to the excess vitamin droplets was negligible in the range of the total vitamin concentration examined. The value at the plateau could therefore be regarded as the solubility of the vitamin in a bile salt at a specific concentration.

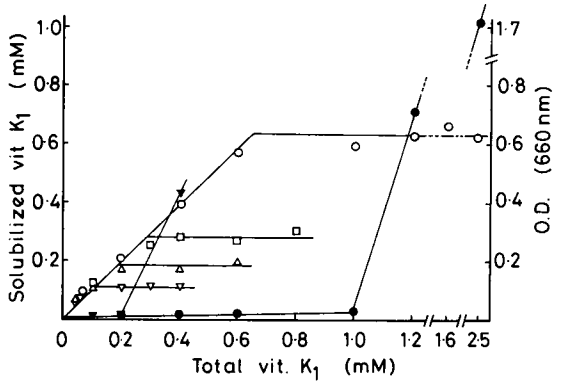

FIG. 1. Solubilization of vitamin K_1 and turbidity change as a function of the total vitamin in SDOC, pH 7·5, μ 0·2 and 25 °C. SDOC (mM): ▽, 2; △, 4; □, 10; ○, 20. Turbidity change was expressed by the corresponding closed symbols to the above. The total vitamin in the system is expressed in mM, regardless of solubilized or droplets.

The turbidity changes showed a sharp inflection point at about 1·0 mM on the vitamin axis (SDOC 20 mM), and at 0·2 mM (SDOC 2 mM), beyond which the excess (unsolubilized) vitamin was detected as droplets. These values were 1·5–2 times larger than those obtained by the filtration method, probably because the turbidity changes reflect droplets with sizes comparable with, or greater than, the wavelength.

Figs 2 and 3 show the solubilization curves for vitamin K_1 in bile salts at various concentrations up to 20 mM where the bile salts were dissolved in water or in buffer (pH 7·5, μ 0·2). The solubilization curves were not always linear with increase in bile salts, but the solubilizing abilities of any bile salts, regardless of whether they were in free or conjugated form, and dihdyroxy or trihydroxy species, were generally higher in water than those in buffer. The salting-out effects and/or the magnitude of dissociation of the bile acids are likely to be responsible for such difference. The glycine conjugates, SGDOC or SGC, generally solubilized the vitamin to a lesser extent than their corresponding free bile salts, but the difference was not large.

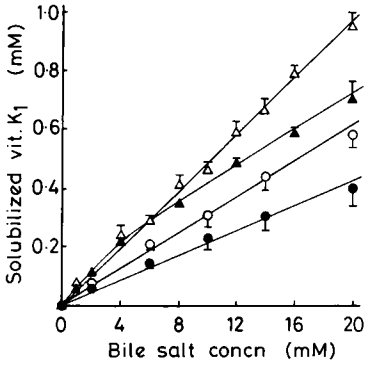

FIG. 2. Solubilization of vitamin K_1 in free bile salts at 25 °C. SDOC, △ (water) and ▲ (buffer, pH 7·5, μ 0·2); SC, ○ (water) and ● (buffer). Bars indicate s.d. (n = 4–6).

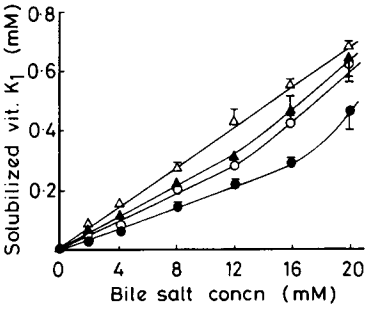

FIG. 3. Solubilization of vitamin K_1 in glycine conjugated bile salts at 25 °C. SGDOC, △ (water) and ▲ (buffer, pH 7·5, μ 0·2); SGC, ○ (water) and ● (buffer). Bars indicate s.d. (n = 4–6).

The CMCs of SDOC and SC were 4–6 and 13–15 mM in water, respectively, and 2–4 and 3–8 mM in electrolyte solution (NaCl 0·15 M) (Yamakawa 1979). Vochten & Joos (1970) examined the effect of pH on the CMCs of SDOC and SC, and found values of 0·1 and 2·7 mM (pH 7·0, NaCl 0·1 M), respectively, and 1 and 3·3 mM (pH 9·0, NaCl 0·1 M). The conjugated salts generally had more polarity, i.e. lower pK_a, giving wider or increasing CMCs over and above those of the corresponding free bile salts (Carey & Small 1972). However, no characteristic change was observed in their corresponding CMC regions of the solubilization curves with SDOC (water), SC (both in

water and in buffer) and SGDOC (water). Other bile salts showed upward curves with increasing bile salt concentration and the extrapolation to the bile salt axis from the upward portion may correspond to their CMC regions. These results suggest that the solubilization of the vitamin by the bile salts is not due to a mechanism according to the simple monomer-micellar model which is often applicable to ordinary surfactant solutions (Type A surfactant according to Helenius & Simons's classification 1975). Thus, vitamin K_1 is appreciably solubilized below the CMCs.

As shown in Table 1, the molar ratios in the solubilization were estimated, and ranged from 0·018 to 0·053 (mol of vitamin K_1 solubilized/mol of bile salt), i.e. approximately 56(SGC)–19(SDOC) moles of bile salt were required to solubilize 1 mole of the vitamin.

Table 1. The solubilization ability of bile salts for vitamin K_1.

Bile salt	Molar ratio[a] (mol of vitamin K_1/mol of bile salt) Water	Buffer
SDOC	0·047	0·053 (0–4) 0·029 (4–20)
SGDOC	0·031	0·025 (0–12) 0·041 (12–20)
SC	0·029	0·021
SGC	0·023 (0–12) 0·041 (12–20)	0·018 (0–16)

[a] Calculated from the slope of the solubilization curve.
Numbers in parentheses indicate the concentration range of bile salts (mM).
Buffer: pH 7·5, μ 0·2 (25 °C).

It is also evident that the solubilizing ability of the dihydroxy bile salts is greater than that of the trihydroxy bile salts. This may be due to the difference of the molecular surface available for the hydrophobic interaction between the vitamin and bile salt.

Solubilization of PC by bile salts

Bile salts form mixed micelles with PC and it is necessary to know how much PC is solubilized. Structural aspects of the mixed micelles have been extensively studied and different types of complex PC-bile salt arrangements have been proposed as a function of the molar ratio (Claffey & Holzbach 1981; Müller 1984; Schurtenberger & Lindman 1985). With further increase in PC, there is a boundary state (or molar ratio) where bile salt micelles are saturated by PC molecules and lamellar aggregates form in solution. This ratio would be also an important parameter related to the disintegration kinetics of liposomes by bile salts.

Figs 4 and 5 indicate the turbidity changes of liposomal suspensions as a function of the PC concentration in SDOC (in water and in pH 7·5 PBS, μ 0·2, respectively). Zero turbidity assumes the vesicles to be completely solubilized, especially in water, and the turbidity increases abruptly where excess lipid forms vesicles. The turbidity was measured at 660 and 800 nm and little difference in the inflection point was found.

There was, however, a clear difference in the turbidity change with increasing PC. In water almost zero turbidity

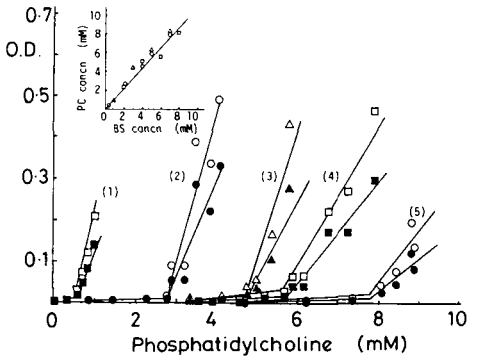

FIG. 4. Turbidity change of egg phosphatidylcholine (PC) dispersed in water with various SDOC concentrations at 25 °C. SDOC (mM): (1), 0·52; (2), 2·1; (3), 4·0; (4), 5·0; (5), 7·0. Open symbols, absorbance at 660 nm; closed symbols, absorbance at 800 nm. Inset: the relationship between PC concentration at the inflection point and SDOC concentration. ○, SDOC; △, SGDOC; □, STDOC.

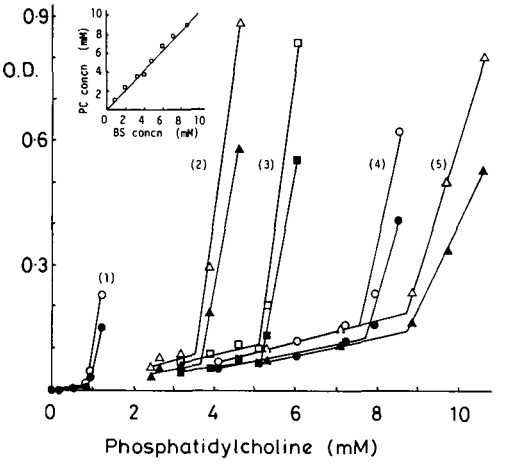

FIG. 5. Turbidity change of egg phosphatidylcholine (PC) dispersed in buffer (pH 7·5, μ 0·2) with various SDOC concentrations at 25 °C. SDOC (mM): (1), 0·85; (2), 2·3; (3), 4·8; (4), 7·2; (5), 8·8. Open symbols, absorbance at 660 nm; closed symbols, absorbance at 800 nm. Inset: the relationship between PC concentration at the inflection point and SDOC concentration. ○, SDOC; □, STDOC.

remained up to the inflection point, while in the buffer solution the turbidity gradually increased, but gave a clear inflection point. The results indicate that the growth of mixed micelles or secondary aggregation of the mixed micelles is caused by coexisting electrolytes. Because each component bears its own specific aggregation property to which a mixture would add further complexity, it is difficult to explain the increased turbidity but it is conceivable that sodium ions adsorbed reduce the negative charge on the surface of the mixed micelles thereby favouring their aggregation of forming larger mixed disc micelles.

Figs 4 and 5 (insets) show the concentrations of PC at the inflection point plotted against various bile salt concentrations. The slope represents the molar ratio between PC and the bile salt in its maximally solubilized system. The molar ratio (PC/SDOC) calculated was equal to about 1 irrespective of the different aqueous environments. The same tendency for the turbidity to change was also seen for the STDOC and SGDOC solubilized systems (data not shown), giving the same slope, i.e. molar ratio as that of SDOC ($\simeq$1).

As PC becomes incorporated into initially globular bile salt micelles, a difference in size and shape of mixed micelles is likely to occur between those formed in water and those in buffer (Figs 4, 5); however, the experimental data give a molar ratio of 1 both in water and in buffer suggesting that the mixed micelle formation occurs in the same molecular packing mode for PC and bile salt. Another important aspect is that the molar ratio of 1 was common to SDOC, STDOC and SGDOC which are all dihydroxy bile salts. However, the molar ratios (PC/bile salt) of the trihydroxy bile salts, SC and STC, were 1·8 and 2, respectively (Yotsuyanagi et al 1983; Schurtenberger & Lindman 1985). That the solubilizing ability of trihydroxy bile salts is greater than that of dihydroxy bile salts would be expected but it is of interest that this is contrary to the solubilizing ability of the bile salts for vitamin K_1.

Solubilization of vitamin K_1 by PC-bile salt mixed micelles

Fig. 6 shows the effect of PC incorporated into the mixed micelles on the solubilization of vitamin K_1. The mixed micelles were formed with SDOC, SC and their corresponding glycine conjugates where the bile salts were always maintained at 20 mM.

It was observed that vitamin K_1 was linearly solubilized with increasing incorporation of PC and there was little difference in the solubilizing ability among these mixed micelles. At the molar ratio of 1:1 (PC/bile salt), the amount of vitamin solubilized was about 25–30 times tha solubilized by bile salts alone.

Coleman et al (1979) reported that the contents of phospholipid and bile salt in gall bladder biles are considerably different among various mammalian species and the molar ratio of phospholipid/bile salt ranges from 0·004 (guinea-pig), 0·013 (rabbit) to 0·2 (man). The content of phospholipid in biles is generally much smaller than that of bile salt on a molar basis under normal physiological conditions. Although biles must be diluted to some extent with intestinal water after flowing into the intestine, the molar ratio of phospholipid and bile salt will remain almost unchanged.

Assuming that the solubilization curves for vitamin K_1 as a function of PC are applicable to the phospholipid-bile salt mixed micelle system in rabbit and in human biles, the magnitudes of solubilized vitamin K_1 have been compared at 20 mM of the bile salts. Because of the molar ratio of 0·013 in rabbit biles, the content of phospholipid is low and its contribution almost negligible. In human biles with a molar ratio of 0·2, the vitamin is solubilized about 7 times more than by bile salt alone (see Fig. 6). If vitamin K_1 is orally administered in liposomal form, a large amount of phospholipid will be also supplied to the autologous mixed micelle system in the intestine, where such exogenous phospholipid possibly enhances solubilization of the vitamin. Especially, where the phospholipid-bile salt ratio is as small as in rabbit biles, exogenous phospholipid may increase the solubilization of vitamin K_1, resulting in more effective transport across the unstirred water layer to the enterocyte. It is therefore conceivable that this particular characteristic of the PC-bile salt system is responsible, in part, for the enhanced recovery of blood coagulation by oral administration of liposomal vitamin K_1 (Nagata et al 1984). Simultaneously, kinetics of the liposome-bile salt or mixed micelles interaction will be also an important factor before the magnitude of solubilization of the vitamin.

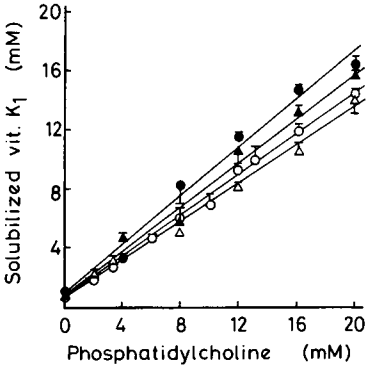

FIG. 6. The effect of egg phosphatidylcholine (PC) incorporated in the mixed micelles on the solubilization of vitamin K_1 pH 7·5 μ 0·2 and at 25 °C. Bile salts (20 mM throughout): △, SDOC; ○, SC; ▲, SGDOC; ●, SGC. Bars indicate s.d. (n = 6).

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