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EFFECTS OF FREE FATTY ACIDS AS MEMBRANE COMPONENTS ON PERMEABILITY OF DRUGS ACROSS BILAYER LIPID MEMBRANES

A MECHANISM FOR INTESTINAL ABSORPTION OF ACIDIC DRUGS

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Summary

Studies of the influence of fatty acids, which were the component of intestinal mucosal lipids, on the permeability of several drugs across bilayer lipid membranes generated from egg phosphatidylcholine and intestinal lipid have been pursued. The permeability coefficients of *p*-aminobenzoic acid, salicylic acid and *p*-aminosalicylic acid (anionic-charged drug) increased when fatty acids such as lauric, stearic, oleic, linoleic and linolenic acid were incorporated into the bilayer lipid membranes generated from phosphatidylcholine. In the presence of methyl linoleate and oleyl alcohol, no enhancing effect on *p*-aminobenzoic acid transfer was obtained. The effect of fatty acids was more marked at pH 6.5 than at pH 4.5. In contrast, upon the addition of fatty acids to intestinal lipid membranes which originally contained fatty acids, the permeability coefficient of *p*-aminobenzoic acid tended to decrease, though the permeability through intestinal lipid membranes was larger than that of phosphatidylcholine membranes. The permeability of *p*-aminobenzoic acid across bilayer lipid membranes from intestinal phospholipids was significantly decreased to about equal that of phosphatidylcholine membranes, and reverted to the value of intestinal lipid membranes when fatty acids were added to intestinal phospholipids. It seemed reasonable to assume that free fatty acids in the intestinal neutral lipid fraction could contribute to the increase in the permeability of *p*-aminobenzoic acid. On the basis of above results, possible mechanisms for good absorbability of weakly acidic drugs from the intestine are discussed.

Introduction

A drug introduced into the body must cross several cellular and subcellular membrane structures when it is absorbed and distributed. There is much

evidence to suggest that the biological membranes, lipoid in nature, play an important role in determining the rate and specificity of the drug transport process.

Artificial lipid membranes such as phospholipid vesicles and bilayer lipid membranes have been used recently to investigate the membrane phenomena normally inaccessible to experiments in natural systems on account of technical reasons or simply biological complexity. In a preceding paper [1], we investigated the transport of drugs across bilayer lipid membranes generated from the intestinal lipid and egg phosphatidylcholine in order to obtain further information on the molecular mechanism underlying drug absorption from the intestine. It has been found that the permeability coefficients of acidic drugs such as *p*-aminobenzoic acid and salicylic acid were much larger than comparable values predicted from the partition coefficients, and the permeability for these drugs through intestinal lipid membranes was even larger than that of phosphatidylcholine membranes. Thereby, it was suggested that the phospholipids and other lipid components of the small intestine might play an important role in membrane permeability to acidic drugs. This result seemed to correspond to the well known phenomena that weak acids, those with a p*K*a of 3 or higher, are absorbed relatively rapidly from the small intestine at physiological pH [2—4]. On the other hand, the composition of intestinal lipid was mainly phospholipid, cholesterol, fatty acid and glyceride by thin layer chromatography, and the high content of free fatty acid was noteworthy [1]. Similar results have been reported on the intestinal mucosa [5,6] and microvillus membrane [7,8].

The purpose of the present study was to examine further the transport of drugs across bilayer lipid membranes formed from intestinal lipid and egg phosphatidylcholine, and to explore the effects of fatty acids, which are intestinal lipid components, on drug transfer.

Materials and Methods

Materials

L-α-Phosphatidylcholine (Type III E, from egg yolk), oleic acid, linoleic acid, linolenic acid, monolein, methyl linoleate and oleyl alcohol were purchased from Sigma Chemical Co. Cholesterol, lauric acid, stearic acid and *n*-decane were obtained from Nakarai Chemicals, Ltd. These chemicals were certified as at least 99% pure by the suppliers and confirmatory tests with thin layer chromatography using a solvent mixture of hexane/ethyl ether/acetic acid (70 : 30 : 1, by vol.) or chloroform/methanol/water (65 : 25 : 4, by vol.) were in close agreement with the stated purities. Silicic acid (100 mesh) was obtained from Mallinckrodt Chemical Works and silica gel G (type 60) from E. Merck Japan, Ltd. All other chemicals used were of analytical grade and were obtained commercially.

Extraction of lipids and separation of neutral lipids and phospholipids

Total lipids from the mucosa of the small intestine of male Wistar albino rats, 150—180 g and fasted for 16—20 h, were prepared according to Folch et al. [9]. In some experiments, lipids (100 mg) in 1 ml of chloroform were

applied to columns containing 5—8 g silicic acid, activated at 120 °C for 12 h. Neutral lipids were eluted with 200 ml of chloroform, and phospholipids with 200 ml of methanol. All lipids were dissolved in chloroform, stored below 0 °C for not longer than 4 weeks.

Membrane formation and permeability measurement

The procedures of membrane formation and permeability measurement were similar to those described previously [1]. Bilayer lipid membranes were formed by brushing lipid solutions across a 1.5 mm diameter hole in a Teflon cup separating two compartments containing either pH 4.5 (citric acid/Na_2HPO_4) or pH 6.5 (NaH_2PO_4/Na_2HPO_4) isotonic buffer. The volume of the inner compartment was about 7 ml and that of the outer one about 73 ml. Phosphatidylcholine (10 mg) or intestinal lipid (10 mg) in chloroform solution was mixed with desired amounts of additive lipids such as cholesterol and fatty acids, dried under N_2 gas and finally dissolved in 1 ml of *n*-decane for membrane-forming solution. Cholesterol (0.3—0.5% w/v) was incorporated into all membrane-forming solutions to facilitate the thinning and increase the stability of their membranes [1]. The electrical resistance of the membranes was measured with an electrometer (Keithley, 610 C).

The drugs used in the permeability measurements were dissolved in isotonic buffer (1.5—15.0 mg/ml). After the membranes turned completely black and gave a constant resistance, the drug solution (0.2 ml) was added to the inner compartment and buffer solution (1.25 ml) was added to the outer one. The inner compartment was stirred gently with a microspatula at the beginning. Upon completion of the experiment, the hole was quickly sealed by the brush with a small quantity of liquid paraffin to prevent mixing of the inner and outer solutions, and the incubation solutions were withdrawn from both compartments into volumetric cylinders by suction. After each volume was measured, the aliquots were assayed spectrophotofluorometrically as described below. The experiments were carried out at 25°C.

Total flux, J, and apparent permeability coefficient, P, were calculated from the relation:

$$J = \frac{C_0 V_0}{A t} = P(C_i - C_0)$$

where A is membrane area in cm^2, V_0 is the volume of the outer compartment, C_i and C_0 the drug concentrations of the inner and outer compartments, respectively, and t, the time of diffusion.

Apparent partition coefficients

5 ml of the buffered drug solution (pH 6.5) was added to an equal volume of chloroform in a glass stoppered tube, and equilibrated at 25°C by vigorous shaking. The separated aqueous phase was analysed. The apparent partition coefficient of a drug was calculated from the decrease of concentration in the aqueous phase.

Analytical methods

All drugs were determined spectrophotofluorometrically [10—13]. The pH

of the samples was adjusted to a pH appropriate to the analysis of the drug. The maximum activation and emission wavelengths for salicylamide, salicylic acid, *p*-aminobenzoic acid, *p*-aminosalicylic acid and aniline were 330 and 415 nm, 300 and 400 nm, 295 and 345 nm, 300 and 390 nm, and 280 and 340 nm, respectively. Cholesterol was determined by the method of Zlatkis and Zak [14]. Fatty acids were determined by the method of Duncombe [15].

Results and Discussion

Effects of fatty acids on the permeability of drugs across egg phosphatidylcholine membranes

In order to assess the relative importance of the various membrane constituents in determining the drug permeability across bilayer lipid membranes, we conducted a series of experiments in which concentrations of the individual components, particularly fatty acids in this study, in the membrane forming solutions have been varied. Table I shows the effect of stearic acid on the permeability coefficients for various drugs across bilayer lipid membranes formed from phosphatidylcholine-cholesterol at pH 6.5. In the presence of stearic acid, the permeability coefficient of salicylamide, uncharged (drug) appeared to be identical to the control value, and that of aniline, weak base, was decreased. On the other hand, the permeability coefficients of *p*-aminobenzoic acid, salicylic acid and *p*-aminosalicylic acid, (anionic-charged drug) significantly increased when stearic acid was incorporated.

The above results correlate well with the difference of the drug permeability between phosphatidylcholine and intestinal lipid membranes [1]. In view of the fact that fatty acid was a constituent of intestinal lipid, it seemed possible that incorporation of fatty acid into the bilayer lipid membranes increased the permeability coefficients of *p*-aminobenzoic acid and salicylic acid.

To further examine the enhancing effect of fatty acids on the permeability of acidic drugs, it was of interest to compare the potency of various fatty acids,

TABLE I

EFFECT OF STEARIC ACID ON THE PERMEABILITY COEFFICIENTS OF DRUGS ACROSS BILAYER LIPID MEMBRANES GENERATED FROM EGG PHOSPHATIDYLCHOLINE

Chloroform/water partition coefficients were calculated from the distribution of a drug after shaking an aqueous solution of drug (pH 6.5) with the organic solvent. The membranes were generated at 25°C from solutions containing either 1% (w/v) egg phosphatidylcholine/0.5% (w/v) cholesterol or 1% (w/v) egg phosphatidylcholine/0.3% (w/v) cholesterol/0.5% (w/v) stearic acid in *n*-decane. The aqueous phase was pH 6.5 isotonic phosphate buffer. The period of drug diffusion was 30 min for salicylic acid, and for others 60 min. Each value is the mean ± S.E. of 3—7 experiments.

Drug	pK_a	Partition coefficient	Permeability coefficient (10^{-5} cm/s)	
			Control	Stearic acid (0.5% w/v)
Aniline	4.6	24.8	43.7 ± 4.5	30.5 ± 2.2
Salicylamide	8.5	2.38	22.7 ± 0.6	21.4 ± 0.3
Salicylic acid	3.0	0.01	8.9 ± 1.1	11.8 ± 1.3
P-Aminobenzoic acid	4.8	0.03	7.4 ± 0.4	17.5 ± 1.4
P-Aminosalicylic acid	4.0	0.02	1.7 ± 0.3	3.6 ± 0.3

fatty acid ester and alcohol in this system. Table II gives the effects of fatty acids, methyl linoleate and oleyl alcohol on the permeability coefficient of *p*-aminobenzoic acid across bilayer lipid membranes generated from egg phosphatidylcholine. Saturated fatty acids such as lauric acid and stearic acid at 0.5% (w/v) increased the permeability of *p*-aminobenzoic acid, and at 0.1% (w/v) exerted no noticeable effect, while at 1.0% (w/v) the enhancing effect was reduced. Unsaturated fatty acids such as oleic acid, linoleic acid and linolenic acids at 0.1% (w/v) increased significantly the permeability of *p*-aminobenzoic acid. In the presence of methyl linoleate and oleyl alcohol, however, no enhancing effect on the permeability of *p*-aminobenzoic acid. In the presence of methyl linoleate and oleyl alcohol, however, no enhancing effect on the permeability of *p*-aminobenzoic acid was obtained. On the basis of these data, it seemed reasonable to assume that the permeability of *p*-aminobenzoic acid was increased in the presence of an appreciable amount of fatty acid as a membrane component.

Korepanova et al. [16] reported that the presence of linoleic acid in phosphatidylcholine-cholesterol bilayer membranes only slightly increased the membrane electrical conductivity, even at a phosphatidylcholine : cholesterol : linoleic acid molar ratio of 1 : 1 : 3, though the presence of linoleic acid in membranes at the above molar ratio increased the charged density on the membranes. In this study, as shown in Table III, the properties of bilayer lipid membranes incorporated with fatty acids appeared to be identical to control values. Thus, the lack of decreasing the electrical resistance in the presence of fatty acids might suggest that the effect of fatty acids on *p*-aminobenzoic acid transfer was not due to a change of ion permeability.

Furthermore, in order to study the pH dependence for the effect of fatty

TABLE II

EFFECT OF FATTY ACIDS ON THE PERMEABILITY COEFFICIENT OF *P*-AMINOBENZOIC ACID ACROSS BILAYER LIPID MEMBRANES GENERATED FROM EGG PHOSPHATIDYLCHOLINE

The membranes were generated at 25°C from solutions containing either 1% (w/v) egg phosphatidylcholine/0.5% (w/v) cholesterol or 1% (w/v) egg phosphatidylcholine/0.3% (w/v) cholesterol/fatty acid in *n*-decane. The aqueous phase was pH 6.5 isotonic phosphate buffer. Each value is the mean ± S.E. of 3—6 experiments.

		Permeability coefficient (10^{-5} cm/s)
Control		7.4 ± 0.4
Lauric acid	0.1%	7.0 ± 1.5
	0.5%	17.1 ± 1.0
Stearic acid	0.05%	10.3 ± 0.7
	0.1%	9.4 ± 0.9
	0.5%	17.5 ± 1.4
	1.0%	14.1 ± 1.4
Oleic acid	0.05%	12.1 ± 0.4
	0.1%	13.9 ± 1.6
	0.5%	15.7 ± 0.5
Linoleic acid	0.1%	15.1 ± 1.4
Linolenic acid	0.1%	13.7 ± 1.3
Methyl linoleate	0.5%	5.7 ± 0.7
Oleyl oleate	0.5%	6.8 ± 0.8

TABLE III

EFFECT OF FATTY ACIDS ON THE VARIOUS PROPERTIES OF BILAYER LIPID MEMBRANES

The conditions were as described in Tables I and II.

	Time of formation (min)	Breakdown voltage (mV)	Membrane resistance (Ω/cm^2)
Control	1—2	250—300	$5.9 \cdot 10^7$
Lauric acid 0.5%	0.5—1	250—300	$7.5 \cdot 10^7$
Stearic acid 0.5%	0.5	250—300	$7.7 \cdot 10^7$
Oleic acid 0.5%	0.5	250—300	$4.9 \cdot 10^7$

acids on *p*-aminobenzoic acid transfer, the effect of linolenic acid (0.1% w/v) on the permeability of *p*-aminobenzoic acid across phosphatidylcholine membranes formed at pH 4.5 was examined (control, $20.1 \pm 0.4 \cdot 10^{-5}$ cm/s; linolenic acid, $22.2 \pm 0.7 \cdot 10^{-5}$ cm/s). Consequently, the enhancing effect of linolenic acid was more marked at pH 6.5 (Table II), where *p*-aminobenzoic acid was mainly ionized form, than pH 4.5. This result may correspond to the well known phenomena that the weak acids are absorbed more largely from the small intestine at physiological pH than comparable values predicted from the partition coefficients [2—4].

Peters [17] showed that long-chain fatty acids at a water/benzene interface were only 50% ionized when the bulk pH was 3 units above their pK_a values. Gebicki and Hicks [18] have reported that microscopic particles, resembling phospholipid liposomes, were obtained from long-chain fatty acids in the presence of 0.1 M Tris buffer at pH 8, and the bilayers were stabilized by ionization of a small proportion of the weak acid groups because the surface pH was expected to be about 3 units below the bulk pH of 8. It is reasonable to consider that the process of drug transport includes three stages: (1) partition into the membrane, (2) diffusion through the membrane and (3) release from the membrane. With the limited data available, it is not now possible to establish unequivocally the mechanisms by which fatty acids alter the permeability of acidic drugs in the above stages. However, based on the result of Gebicki et al. and the above data concerning the effects of fatty acid derivatives (Tables I and II) and pH dependency, it may be inferred that fatty acids oriented in the membranes lower the microclimate pH or unstirred layer of pH of the bilayer lipid membranes below the bulk pH, therefore, the unionized form of *p*-aminobenzoic acid is increased, and the partition of acidic drugs into the membranes is enhanced. Also the difference in the fatty acid effect between *p*-aminobenzoic acid (pK_a 4.8) and salicylic acid (pK_a 3.0) might be explained in terms of pK_a values.

Effect of fatty acids on the permeability of drugs across intestinal lipid membranes

The effect of fatty acid addition on the permeability of *p*-aminobenzoic acid across bilayer lipid membranes from intestinal lipid, which originally contains fatty acids as a lipid constituent, was also studied. Upon the addition of fatty acids to intestinal lipid membranes, the permeability coefficient of *p*-amino-

benzoic acid tended to decrease, though the permeability coefficient of *p*-aminobenzoic acid through intestinal lipid membranes was 1.9 times larger than that obtained from phosphatidylcholine membranes (control, $14.4 \pm 0.9 \cdot 10^{-5}$ cm/s; stearic acid 0.1%, $10.8 \pm 1.7 \cdot 10^{-5}$ cm/s; stearic acid 0.5%, $8.9 \pm 0.6 \cdot 10^{-5}$ cm/s; oleic acid 0.1%, $12.8 \pm 0.7 \cdot 10^{-5}$ cm/s). The effect appeared in higher concentrations of fatty acids and may result from other factors such as fluidity change of lipid bilayers, but it remains unsolved.

In order to clarify the contribution of fatty acid to *p*-aminobenzoic acid transfer across bilayer lipid membranes, intestinal total lipid was fractionated. Silicic acid column chromatography was utilized for the separation of total lipids into neutral lipid and phospholipid fractions, and thin layer chromatography for separation and determination of the individual lipid classes in the fractions obtained from silicic acid columns. Content of fatty acid was approximately 30% of the total neutral lipid.

As shown in Table IV, the permeability coefficient of *p*-aminobenzoic acid across bilayer lipid membranes formed from intestinal phospholipids was markedly decreased as compared to that of intestinal total lipid membranes, and was about equal to that of phosphatidylcholine membranes. In the intestinal phospholipid membrane-incorporated oleic acid or linolenic acid, however, the permeability coefficient of *p*-aminobenzoic acid was increased, and reverted to that observed in the intestinal lipid membranes.

In an effort to determine the contribution of neutral lipid containing fatty acids to drug transport, the effect of neutral lipid fraction on the permeability of *p*-aminobenzoic acid was also studied. As indicated in Table IV, the permeability coefficient of *p*-aminobenzoic acid across the membrane-incorporated neutral lipid fraction was significantly increased when compared with phosphatidylcholine membranes. The neutral lipid fraction eluted from a silicic acid column was applied to thin layer plates and developed with hexane/

TABLE IV

EFFECT OF FATTY ACIDS, INTESTINAL NEUTRAL LIPIDS AND MONOLEIN ON THE PERMEABILITY COEFFICIENT OF *p*-AMINOBENZOIC ACID ACROSS BILAYER LIPID MEMBRANES GENERATED FROM EGG PHOSPHATIDYLCHOLINE AND INTESTINAL PHOSPHOLIPIDS

Silicic acid column chromatography was utilized for the separation of total lipids into neutral lipid and phospholipid fractions as indicated under Methods. Specified amounts of fatty acids, monolein and intestinal neutral lipids were added into the membrane-forming solutions containing either egg phosphatidylcholine or intestinal phospholipids. Cholesterol (0.3 w/v) was incorporated into each membrane-forming solution. The aqueous phase was pH 6.5 isotonic phosphate buffer. Each value is the mean ± S.E. of 3—7 experiments.

		Permeability coefficient (10^{-5} cm/s)
Egg phosphatidylcholine		7.4 ± 0.4
Intestinal total lipids		14.4 ± 0.9
Intestinal phospholipids		7.6 ± 0.6
+ Oleic acid	0.1%	14.9 ± 1.0
+ Linoleic acid	0.1%	15.3 ± 0.9
Egg phosphatidylcholine		
+ Intestinal neutral lipids	0.3%	15.9 ± 0.6
+ Monolein	0.1%	9.9 ± 0.5

ethyl ether/acetic acid (70 : 30 : 1, by vol.). The major spots identified were cholesterol, free fatty acids, triglycerides and monoglycerides. Within the concentration range (0—1.5% w/v) employed, the addition of cholesterol had a small effect, usually tending to decrease the permeability of the drug [1]. Then, monolein which was selected as one of glycerides, was added to the membrane-forming solutions, but little effect on the permeability of *p*-aminobenzoic acid across their membranes was obtained. On the basis of these data, it seemed reasonable to assume that free fatty acids in the intestinal neutral lipid fraction could contribute to the increase in the permeability coefficient of *p*-aminobenzoic acid.

In the course of work designed to obtain a model for in vitro absorption from the gastrointestinal tract, Stricker [19] has described that the rate constants of the diffusion of acidic drugs through the barrier 1180 containing caprylic acid was in good accordance with the data on in vivo studies. It is possible that this lipid barrier might be different from the structure of biological membranes and their properties [20]. However, in view of the theoretical model [3,21] that the luminal surface of the intestinal-blood barrier is slightly acidic and there exists an accumulation of positively charged ions including H^+ at the aqueous-lipid interface, the fixed anion of caprylic acid in the lipid barrier may contribute towards maintaining the acidic microclimate resemblance to the characteristics of the mucosal cell membranes.

Interest in the fact that fatty acid was a component of intestinal mucosal lipid prompted the present study, which was designed to assess the effect of fatty acid on the permeability of drugs across bilayer lipid membranes. On the basis of the above results, possible mechanisms for the good absorbability of weakly acidic drugs from the intestine might be interpreted, at least in part, in terms of the contribution of phospholipids [1] and free fatty acids located in the microvillus membranes.

Recently, Winne [22] has investigated theoretically the conditions for deviations of the pH absorption curves from the course predicted by the simple pH partition theory, and has concluded that apart from provided pH absorption curves for the analysis, additional and independent experimental information about particular aspects such as microclimate pH and unstirred layer is needed to solve the problem. Lucas et al. [23] have shown the existence of an acidic microclimate pH at the surface of the small intestine by pH-sensitive microelectrodes, as originally proposed by Hogben et al. [3] (virtual pH). The results of our study also appear to lend some support to the existence of a more acid pH region adjacent to the intestinal cell surface.

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