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Effect of Mucosal Lipid Components on the Membrane Permeation of Water-Soluble Drugs

MASAYUKI MIZUNO,[a] MAMI YOSHIDA,[a] MITSURU HASHIDA,[a] TOSHIKIRO KIMURA,[b] and HITOSHI SEZAKI*,[a]

Faculty of Pharmaceutical Sciences, Kyoto University,[a] Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto 606, Japan and Faculty of Pharmaceutical Sciences, Okayama University,[b] 1-1-1, Tsushima-naka, Okayama 700, Japan

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The contribution of intestinal mucosal lipid components to the membrane permeation process of water-soluble drugs was examined by using liposomes as a model membrane. Mucosal lipids extracted from the rat small intestine were fractionated into several lipid classes by column chromatography and each lipid fraction was reconstituted into the liposomal membrane. The release rate of drugs incorporated in liposomes was measured (pH 6.5) as an index of the membrane permeability.

6-Carboxyfluorescein (6-CF), procainamide ethobromide (PAEB) and sulfanilic acid (SA) were employed as model drugs. Their absorption percentages from rat small intestine in one hour were 2.4%, 3.2% and 7.5%, respectively, as determined by the *in situ* recirculation technique (pH 6.5). These drugs were more permeable through the liposomal membranes containing intestinal free fatty acids than egg lecithin liposomes, with the exception of SA.

Arrhenius plots of the release rate constants indicated that increased liposomal membrane fluidity was partly responsible for this accelerated release in the case of PAEB, but no significant change was observed in the activation energy for the membrane permeation of 6-CF.

From these observations, it is concluded that lipid components, especially free fatty acids of the intestinal mucosa, play an important role in the absorption process of these water-soluble drugs.

Keywords——water-soluble drug; intestinal absorption; mucosal lipid component; free fatty acid; liposome; membrane permeability; Arrhenius plot; membrane fluidity

In recent years, many efforts have been made to elucidate the intestinal absorption mechanisms of water-soluble drugs.[1] In particular, some basic studies have been carried out in order to characterize the absorption mechanisms for amino-β-lactam antibiotics.[2] On the basis of these previous findings, many investigators have proposed several transport mechanisms within the intestinal mucosal membrane and have discussed the characteristics of carrier proteins. However, less attention has been paid to the other major function of epithelia, *i.e.*, their ability to serve as a barrier. Several kinds of *in vitro* model membranes have been devised to clarify the *in vivo* absorption mechanisms.[3] Such model membranes include, for example, the Sartorius absorption simulator, black lipid membranes, and liposomes. Because of the morphological similarity to native biological membranes (lipid bilayer) in addition to ease of preparation, high reproducibility, high stability and so on, liposomes have been used by a number of investigators as a model membrane system and more recently as a drug delivery system.[4] For such reasons, the present research was aimed at the elucidation of the effect of rat intestinal lipid components on the membrane permeability to poorly absorbable drugs by using liposomes as a model membrane. Intestinal lipids were reconstituted into liposomes and the release rate of drugs from liposomes was measured as an index of the membrane permeability.

Three poorly absorbable drugs, 6-carboxyfluorescein (6-CF), procainamide ethobromide (PAEB) and sulfanilic acid (SA), were used as model drugs and the role of intestinal mucosal lipid components in determining the membrane permeability to these drugs was investigated.

Experimental

Materials——6-CF was purchased from Eastman Kodak, Rochester, N.Y. Impurities in 6-CF were checked according to the method of Ralston *et al.*[5] Since no impurity was detected, 6-CF was used without further purification. PAEB was kindly supplied by Squibb Co., Princeton, NJ. Egg phosphatidylcholine was prepared from hen egg yolks according to the method of Rhodes and Lea.[6] Stearic acid and oleic acid were obtained from Nakarai Chemicals Co. (Kyoto). [^{14}C]Glucose (4.8 mCi/mmol) was obtained from New England Nuclear, Boston, MA. All other reagents were of the highest grade available.

Preparation of Drug Solution——For liposomal and absorption studies, drugs were dissolved in buffered saline (Na_2HPO_4–NaH_2PO_4–NaCl, pH 6.5) and isotonic phosphate buffer (Na_2HPO_4–NaH_2PO_4, pH 6.5), respectively. A 6-CF solution of 200 mM was prepared by titrating the appropriate amount of 6-CF with 1 M NaOH until the pH reached 6.5.

Separation of Intestinal Lipids——Intestinal total lipids were extracted from the scraped mucosa of the rat small intestine by the method of Folch *et al.*[7] Intestinal total lipids were then fractionated stepwise. Firstly, they were applied to a silicic acid (Mallinckrodt, Inc.) column. Intestinal neutral lipids were separated efficiently by elution with chloroform. After this process, intestinal neutral lipids were further separated into three fractions. Using a Florisil[8] (Nakarai Chemicals Co.) column, intestinal neutral lipids were eluted with 50% ether in hexane, 2% methanol in ether and 4% acetic acid in ether, successively. The eluates were collected and termed fractions A, B and C, respectively. Figure 1 illustrates the thin-layer chromatogram (TLC) of intestinal mucosal lipids. Intestinal total lipids were chiefly composed of phospholipids, cholesterol, free fatty acids and triglycerides. Triglycerides and cholesterol were included in fraction A and free fatty acids in fraction C. Monoglycerides, which are minor components in intestinal mucosal lipids, should be eluted in fraction B, but in this case they could not be identified distinctly as a single spot on TLC. These findings suggest that the separation procedure is a satisfactory one.

Preparation of Liposomes——Liposomes were prepared according to the method employed in our previous paper.[9] A Sephadex G-75 column was used to separate the liposomal fraction from free drugs and the liposomal suspension was immediately used for the release experiments.

Measurement of the Release Rate from Liposomes——For PAEB and SA, the overall release rate of drugs from liposomal suspensions was determined by the modified dynamic dialysis method of Klein *et al.*[10] Briefly, 5 ml of liposomal suspension was added to Visking cellulose tubing (18/32) and 10 ml of isotonic phosphate-buffered saline was used as the external solution. As the diffusion rate of free drugs through the Visking dialysis tube was rapid, the permeation across the lipid bilayer of liposomes could be taken as the rate-limiting process in the release to the external medium. For 6-CF, the efflux from liposomes was measured spectrofluorometrically according to the method of Szoka *et al.*[11] The efflux of 6-CF was directly measured as an increase in the fluorescence intensity of 6-CF. The total fluorescence of the sample was determined by adding 0.1 ml of 10% Triton X-100 to the cuvette to release all of the 6-CF. Unless otherwise stated, the temperature was maintained at 25 °C. A semilogarithmic plot of the percentage of the drug remaining in liposomes against time was linear, and the release rate constant was determined from the slope.

Absorption Experiments——Male Wistar albino rats weighing 150—200 g were used as described by Kakemi *et al.*[12] The bile duct was ligated in all experiments. Rectal temperature was maintained at 37 °C. Forty ml of drug solution (0.1 mM, pH 6.5) was recirculated in the small intestine at the rate of 5 ml/min. After 1 h, the perfusate was

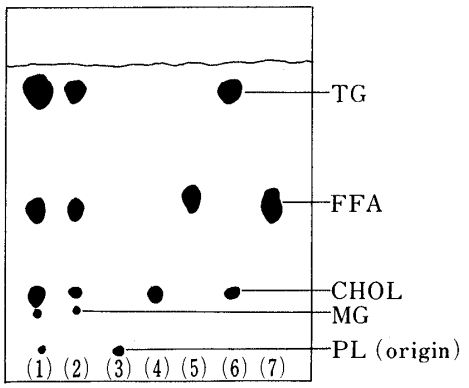


Fig. 1. Thin-Layer Chromatogram of Intestinal Mucosal Lipids

Total lipids, neutral lipids and fractions A and C were obtained as described in Experimental.
Solvent: hexane–ether–acetic acid (70 : 30 : 1, v/v). Detection of spots: H_2SO_4.
(1) intestinal total lipids (100 μg), (2) intestinal neutral lipids (100 μg), (3) intestinal phospholipids (50 μg), (4) cholesterol (25 μg), (5) stearic acid (25 μg), (6) fraction A (50 μg), (7) fraction C (25 μg). PL, phospholipids; MG, monoglycerides; CHOL, cholesterol; FFA, free fatty acids; TG, triglycerides.

removed and the intestine was washed with pH 6.5 phosphate buffer. The amount absorbed was calculated from the difference in the amount of the drug between the initial and the final solutions.

Analytical Methods——Determinations of PAEB and SA were carried out by a colorimetric method as follows. Aliquots of dialysate samples were acidified with 0.5 ml of concentrated HCl, diazotized in the regular manner, and coupled with Tsuda's reagent, then optical densities were determined at 550 nm using a Hitachi double beam spectrophotometer (type 220). 6-CF fluorescence was measured with a Shimadzu RF-540 spectrofluorometer equipped with a jacketed cuvette holder in which the temperature could be adjusted by using circulating water. The excitation wavelength was 490 nm and the emission wavelength was 520 nm. [^{14}C]Glucose was measured with a liquid scintillation counter (Aloka LSC-900, Tokyo).

Results

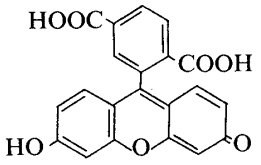
Chemical Structures and Physicochemical Properties of Drugs

Table I summarizes the chemical structures and physicochemical properties of the three drugs. Considering their pK_a values, the drugs are thought to exist as fully ionized forms at the physiological pH of the small intestine (pH 6.5). The apparent partition coefficients of these drugs were virtually zero and these poorly lipophilic drugs were absorbed from the small intestine to the extents of only 2.4%, 3.2% and 7.5% in 1 h, respectively.

Relationship between the Absorption Rate Constants and the Release Rate Constants

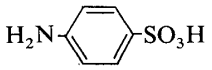
Figure 2 shows the relationship between the absorption rate constants and the release

TABLE I. Chemical Structures and Physicochemical Properties of Drugs



6-carboxyfluorescein (6-CF)

procainamide ethobromide (PAEB)



sulfanilic acid (SA)

	M_r	pK_a	PC[a]	% absorbed in 1 h[b]
6-CF	376.32	3.5, 4.4, 6.7	<0.01	2.4
PAEB	344.20	Quaternary ammonium	0.012	3.2
SA	173.84	Strong 3.23	0.013	7.5

a) Apparent partition coefficient to chloroform at pH 6.5, 37 °C. *b*) The *in situ* recirculation method (pH 6.5).

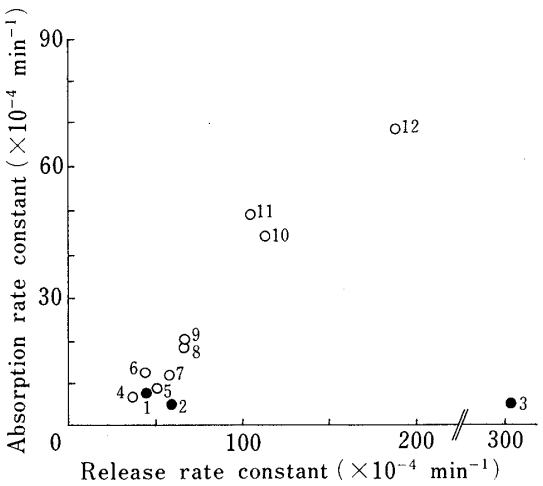


Fig. 2. Relationship between the *in Situ* Absorption Rate Constants and the Release Rate Constants from Intestinal Total Lipid Liposomes

●: 1, SA; 2, 6-CF; 3, PAEB. ○: 4, phenol red; 5, cefazolin; 6, cephaloridine; 7, amoxicillin; 8, ceftezol; 9, bromphenol blue; 10, cephalexin; 11, quinine; 12, cephradine.

rate constants of 6-CF, PAEB and SA from liposomes consisting of intestinal total lipids. Other water soluble drugs dealt with in our previous report[9] were also plotted for reference. Liposomes were prepared from 32 mg of total lipid extracts in the experiments on PAEB and SA, and 3.2 mg for that on 6-CF. As is evident from Fig. 2, poorly-absorbable drugs, except PAEB, are released slowly compared with well-absorbable drugs, *e.g.*, cephalexin, cephradine, quinine and so on, which were reported to be released rapidly from liposomes consisting of intestinal total lipids.[9]

Effect of Intestinal Lipid Components on the Release Rate from Liposomes

In order to examine the effect of the intestinal neutral lipids on the liposomal permeability to these drugs, the release rates from liposomes were examined in the presence of various neutral lipids. The release rate constants of 6-CF, PAEB, and SA are shown in Table II. Six kinds of liposomes were prepared; their lipid compositions are included in Table II. The release rate constants from composition (B), (C), (D), (E), and (F) were compared with those

TABLE II. Effect of Lipid Composition on the Release Rate of Drugs from Liposomes

Lipid composition	Release rate constant ($\times 10^{-4}$ min^{-1})		
	6-CF	PAEB	SA
(A)	125.7± 2.7 (3)	7.01± 0.63 (5)	3.24±0.21 (3)
(B)	46.1± 0.9 (4)[a]	3.56± 0.39 (4)	2.87±0.28 (4)
(C)	58.4± 2.1 (4)[a]	4.57± 0.58 (4)	3.58±0.65 (4)
(D)	93.4± 1.8 (4)[a]	6.48± 0.60 (4)	2.96±0.40 (4)
(E)	85.0±12.6 (4)	3.35± 0.32 (4)	3.37±0.73 (4)
(F)	284.5± 7.6 (3)[a]	166.0 ±14.8 (8)[a]	3.57±0.23 (3)

Lipid composition of liposomes: (A), egg lecithin; (B), egg lecithin : cholesterol = 4 : 1 (molar ratio); (C), egg lecithin : cholesterol : dicetylphosphate = 16 : 4 : 1 (molar ratio); (D), egg lecithin : intestinal neutral lipids = 8 : 1 (weight ratio); (E), egg lecithin : fraction A = 4 : 1 (weight ratio); (F), egg lecithin : fraction C = 4 : 1 (weight ratio). Results are expressed as the mean ± S.E. with the number of experiments in parentheses. The data were analyzed by means of Student's *t*-test. *a*) $p<0.001$.

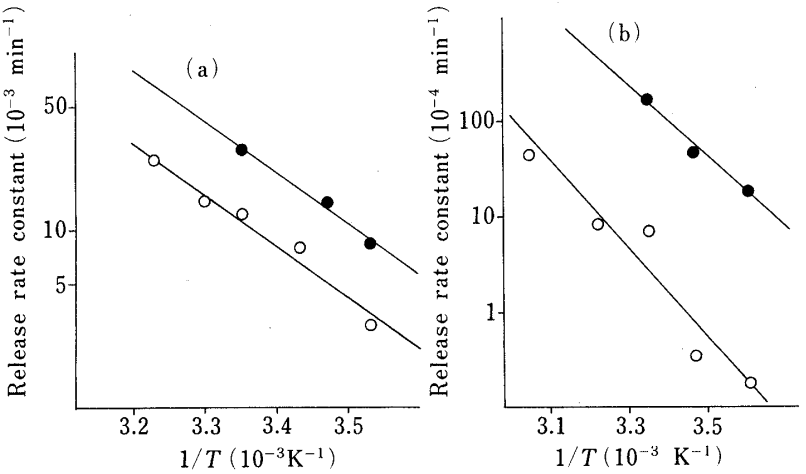


Fig. 3. Arrhenius Plots of the Release Rate Constants of 6-CF (a) and PAEB (b) from Liposomes with Various Lipid Compositions

Each point is a mean value of at least four experiments. Regression lines were obtained from a least-squares fit of the points. Activation energies were calculated from the slopes of these lines. Liposomal lipid compositions are "composition (A)" (○) and "composition (F)" (●) of Table II.

from composition (A) liposomes as the control. For three drugs in Table II, the release rate constants from (B), (C), (D) or (E) were not more than that from (A). However, the release rate constants for 6-CF and PAEB were significantly increased by the addition of fraction C (F), though no significant effect was observed on the release rate of SA.

Arrhenius Plot of the Release Rate Constants of 6-CF and PAEB

The release rates of 6-CF and PAEB were investigated at various temperatures. The release rate constants increased with increasing temperature. When the logarithm of the release rate constants was plotted against the inverse of the absolute temperature, a straight line was obtained for each composition of liposomes. From the slopes of the lines, the activation energies for the permeation process of 6-CF and PAEB through liposomal membranes were calculated. The activation energies were 13.12 kcal/mol (control) and 13.38 kcal/mol (composition (F)) for 6-CF, and 21.33 kcal/mol (control) and 17.15 kcal/mol (composition (F)) for PAEB.

Effect of Fatty Acids on Release Rates from Liposomes

A synthetic saturated fatty acid (stearic acid) and an unsaturated fatty acid (oleic acid) were employed in order to clarify the roles of free fatty acids in the liposomal membrane permeability. As shown in Table III, a marked increase of the release rate of 6-CF was observed on the addition of either fatty acid.

Examination of the Interaction between PAEB and the Membrane Lipids

Table IV shows apparent partition coefficients of PAEB between chloroform containing

TABLE III. Effect of Fatty Acids on the Release Rates of 6-CF and PAEB from Liposomes

Lipid composition	Release rate constant ($\times 10^{-4}$ min^{-1})	
	6-CF	PAEB
Control (egg lecithin)	125.7 ± 2.70 (3)	7.01 ± 0.63 (5)
+ fraction C 20% (w/w)	284.5 ± 7.60 (3)	166.0 ± 14.8 (8)
+ stearic acid 20% (w/w)	249.8 ± 11.6 (3)	36.5 ± 1.22 (6)
+ oleic acid 20% (w/w)	231.1 ± 11.6 (3)	234.3 ± 3.29 (4)

Liposomes consisted of 80% (w/w) egg lecithin and 20% (w/w) fatty acid. Results are expressed as the mean ± S.E. with the number of experiments in parentheses.

TABLE IV. PAEB Partitioning to Chloroform Containing Fatty Acids

Fatty acid	Partition coefficient
None	0.031[a]
Fraction C	0.041[b]
Stearic acid	0.029[b]
Oleic acid	0.034[b]

a) Chloroform to phosphate-buffered saline (pH 6.5, 25 °C) partition coefficient. *b*) Chloroform containing 0.3 mg/ml free fatty acid to phosphate-buffered saline (pH 6.5, 25 °C) partition coefficients. The initial aqueous phase concentration of PAEB was 0.1 mM.

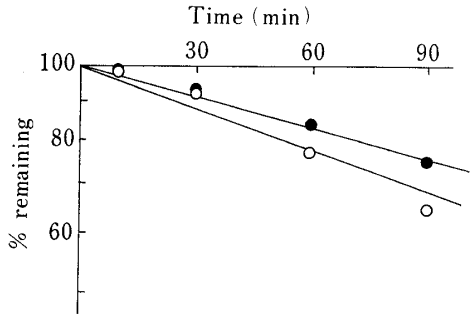


Fig. 4. Effect of Coexistence of PAEB on the Release Rate of [^{14}C]Glucose from "Composition (F)" Liposomes

PAEB (20 mM) was added at the time of preparing the liposomes.
○, control; ●, PAEB. Regression lines were obtained from a least-squares fit of the points. Each point is the mean value of four experiments.

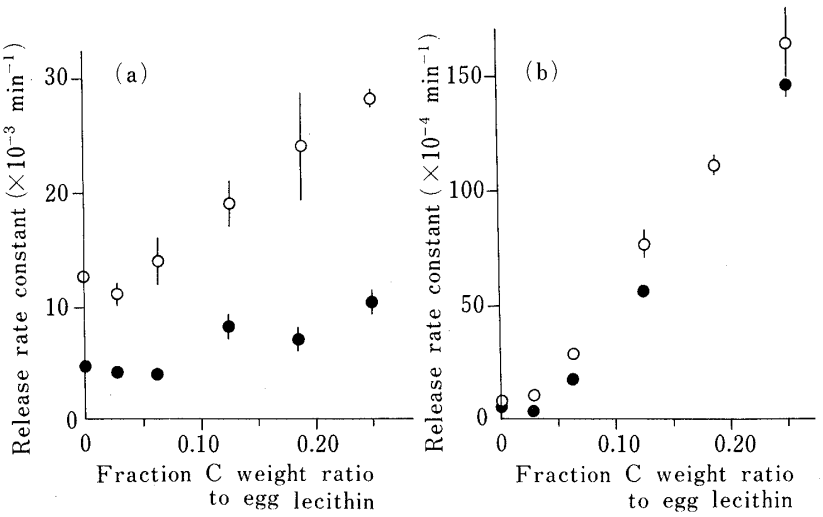


Fig. 5. Release Rate Constants of 6-CF (a) and PAEB (b) through Liposomal Membranes Containing Various Amounts of Fraction C

Liposomes were prepared from egg lecithin (○) or egg lecithin plus cholesterol (4:1 in molar ratio) (●). Fraction C was added to the liposomes with the stated weight ratio to egg lecithin. Vertical bars represent the mean ± S.E. of at least three experiments.

various fatty acid classes and phosphate-buffered saline (pH 6.5). As is evident from Table IV, the partition coefficient of PAEB was not influenced by the addition of any fatty acid to the organic phase.

In order to investigate the effect of PAEB on the permeability of liposomes, the release rates of [^{14}C]Glucose from liposomes (composition (F)) were examined in the presence and absence of PAEB. As shown in Fig. 4, the release rate of glucose was not greatly affected by PAEB. Namely, the release rate constants of glucose with and without PAEB were 3.12×10^{-3} min^{-1} and 4.96×10^{-3} min^{-1}, respectively.

Permeability of Liposomal Membrane Incorporating Fraction C

Figure 5 shows the release rate constants of 6-CF (a) and PAEB (b) through liposomal membrane containing various amounts of fraction C. It is evident that the release rate constants of both drugs were almost proportional to the amount of fraction C.

Discussion

Concerning the absorption mechanism of polar drugs, Kimura *et al.* suggested that the rate-limiting step of intestinal absorption of the drug exists in the permeation process through the lipid bilayer membrane in the brush border membrane.[9] It seems reasonable to consider that the membrane permeability is greatly affected by the membrane constituents. The present study was aimed at elucidating the contribution of intestinal mucosal lipid components to the intestinal mucosal barrier. In particular, our attention was focussed on the role of intestinal free fatty acids in the membrane permeation process of polar drugs.

The validity of employing 6-CF, PAEB and SA as model drugs was investigated at the beginning of this study. Absorption experiments using the *in situ* recirculation technique revealed that these drugs were absorbed only slightly from the rat small intestine (Table I). While 6-CF is known to have no interaction with lipids,[13] cationic PAEB might form complexes (for example ion pairs) with anionic lipid components. In order to investigate the possible interaction, partition coefficients and the release rate constants of glucose were examined (Fig. 4, Table IV). It is well known that the permeation of highly-water soluble

glucose through liposomal membranes is limited by the lipid bilayers.[14] Therefore, if any changes in the membrane structure or properties should be induced by PAEB, the release rate of glucose would change as well.[9] Figure 4 suggests that PAEB does not form complexes with lipids, because the two regression lines have similar slopes. The result that no enhancement effect of PAEB was observed on the release rate of glucose indicates that PAEB may not influence the membrane permeability. Thus, it would appear that 6-CF, PAEB and SA can be used as model poorly-absorbable drugs. In Fig. 2, the release rate of PAEB was much faster than was predicted from its absorbability. The reason for this is not well understood, but some of the lipid components, such as glycolipids and monoglycerides, which were not studied in the present research, may influence the permeation of PAEB across liposomal membranes.

6-CF has been widely accepted as an aqueous space marker of liposomes since Weinstein *et al.* first introduced it,[15] and this compound is very valuable for investigations of the structure and properties of membrane systems.[16] 6-CF is known to be a trivalent anion at neutral pH and electrically neutral in the acidic pH region.[17]

Hori *et al.*[18] examined the effect of free fatty acids on the permeability of black lipid membranes to acidic drugs and concluded that the good absorbability of weakly acidic drugs from the small intestine might be explained, at least in part, in terms of the contribution of the free fatty acids, which produced a more acidic pH region adjacent to the intestinal cell surface. Szoka *et al.*[11] and Straubinger *et al.*[17] reported that the leakage of 6-CF from liposomes drastically increased on lowering the pH value from neutral pH to pH 5.0 or so. Taking these results into consideration, it may be reasonable to consider that free fatty acids arranged in liposomal membranes lower the pH of the area adjacent to the membrane and in this lowered pH region, the nonionic form of weakly acidic 6-CF increases and easily passes through the liposomal membranes. On the other hand, the release rate of SA, a strongly acidic drug, was not affected by the incorporation of fraction C. It is speculated that fully ionized SA at even the lowered pH region may be prevented from gaining access to the membranes by electrostatic repulsion between the anion and an anionic layer of fatty acids on the surface of the membrane.[19] Muranushi *et al.*[19] indicated that fatty acids not only disorder the hydrophobic region of the lipid bilayer but also interact with the polar head groups of phospholipids and that unsaturated fatty acids are more effective than saturated ones as regards the disordering effect on the membrane structure. As shown in Table III, the release rate of 6-CF was increased by the incorporation of saturated or unsaturated fatty acids. Although the fatty acids have some effects on the membrane properties, as described by Muranushi *et al.*,[19] it seems that the lowered pH value may play an important role in the permeation process of weakly acidic drugs such as 6-CF.

On the other hand, in the case of PAEB, which is a quaternary ammonium compound, the increased release rate induced by the incorporation of fraction C cannot be explained only in terms of the decreased pH region adjacent to the membrane. Blok *et al.*[20] investigated the relationship between the activation energy of water permeation through liposomal membranes and the membrane fluidity. Similarly, we examined the temperature dependency of the release rate, and an Arrhenius plot of the release rate constants was prepared (Fig. 3). In the case of PAEB, the activation energy decreased from 21.33 kcal/mol to 17.15 kcal/mol upon the addition of fraction C, suggesting that the liposomal membrane fluidity is increased by the addition of free fatty acids fraction. On the other hand, no significant change was observed in the activation energy for 6-CF permeation with or without fraction C. From these observations, it may be assumed that either the change in the membrane fluidity (PAEB) or the decreased pH region (6-CF) apparently increases the release rate of each drug. However, it is likely that the change in the membrane fluidity plays some part in the release of 6-CF. Since the fluidity of membranes is regarded as an inherent property of membranes themselves, the reason why the activation energy did not change is not clear.

As shown in Table III, the release rate of PAEB was greatly increased by the addition of an unsaturated fatty acid (oleic acid), while less effect on the release rate was caused by the addition of a saturated fatty acid (stearic acid). Increased permeability to PAEB was also observed after the incorporation of intestinal free fatty acids fraction (fraction C) (Table II). These findings suggest that intestinal mucosal membrane contains a considerable amount of unsaturated free fatty acids. It has been reported that free fatty acids account for 2.6%[21] or 14.2%[22] of the total lipid content of the microvillus membrane of the rat small intestine. On the basis of the above results, it can be concluded that lipid components, especially the free fatty acids of the intestinal mucosa, may play an important role in the permeation of these polar drugs.

Recently intestinal drug absorption has been suggested to include many potentially rate-limiting steps.[23] These barriers are the aqueous stagnant layer, mucous layer, apical membrane, epithelial cell contents, basal membrane, basement membrane and capillary wall membrane, as well as the blood flow. Furthermore, the intestinal brush border membrane consists of not only lipids but also membrane proteins and glycocalyx, glycoproteins. Although complex mechanisms appear to be operating in the drug absorption process, further investigation of the role of fractionated intestinal mucosal lipids should provide useful information about the intestinal absorption of polar drugs.

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