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MECHANISM FOR THE INDUCEMENT OF THE INTESTINAL ABSORPTION OF POORLY ABSORBED DRUGS BY MIXED MICELLES II. EFFECT OF THE INCORPORATION OF VARIOUS LIPIDS ON THE PERMEABILITY OF LIPOSOMAL MEMBRANES

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SUMMARY

The mechanism for the enhancement of the intestinal absorption of drugs in the presence of mixed micelles was investigated using liposomal membranes as a biomembrane model. The effect of the incorporation of various lipids on the permeability of drugs through liposomal membranes was studied. Liposomes were prepared from egg phosphatidylcholine. Lipids used were fatty acids, glycerides, oleyl alcohol and methyl oleate, and drugs were phenol red, bromphenol blue, cefazolin, sulfanilic acid and procainamide ethobromide (PAEB). The absorption of these drugs in the large intestine was enhanced by the addition of monoolein–bile salt mixed micelles. The incorporation of monoolein into liposomal membranes markedly increased the release rate of these drugs through the membranes. Unsaturated fatty acids such as oleic acid and linoleic acid markedly enhanced the release rate of PAEB, while saturated fatty acids caused a small increase in the release rate. Diolein, triolein, oleyl alcohol, methyl oleate, oleic acid and linoleic acid had no enhancing effect on the release of phenol red. The effect of the treatment of liposomes with various solutions on the permeability of drugs through the membranes was investigated. Treatment solutions were a lipid–bile salt mixed micellar solution, a lipid-emulsion and a bile salt micellar solution. The treatment with a mixed micellar solution increased the release and the uptake of drugs. Temperature-dependent studies demonstrated that the incorporation of monoolein caused a decrease in the activation energy for the permeation process of phenol red through liposomal membranes from 17.5 to 14.3 kcal/mol.

INTRODUCTION

We have reported that the intestinal absorption of poorly absorbed drugs such as aminoglycosides (Muranishi et al., 1979) and heparin (Tokunaga et al., 1978) was greatly

enhanced in the presence of monoolein– or oleic acid–bile salt mixed micelles. The enhancing effect of unsaturated fatty acid mixed micelles was larger than that of saturated fatty acid mixed micelles and it was suggested that the enhanced absorption of drugs by the addition of mixed micelles was mostly due to the alteration of the mucosal membrane permeability caused by the incorporation of the lipid component of mixed micelles (the former report).

Liposomes have been extensively investigated as biomembrane models. Membrane permeability properties (Blok et al., 1977; Naoi et al., 1977), the alteration of membrane permeability (Inoue and Kitagawa, 1976; Fukuzawa et al., 1979) and the fragility of membranes (Richards and Gardner, 1978; Sunamoto et al., 1978) were studied using liposomal membranes. The transport of drugs across black lipid membranes (Inui et al., 197 ; Hori et al., 1978) or liposomal membranes (Yasuhara et al., 1977) was investigated in order to elucidate the mechanism for the intestinal absorption of drugs.

In the present study, phenol red, bromphenol blue, cefazolin, sulfanilic acid and procainamide ethobromide were chosen as models of poorly absorbed drugs, and the effect of the incorporation of various lipids on the permeability of liposomal membranes was studied in relation to the mechanism for the enhancement of the intestinal absorption of drugs in the presence of mixed micelles.

MATERIALS AND METHODS

Materials. Egg phosphatidylcholine was prepared according to the method of Rhodes and Lea (1957). Egg phosphatidylcholine was purified from egg yolk by acetone precipitation and subsequent chromatography over alumina oxide and silica gel. The purity of egg lecithin was checked by thin-layer chromatography on silica gel plate with chloroform–methanol–water (65 : 25 : 4) as solvent. Synthetic dioleoyl phosphatidylcholine was obtained from Sigma Chemicals. Monoolein of high purity grade (Nikko Chemicals) was used. Procainamide ethobromide was provided by Squibb Institute for Medical Research. Sodium cefazolin was a gift from Fujisawa Pharmaceutical Industry, Osaka, Japan. Sodium taurocholate was synthesized according to the method of Norman (1955). All other chemicals were of analytical grade and were obtained from Nakarai Chemicals.

Preparation of liposomes and measurement of permeability. Chloroform solution in which egg phosphatidylcholine (80 μmol) and cholesterol (20 μmol) with or without other lipids were dissolved was transferred to a round-bottomed flask. The chloroform was removed on a rotatory evaporator to form a thin lipid film on the wall of the flask. After 2 h evaporation, 5 ml of pH 6.5 isotonic phosphate buffer solution (NaH_2PO_4–Na_2HPO_4) containing the drug (20 mM) was added to the flask and the atmosphere was replaced with N_2, vigorously mixing on a vortex mixer for 10 min. The milky suspension was subjected to ultrasonic radiation for 30 min at 0°C using an Ohtake 5202 sonicator. After sonication an almost optically clear suspension of microvesicles was obtained and allowed to stand for 30 min at room temperature to anneal damage and imperfections in the newly formed lipid bilayer membranes. The liposomes were separated from the non-entrapped drug by column chromatography at room temperature on Sephadex G-25 (12 X 2 cm) using pH 6.5 isotonic phosphate buffer solution. The liposomes were eluted in the void volume and were made up to a final vol. of 7.5 ml. Immediately, the drug-

entrapped liposome fraction was placed in a water bath at 37°C. After 5, 15, 30 and 60 min, sampled solution was again subjected to gel filtration to separate the liposome-entrapped drug from the released drug and the drugs in both fractions were determined. Release rate constant K was obtained from an equation, $\ln A/A_0 = -Kt$, where A_0 and A are the amounts of the entrapped drug at time 0 and t respectively, and t is the time of diffusion. Release rate constant was calculated from the value of A/A_0 above 0.5.

Treatment of liposomes. The effect of the treatment of liposomes with various solutions on the release of drugs from liposomes was studied in the following manner. Treatment solution (0.25 ml) was added to 5 ml of pH 6.5 buffer solution with vigorous mixing at 37°C. Immediately, 3 ml of the drug-entrapped liposome fraction which was prepared as described above, was added with vigorous mixing. Mixed micellar solution and emulsion which were the treatment solutions, were prepared by sonicating the solution for 3 min at 37°C. The release rate constant was calculated as described above.

The effect of the treatment of liposomes with various solutions on the uptake of drugs into liposomes was studied in the following way: treatment solution (0.25 ml) was added to 5 ml of pH 6.5 isotonic drug solution (20 mM) with vigorous mixing at 37°C. Immediately, 3 ml of the no-drug-entrapped liposome fraction was added with mixing. After a definite time, the sampled solution was subjected to gel filtration and the liposome-entrapped drug was determined.

In situ absorption experiment. Male Wistar albino rats weighing 200–250 g were used. The procedure of absorption experiment in the large intestine was the same as those reported before (Muranishi et al., 1979; the former report). Mixed micellar solutions were composed of 10 mM lipid and 10 mM sodium taurocholate (NaTC). The concentration of drugs in the test solution was 1 mM except for cefazolin. Administered dose of cefazolin was 1.5 mg/200 g rat.

Analytical methods. Phenol red: after the addition of 20% Triton X-100 to 4 ml of the sample solution to dissolve the lipid, the solution was alkalinized with N NaOH and concentration determined spectrophotometrically at 560 nm. Bromphenol blue: after the addition of Trixon X-100, the drug concentration was determined at 603 nm. Cefazolin: lipids in the sample solution were extracted with chloroform by rocking at 14 cpm for 90 min. After centrifugation, the supernatant was determined at 271 nm. In the absorption experiment, the antimicrobial activity of cefazolin was determined by the paper disc method using *Bacillus subtilis* PCI 219 as the test organism. Procainamide ethobromide and sulfanilic acid: after the addition of Triton X-100, the drugs were diazotized, coupled with 2-diethylamino-ethyl-1-naphthylamine and concentration determined at 550 nm. In the absorption experiment, the colored material was extracted with isoamyl alcohol by the addition of sodium chloride and concentration determined at 560 nm.

RESULTS

Permeability of liposomal membranes incorporated with various lipids

In the previous papers (Muranishi et al., 1979; the former report), aminoglycosides were chosen as poorly absorbed drugs. In this investigation, phenol red, bromphenol blue, cefazolin, sulfanilic acid and procainamide ethobromide (PAEB) were used as impermeable drugs to liposomal membranes because their determinations are very facile

TABLE 1

RELEASE RATE CONSTANT, K, FOR THE RELEASE OF PHENOL RED THROUGH LIPOSOMAL MEMBRANES, CONTAINING VARYING AMOUNTS OF MONOOLEIN

Liposomes were prepared from egg phosphatidylcholine (80 μmol) and cholesterol (20 μmol)

Monoolein (μmol)	K (10^{-3} min^{-1}) ± S.D. [a]
0	2.06 ± 0.40
1	4.60 ± 0.23
2.5	6.19 ± 0.34
5	8.42 ± 0.39
10	13.62 ± 0.91
20	22.61 ± 0.71

[a] Figures represent the mean ± S.D. of 3 experiments.

and sensitive. Table 1 shows the release rate constant of phenol red through liposomal membranes containing varying amounts of monoolein. The incorporation of monoolein markedly enhanced the release rate of phenol red. This enhancement may not be due to the imperfections of liposomes since monoolein- (10 μmol) incorporated liposomes which were allowed to stand for 20 h at 4°C showed a similar value for release rate constant, 13.10 ± 0.66 (10^{-3} min^{-1}). The release rate constant was almost proportional to the amount of monoolein. Monoolein (10 μmol) caused a considerable increase in the release rate, and subsequent experiments were performed by the incorporation of 10 μmol lipid.

Table 2 shows the effect of monoolein incorporation on the release of various impermeable drugs. The release rate of all drugs was enhanced by the incorporation of monoolein. The absorption of these drugs in the large intestine was enhanced in the presence of the monoolein–bile salt mixed micelles as shown in Table 3. Phenol red and PAEB for which the increase in release rate was noticeable are chosen as representative models of anionic and cationic drug, respectively, in subsequent experiments.

TABLE 2

EFFECT OF THE INCORPORATION OF MONOOLEIN (10 μmol) INTO LIPOSOMAL MEMBRANES ON THE RELEASE OF VARIOUS DRUGS

Drug	K (10^{-3} min^{-1}) ± S.D.	
	None	Monoolein
Phenol red	2.06 ± 0.40	13.6 ± 0.9
Bromphenol blue	5.96 ± 0.42	16.2 ± 2.4
Sulfanilic acid	0.00 ± 0.00	2.26 ± 0.11
Cefazolin	0.00 ± 0.00	2.82 ± 0.64
Procainamide ethobromide	0.00 ± 0.00	13.1 ± 0.3

TABLE 3

EFFECT OF MIXED MICELLES ON THE DISAPPEARANCE OF DRUGS FROM THE LARGE INTESTINAL LOOP FOR 15 MIN

Figures in parentheses refer to the number of animals.

Drug	% Disappeared ± S.D.		
	None	NaTC + monoolein	NaTC + oleic acid
Phenol red	2.8 ± 1.1 (5)	18.4 ± 3.4 (4)	35.5 ± 2.4 (5)
Bromphenol blue	3.1 ± 1.4 (4)	16.1 ± 4.3 (4)	–
Sulfanilic acid	4.6 ± 1.0 (3)	22.0 ± 3.9 (3)	–
Cefazolin	5.4 ± 3.4 (4)	20.7 ± 7.4 (4)	–
PAEB	3.4 ± 1.1 (5)	17.1 ± 3.8 (4)	29.1 ± 6.0 (4)

The effect of the incorporation of various fatty acids on the release of PAEB are shown in Table 4. Unsaturated fatty acids markedly enhanced the release rate of PAEB, while saturated fatty acids caused a small increase in the release rate. The lower the melting point of the fatty acid is, the more the permeability of PAEB increased. This result gives a close correlation with the results of the absorption experiments (the former report), that is, the degree of the increase in the permeability of liposomal membranes by the incorporation of various fatty acids corresponds to the extent of the enhancement of the intestinal absorption in the presence of fatty acid mixed micelles. The intestinal absorption of PAEB was also enhanced by oleic acid–bile salt mixed micelles (Table 3).

Table 5 shows the effect of the incorporation of various lipids on the release of phenol red from liposomes. Methyl oleate, oleyl alcohol, diolein and triolein did not produce an

TABLE 4

EFFECT OF THE INCORPORATION OF VARIOUS FATTY ACIDS (10 μmol) ON THE RELEASE OF PROCAINAMIDE ETHOBROMIDE

Fatty acid	$C_{m:n}$ [a]	K (10^{-3} min^{-1}) ± S.D.
None		0.00 ± 0.00
Caprylic acid	$C_{8:0}$	0.27 ± 0.05
Lauric acid	$C_{12:0}$	7.83 ± 0.92
Myristic acid	$C_{14:0}$	5.38 ± 0.91
Palmitic acid	$C_{16:0}$	3.78 ± 0.97
Stearic acid	$C_{18:0}$	3.66 ± 0.21
Palmitoleic acid	$C_{16:1}$	9.19 ± 0.79
Oleic acid	$C_{18:1}$	11.7 ± 1.5
Linoleic acid	$C_{18:2}$	13.0 ± 1.5
Linolenic acid	$C_{18:3}$	15.5 ± 1.1

[a] m = number of carbon; n = number of double bond.

TABLE 5

EFFECT OF THE INCORPORATION OF VARIOUS LIPIDS ON THE RELEASE OF PHENOL RED

Lipid (μmol)	K (10^{-3} min^{-1}) ± S.D.
None	2.06 ± 0.40
Monoolein (10)	13.62 ± 0.91
Diolein (5)	1.47 ± 0.15
Triolein (5)	1.32 ± 0.25
Methyl oleate (10)	2.52 ± 0.33
Oleoyl alcohol (10)	2.26 ± 0.04
Oleic acid (10)	2.03 ± 0.14
Linoleic acid (10)	1.40 ± 0.27
Dioleoyl phosphatidylcholine (5)	4.36 ± 0.60

increase in the permeability of the membranes. Dioleoyl phosphatidylcholine induced a small increase in the release rate. It is considered that simple penetration of an unsaturated carbon-chain into the membrane may not induce an increase in the permeability of the lipid bilayer. These results are in agreement with the results of the former report that these lipids had no enhancing effect on the intestinal absorption of streptomycin. Oleic acid and linoleic acid did not cause an increase in the permeability of phenol red and also oleic acid did not cause an increase in the release rate of sulfanilic acid which is an anionic drug. However, these fatty acids caused an increase in the mucosal membrane permeability to anionic drugs as well as to cationic drugs in the in situ experiments (Table 3; the former report). This disagreement will be discussed in Discussion.

Effect of the treatment with various solutions on the permeability of liposomal membranes

In the absorption experiment, intraluminal mixed micelles exert an action on the mucosal membrane. To make up a similar situation, a treatment study was performed. Table 6 shows the effect of the treatment with various solutions on the release of drugs. The treatment with mixed micellar solution increased the release rate of drugs most. Fig. 1 shows the effect of the treatment with various solutions on the uptake of drugs into liposomes, and also that the treatment with mixed micellar solution most enhanced the uptake of drugs.

The treatment with sodium taurocholate (NaTC) micellar solution caused only a small increase in the permeability of drugs. Therefore, it is suggested that the lipid component of mixed micelles may play a critical role in the alteration of the membrane permeability. Since the treatment with mixed micellar solution enhanced the permeability of the membrane more than the treatment with emulsion, it is considered that lipids in the solution treated with mixed micellar solution may disperse finely almost in the form of a monomer and be incorporated into the lipid bilayer. Only oleic acid may disperse finely to some extent, for the treatment of oleic acid-emulsion enhanced the permeability of the membranes to PAEB appreciably.

TABLE 6

EFFECT OF THE TREATMENT OF LIPOSOMES WITH VARIOUS SOLUTIONS ON THE RELEASE OF PHENOL RED AND PROCAINAMIDE ETHOBROMIDE

Treatment solution	K (10^{-3} min^{-1}) ± S.D.
Phenol red	
Buffer solution	2.06 ± 0.40
10 mM NaTC	3.20 ± 0.18
10 mM monoolein	4.47 ± 0.43
10 mM NaTC + 10 mM monoolein	9.78 ± 0.69
PAEB	
Buffer solution	0.00 ± 0.00
10 mM NaTC	0.31 ± 0.31
10 mM oleic acid	3.48 ± 0.86
10 mM NaTC + 10 mM oleic acid	6.88 ± 0.98

Temperature-dependent study

To examine the change of the activation energy for the permeation process of phenol red through liposomal membranes by the incorporation of monoolein, temperature-dependent studies were performed. Fig. 2 shows the Arrhenius plot of the release rate constant of phenol red through liposomal membranes and monoolein-incorporated liposomal membranes. The activation energy for the permeation process was obtained assuming the release rate constant, K, obeys an equation, $K = A \exp(-E_a/RT)$, where A is some constant with respect to temperature, E_a is the activation energy, R is the gas

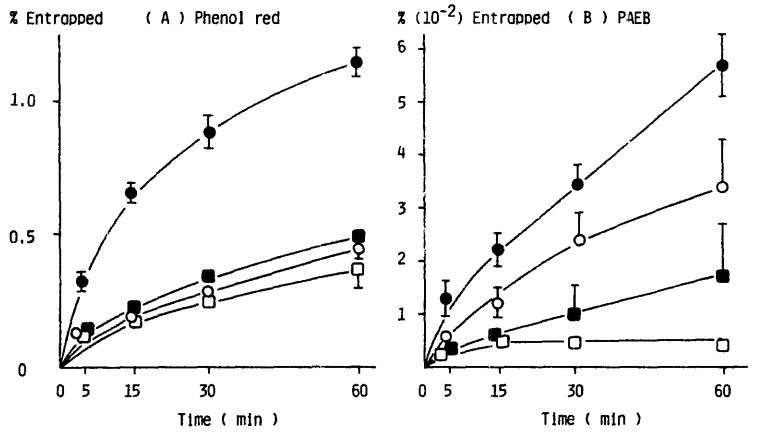

Fig. 1. Effect of the treatment of liposomes with various solutions on the uptake of phenol red and PAEB into liposomes. Each value is the mean ± S.D. of 3 experiments. A: Phenol red: treatment solutions are: •, 10 mM NaTC + 10 mM monoolein; ○, 10 mM monoolein; ■, 10 mM NaTC; and □, buffer solution. B: PAEB: treatment solutions are: •, 10 mM NaTC + 10 mM oleic acid; ○, 10 mM oleic acid; ■, 10 mM NaTC; and □, buffer solution.

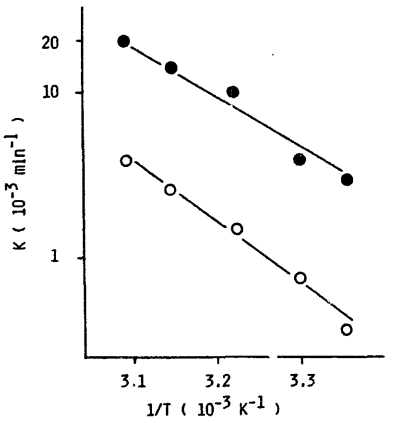

Fig. 2. Arrhenius plot of the release rate constant K, of phenol red through liposomal membranes. The liposomes had the following lipid compositions: •, egg phosphatidylcholine–cholesterol–monoolein (80 : 20 : 10); ○, egg phosphatidylcholine–cholesterol (80 : 20). The activation energies for the permeation process of phenol red through these membranes were 14.3 and 17.5 kcal/mol respectively.

constant and T is the absolute temperature. The incorporation of monoolein caused a decrease in the activation energy from 17.5 to 14.3 kcal/mol.

DISCUSSION

A close correlation was found between the enhancement of the intestinal absorption of drugs induced by mixed micelles and the alteration of the permeability of liposomal membranes by the incorporation of the lipid component of mixed micelles. Namely, lipids which enhanced the intestinal absorption increased the permeability of liposomal membranes and lipids which did not cause an increase of the drug absorption, did not cause the alteration of the permeability of liposomal membranes. Furthermore, the degree of the enhancing effect of various fatty acids on the permeability of liposomal membranes corresponds to the extent of the enhanced intestinal absorption in the presence of fatty acid mixed micelles.

The present study, however, could not designate the alteration of the mucosal membrane permeability to anionic drugs induced by unsaturated fatty acid mixed micelles, since oleic acid and linoleic acid did not enhance the permeability of liposomal membranes to phenol red or sulfanilic acid. Monoolein enhanced the release rate of both anionic and cationic drugs (Table 2) and oleic acid increased the release rate of a cationic drug (Table 4). The pK_as of long-chain fatty acids are known to be about 4.85 (White et al.). Consequently, it is considered that the release of these anions from fatty acid-incorporated liposomes may be inhibited by the repulsion between anion and anionic layer of fatty acid on the surface of the membranes. On the mucosal membrane, the charge of a fatty acid may be eliminated by the acidification of the mucosal membrane (Hogben et al., 1959; Kakemi et al , 1965) or a binding to the membrane protein, and if so, the alteration in the mucosal membrane permeability to these anions may be induced by unsaturated fatty acid mixed micelles.

Richards et al. investigated the effect of sodium taurocholate on the structural integrity of liposomes and reported that liposomes were totally disrupted in the presence of 10 mM bile salts (Richards and Gardner, 1978). The critical micellar concentration of taurocholate is known to be 0.2 mM in the presence of a phospholipid such as lecithin (Carey and Small, 1970). Although the final concentration of taurocholate in the present treatment experiment (Table 6, Fig. 1) was about 0.3 mM, liposomes may not be disrupted for the following reasons. The amount of egg lecithin was 12 times as large as that of bile salt. Not only the release but the uptake of drugs occurred. Linearity was found in the semi-logarithmic plot of A/A_0 to time, and in the uptake experiment the amount of the entrapped drug increased gradually.

It is interesting to note that lipids which enhanced the permeability of liposomal membranes are fusogenic lipids (Ahkong et al., 1973). Lucy et al. investigated the interactions of membrane phospholipids with fusogenic lipids by electron microscopy (Howell et al., 1973) and monolayers (Maggio and Lucy, 1975; 1976). They reported that the presence of a low melting fusogenic lipid in a mixed monolayer with phosphatidylcholine might facilitate the possibilities for molecular movement in the polar region of the phospholipid and fusogenic lipids were able to induce morphological changes with liposomes of phosphatidylcholine. In closing, they suggested that the presence of a low melting fusogenic lipid might produce an increase in the permeability of the lipid bilayer.

Temperature-dependent studies demonstrated that the incorporation of monoolein caused a decrease in the activation energy for the permeation process of phenol red from 17.5 to 14.3 kcal/mol (Fig. 2). The incorporation of dioleoyl lecithin caused a small increase in the release rate (Table 4). Blok et al. indicated that the activation energy of the water permeation through liposomal membranes decreased as the unsaturation of the acyl chain of lecithin increased (Blok et al., 1977). Therefore, it is considered that the incorporation of monoolein may increase the fluidity of the membranes.

The mechanism of the intestinal fat absorption has been studied (Ockner and Isselbacher, 1974). Westergaard et al. reported that the principle role of mixed micelles in facilitating lipid absorption is to overcome unstirred layer resistance while the actual process of fatty acid absorption occurs through a monomer phase in equilibrium with mixed micelles (Westergaard and Dietschy, 1976).

Based on the above studies, the mechanism for the inducement of the intestinal absorption of poorly absorbed drugs by mixed micelles is speculated as the following. The micellar state may facilitate the incorporation of the lipid component of mixed micelles into the mucosal membrane. The incorporated lipid interacts with the polar region of the membrane phospholipids and enhances the fluidity and permeability of the mucosal membrane. Consequently, poorly absorbed drugs can transfer across the mucosal membrane easily.

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