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Intestinal absorption enhanced by unsaturated fatty acids: inhibitory effect of sulfhydryl modifiers

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In vitro absorption of carboxyfluorescein was performed with everted colonic segments of rats. Oleic acid solubilized by a nonionic surfactant HCO-60, but not HCO-60 alone, markedly enhanced the permeation of the otherwise poorly permeant carboxyfluorescein through the colonic mucosa. The effect produced by oleic acid was reduced to different extents by pretreating the mucosa with several SH reagents, with *N*-ethylmaleimide being the most effective. The inhibitory effect of *N*-ethylmaleimide was concentration dependent, with more than 5 mM completely blocking the enhancement of transmucosal permeability by oleic acid. The in vitro effect of *N*-ethylmaleimide in the absorption experiments was also observed in situ. The inhibitory effects of $HgCl_2$ and iodoacetamide were comparable to that of *N*-ethylmaleimide, whereas PCMPS, an impermeant SH blocker, had no effect on the enhanced permeation. Various other amino-group modifiers had also no effect on the enhancement. On the other hand, the inhibition was accompanied by a significant reduction in the level of non-protein thiols as well as protein SH groups. Diethyl maleate, which reduced only the non-protein SH level, had no pronounced effect on the oleic acid-induced permeability change. These results suggest that the intact SH group of membrane-associated protein is necessary for the enhanced permeation of carboxyfluorescein elicited by oleic acid.

Introduction

Among currently used therapeutic drugs, several are only slightly absorbed from the gastrointestinal tract because of their low lipid solubility or large molecular size. Therefore, it is necessary that their intestinal absorption be improved with the aid of an absorption enhancer. Bile salts are one candidate for the role of enhancer of intestinal absorption [1]. Recently, much attention has been focused on the enhancing properties of lipoidal substances such as unsaturated fatty acids and their monoacylglycerols because their enhancing ability is very high and is characterized by a transient and reversible nature [2]. We have reported that among the lipoidal substances, unsaturated long fatty acids were the most reactive, and that the colorectum was the most sensitive portion of the gastrointestinal tract [3–5]. The mechanism of this enhanced permeability through the mucosal membrane has not been explored in detail. Our electron spin resonance (ESR) and ^{1}H-NMR studies demonstrated that lipids which

Abbreviations: DIDS, 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid; SITS, 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid; PCMPS, *p*-chloromercuriphenylsulfonate; PCMB, *p*-chloromercuribenzoic acid; DTNB, 5,5′-dithiobis-2-nitrobenzoic acid; TNBS, 2,4,6-trinitrobenzensulfonate.

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are effective can disturb the hydrophobic region of the membrane's interior and interact with the polar region of the lipid bilayer [6]. Therefore, an increase in membrane permeability associated with membrane perturbation by reactive lipids appears to be one of the essential mechanisms. However, the contribution of membrane-bound proteins to the enhancement of intestinal permeability has not been throughly studied. Hydrophobic molecules such as free fatty acids can interact with the membrane components, i.e., phospholipids and membrane-bound proteins [7].

The purpose of the present work was to clarify the possible roles of membrane-associated proteins in the inducement of permeability change produced by unsaturated fatty acids. We used sulfhydryl-modifying reagents in an attempt to alter the effects of oleic acid on the transmucosal permeability of carboxyfluorescein, a compound which we have used as a dye impermeant to the intestine [8,9].

Materials and Methods

Materials

Oleic acid (99.9% purity) and polyoxygenated (60 molar) hydrogenated caster oil (HCO-60) were kindly supplied by Nippon Oil & Fat Co. (Tokyo, Japan) and Nikko Chemicals Co., Ltd. (Tokyo, Japan), respectively. The fatty acid was stored at −20 °C by dissolving in chloroform and substituting the atmospheric air with nitrogen gas. The fatty acid to stored was used within 4 weeks. 5(6)-Carboxyfluorescein was purchased from Eastman Kodak (Rochester, NY). *N*-Ethylmaleimide, iodoacetamide, mercuric chloride ($HgCl_2$), 4,4′-diisothiocyanatostilbene-2,2′-disulfonic acid (DIDS) and 4-acetamido-4′-isothiocyanatostilbene-2,2′-disulfonic acid (SITS) were obtained from Wako Pure Chemical Industry, Ltd. (Osaka, Japan). *p*-Chloromercuriphenylsulfonate (PCMPS) and *p*-chloromercuribenzoic acid (PCMB) were purchased from the Sigma Chemical Co. (St. Louis, MO). All other chemicals and solvents were of reagent grade quality.

Preparation of the test solution

Carboxyfluorescein titrated with 4 M NaOH and diluted to be 0.01 w/v% with 0.1 M Tris-HCl-buffered solution (pH 7.5). A micellar solution was prepared by dispersing the fatty acid into the carboxyfluorescein-HCO-60 solution (molar ratio of oleic acid/HCO-60, 30 : 4). A clear solution was obtained by sonication at 30 w for 5 min under a stream of nitrogen in an ice-cold water bath (Ohtake model 5202 sonicator, Tokyo, Japan). Prior to dispersion, the fatty acid was neutralized with 1 M NaOH.

In situ absorption experiments

In situ absorption experiments were performed by the single-pass perfusion technique using male Wistar albino rats weighing 230–280 g. The animals were fasted but water was given ad libitum for 16 h prior to the experiments. The rats were anesthetized with an intraperitoneal injection of sodium pentobarbital (30 mg/kg). The intestine was exposed through a midline incision, and an intestinal loop was prepared for silicon tubing cannulation at the proximal and distal ends of the entire large intestine (the colon and the rectum). While the test solution was being perfused, blood samples (approximately 0.2 ml) were collected at definite time intervals via a polyethylene catheter placed into the carotid artery. Perfusion of the test solutions was performed at the constant rate of 1 ml per min using a peristaltic pump (ATTO SJ-1220 miniperista pump, Japan). Perfusion of the carboxyfluorescein solution was carried out for 1 h (control period). Following this, a solution of carboxyfluorescein with 5 mM oleic acid was perfused for 1 h.

For pretreatment, 2 ml of the plain buffered solution or the buffered solution containing 0.1 mM *N*-ethylmaleimide was introduced into the intestinal loop, followed by the closing of the cannulated positions of the silicon tubing using forceps. The entire solution in the closed loop was forced out using the air in a syringe 15 min later and was washed three times with saline prior to the perfusion experiments. All solutions used in these experiments were prewarmed to 37 °C. Carboxyfluorescein in plasma samples was extracted as described in a previous publication [8]. Briefly, 50 μl of plasma were mixed with 0.1 ml Triton X-100 and 3 ml of 1 M HCl. 6 ml of *iso*-amyl alcohol was added to the mixture and carboxyfluorescein was extracted by occasional

shaking for 15 min. After centrifugation, the carboxyfluorescein in the solvent phase was re-extracted with 4 ml of 0.12 M Na_2CO_3/0.08 M $NaHCO_3$ (pH 10) for fluorimetrical assay as described below.

In vitro permeation experiments

The permeability of carboxyfluorescein through the intestinal mucosa was also investigated in vitro using everted large intestinal segments. Immediately after isolating an approximately 4.0-cm long section of the colon from the animal killed, the segment was everted on a glass rod (outer diameter 3 mm) and rinsed with ice-cold saline. Two silicon tubing cannula (internal diameter, 3 mm; outer diameter, 5 mm) were connected to the everted gut by ligation with silk thread at a position 5 mm from each end of the segment. The everted intestinal preparation was mounted onto a perfusion apparatus (LKB MICROPERPEX peristaltic pump, Sweden) and bathed in the test solution (outer compartment) of which 30 ml were warmed at an uniform temperature of 37 °C in a 50-ml glass tube immersed in a thermostat. The 0.1 M Tris-HCl-buffered solution (pH 7.4) containing 10 mM D-glucose was maintained at the same constant temperature and perfused into the serosal side of the loop (inner compartment) at a constant flow rate of 0.7 ml per min (0.70 ± 0.02 ml/min, S.D. $n = 25$) in a single-pass perfusion manner. No adsorption of carboxyfluorescein by the silicon tubing was detected. The mucosal and serosal fluids were gassed with a mild stream of 95% O_2 and 5% CO_2. 5 min aliquots of the perfusate effluent were collected in glass vials during the experimental period. These aliquots were diluted with 0.2 M sodium carbonate buffer (pH 10) for the fluorimetrical assay as described below.

Analytical method

Fluorescence of carboxyfluorescein at an excitation wavelength of 490 nm was measured at an emission wavelength of 520 nm using a Hitachi model 650-10 S spectrofluorometer (Hitachi Ltd., Tokyo, Japan).

The concentrations of protein-bound and non-protein sulfhydryls in the intestinal sections were determined according to the method of Sedlak and Lindsay [10]. After 350 mg of the intestine (wet tissue weight) were weighed out, the mucosal material obtained by scraping with a glass slide was homogenized in 8 ml of 0.02 M ethylenediaminetetraacetic acid disodium salt solution (pH 4.7) using a homogenizer (POLYTRON PT 10/35, KINEMATICA GmbH, Switzerland) in an ice bath. The reduced sulfhydryl concentration in these samples was determined using 5,5′-dithiobis-2-nitrobenzoic acid (DTNB) by reading the absorbance at 412 nm, using glutathione as the standard. The protein-bound SH level in some of these samples was also estimated using mercury orange from the absorbance at 470 nm by the method of Sakai [12].

Results

Effect of oleic acid on the mucosal permeability to carboxyfluorescein in the intestinal segment

The absorption-enhancing effect of oleic acid was estimated in terms of the in vitro permeability of carboxyfluorescein through the wall of the large intestine. The effluent-time profiles of carboxyfluorescein after administration with or without oleic acid are shown in Fig. 1A. A time lag in the elevation of the carboxyfluorescein level was observed in all cases and may be mainly attributed to the transmucosal diffusion of carboxyfluorescein, although it appeared to be shortened by the presence of oleic acid. The carboxyfluorescein concentration in the effluent was markedly increased, beginning approximately 15 min after the administration of the oleic acid micellar solution (with HCO-60), reaching an apparent plateau at approximately 60 min, whereas in the case of free carboxyfluorescein, its concentration increased gradually over time. HCO-60 per se did not show a pronounced adjuvant effect on the permeability of carboxyfluorescein (Table I). The oleic acid emulsion (without HCO-60) also improved the permeability of carboxyfluorescein; however, its effectiveness was about one-half that of the micellar solution (Fig. 1A and Table I). These results are consistent with our previous in situ experiment observations [4,10], and strongly support the hypothesis that solubilized fatty acids have the ability to enhance absorption. The slower increase in in vitro permeation by the emulsion was associated with maximal permeation (about 120 min),

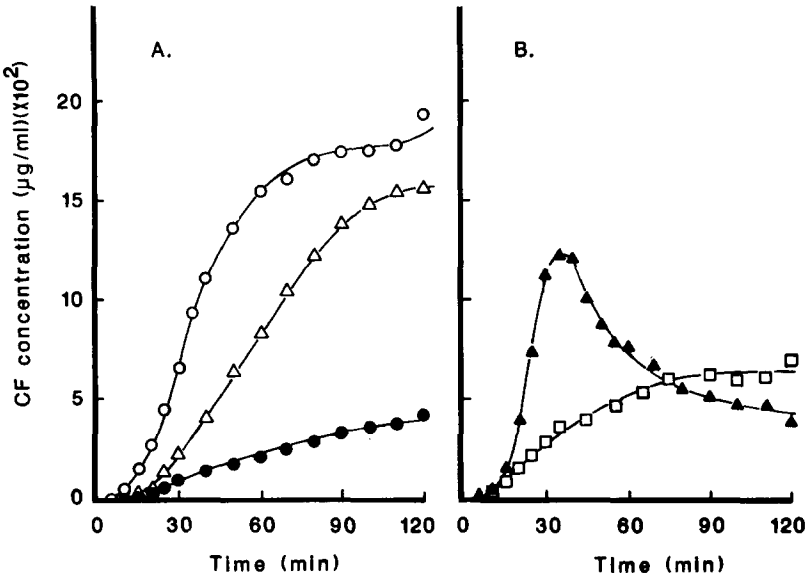

Fig. 1. Appearance of carboxyfluorescein (CF) on the serosal side of everted perfused colonic segments. (A) Administration of 100 μg/ml CF on the mucosal side in the presence (○, △) or absence (●) of oleic acid (10 mM). 0.1 M Tris-HCl-buffered solution containing 10 mM glucose was perfused on the serosal side at a flow rate of 0.7 ml/min for 2 h. Open circles and open triangles denote the micellar solution with HCO-60 and the emulsion of oleic acid, respectively. (B) Effect of *N*-ethylmaleimide on the transmucosal permeability of carboxyfluorescein in the presence of oleic acid. Oleic acid was solubilized with HCO-60 to 10 mM. Coadministration of the micellar solution with 1 mM *N*-ethylmaleimide (▲) and pretreatment of the mucosa with 1 mM *N*-ethylmaleimide for 15 min at room temperature (○). Each point represents the mean for separate experiments in duplicate.

which was closed to that of the micellar solution. However, maximal permeation of both preparations did not correspond to the in situ absorption promoted by oleic acid. Initial absorption is considered to be the most important for in situ and in vitro intestinal permeation induced by fatty acids. Therefore, we used the cumulative amount of permeated carboxyfluorescein for the first perfusion period of 90 min, designated as Q_p, in order to evaluate in vitro permeation. Comparison of the Q_p values is more appropriate for understanding the permeation-enhancing effect of fatty acids. The Q_p values were corrected for the apparent surface area of the colonic serosa and expressed as mg/350 mm^2 per 90 min. As shown in Fig. 2, Q_p increased with an increase in the fatty acid concentration. Although no significant differences were in Q_p observed among the 5, 10 and 30 mM concentrations, Q_p seemed to be maximal for 10 mM. Taking into account the smaller variance of Q_p, 10 mM was chosen as the concentration of oleic acid to be used in the subsequent experiments. On the other hand, when the epithelial cell layer was removed from the colonic segment using deoxycholate, the permeability was markedly increased and no significant differences were ob-

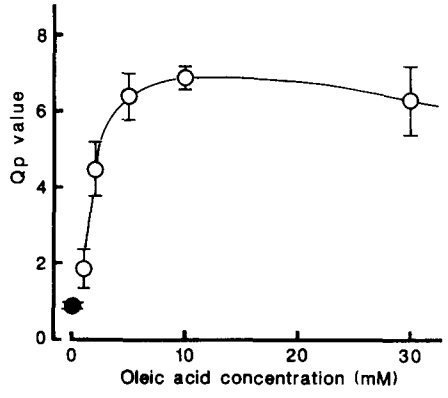

Fig. 2. Relationship between the oleic acid concentration on the mucosal side and the enhancement on the transmucosal permeability of carboxyfluorescein. Q_p values are plotted on the ordinate and are index of the permeability of carboxyfluorescein (see text). Closed circle denotes the Q_p value in the absence of oleic acid. Each value represents the mean with standard error of a group of three to six animals.

served in the Q_p values for the presence and absence of oleic acid (Table I). This suggests that the epithelial cells are the main barrier by which the permeation of carboxyfluorescein is restricted and that they may be the site of action of oleic acid.

Effect of N-ethylmaleimide on the mucosal permeability of carboxyfluorescein in the presence of oleic acid

Among the sulfhydryl-modifying reagents, *N*-ethylmaleimide was chosen first for study. When *N*-ethylmaleimide and oleic acid were coadministered as a micellar solution, the mucosal permeation of carboxyfluorescein was transiently increased up to a nearly maximal level, thereafter decreasing rapidly to the control level (Fig. 1B). On the other hand, in the case of pretreatment of the mucosa with *N*-ethylmaleimide, the enhancing effect of oleic acid was markedly inhibited throughout the entire incubation time period (Fig. 1B). This result indicates that *N*-ethylmaleimide administered to the mucosa inhibits considerably the enhanced permeation elicited by oleic acid, as does the fact that it takes a considerable period of time to demonstrate the inhibitory action. The pretreatment effects of several concentrations of *N*-ethylmaleimide were then tested. *N*-Ethylmaleimide suppressed the enhancing effect caused by oleic acid in a concentration-dependent manner (Fig. 3). 5 mM *N*-ethylmaleimide was the dose which sufficiently fulfilled the requirements of complete inhibition.

In order to confirm the inhibitory effects of *N*-ethylmaleimide, the in situ absorption experiments described earlier were performed. Our previous studies have shown that the absorption-enhancing effect of oleic acid was most prominent at the very early stage after in situ administration in a single dose [4,9]. When the oleic acid solution was continuously perfused in situ, the increased plasma concentration of carboxyfluorescein was prolonged (unpublished data). In this study, the single-pass perfusion technique was also used. As

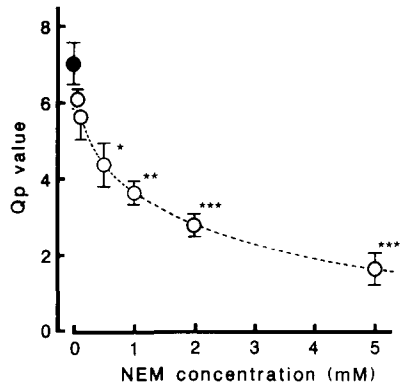

Fig. 3. Effect of various concentrations of *N*-ethylmaleimide (NEM) on the transmucosal permeability of carboxyfluorescein in the presence of oleic acid. Everted colonic segments were pretreated with various concentration of *N*-ethylmaleimide (○) or the medium (●) (control) for 15 min, followed by washing three times with ice-cold saline prior to carboxyfluorescein administration. Each value represents the mean with standard error for a group of three to six animals. Statistical comparison with the control was carried out using Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$, ***, $P < 0.001$.

TABLE I

EFFECT OF OLEIC ACID ON MUCOSAL PERMEABILITY IN VITRO

The in vitro perfusion experiments were carried out in rat everted colon. The concentration of carboxyfluorescein in the serosal perfusate after administration of carboxyfluorescein alone or with oleate into the mucosal compartment was measured in order to estimate the change in mucosal permeability. The cumulative amount of carboxyfluorescein which permeated through the colonic wall from the mucosal compartment per 350 mm^2 of serosal area for the first 90 min is expressed as the Q_p value. Results are represented the mean ± S.E. The concentration of oleate and HCO-60 were 10 mM and 1.3 mM, respectively. Statistical comparison with the control was carried out using the Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

Epithelium	Adjuvant	Q_p (μg/90 min per 350 mm^2)		*n*
Normal	none (control)	1.08 ± 0.115		6
	HCO-60	1.92 ± 0.317	n.s.	3
	oleate	3.58 ± 0.400	***	5
	oleate + HCO-60	7.29 ± 0.526	***	8
Stripped [a]	none	13.90 ± 1.55	n.s.	4
	oleate + HCO-60	14.72 ± 1.05		4

[a] The epithelial cells of the colon were throughly stripped by treating with 1% *N*-acetyl-L-cysteine and 15 mM sodium deoxycholate for 15 min at room temperature. After such a treatment, no epithelial cell can be seen by a light microscopy in the section of the colonic segment stained with hematoxylin and eosin following fixation in a 10% w/v isotonic formalin.

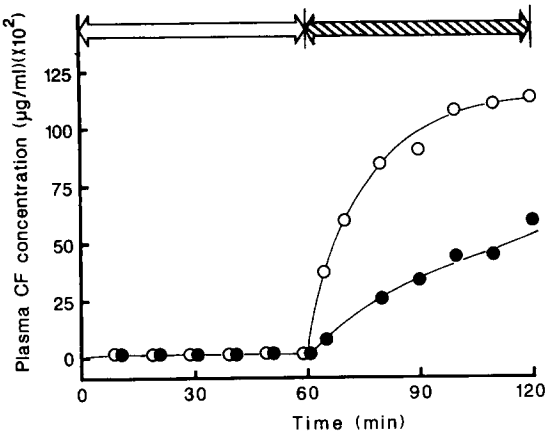

Fig. 4. Plasma concentration time curves during the in situ single-pass perfusion of carboxyfluorescein (CF). Effect of pretreatment of the mucosa with 0.1 mM *N*-ethylmaleimide (●); sham operation with the medium (○). Rats underwent a perfusion period of 1 h (hatched arrow) with 5 mM oleic acid, preceded by a control period of 1 h without any adjuvants (open arrow). Each point represents the mean with standard error for separate experiments in duplicate.

shown in Fig. 4, the increment of plasma concentration of carboxyfluorescein was markedly reduced by pretreating the mucosa with *N*-ethylmaleimide, although this pretreatment did not influence the absorption of free carboxyfluorescein. These results are fairly consistent with those concerning the inhibitory effects in the in vitro studies.

Tissue sulfhydryl content and oleic acid-induced permeability enhancement

The above results suggest that an SH-related substance is possibly involved in the mechanism of permeability enhancement by oleic acid. Therefore, the SH contents in the tissue were measured using DTNB. As shown in Table II, *N*-ethylmaleimide tended to decrease both non-protein SH and protein-bound SH levels as its concentration increased. Non-protein SH and protein-bound SH levels were significantly reduced by concentrations of *N*-ethylmaleimide greater than 2 and 5 mM, respectively. The use of a more specific and hydrophobic SH reagent than DTNB, mercury orange [12], permitted the detection of a significant reduction in protein-bound SH, even with 1 mM *N*-ethylmaleimide. The reduction was similar

TABLE II

EFFECT OF *N*-ETHYLMALEIMIDE ON TISSUE SULFHYDRYL CONTENT

Everted sacs prepared from the midcolon were incubated with various concentrations of *N*-ethylmaleimide solution (pH 7.5) or the medium (control) for 15 min at room temperature, followed by removal of the incubation fluid. After washing with ice-cold saline, the concentrations of protein-bound and non-protein sulfhydryls in the tissue were determined using DTNB according to Sedlak and Lindsay's method [10]. Protein content in the homogenate was determined according to Lowry's method modified by Bensadoun and Weinstein [11]. These assays were performed with regard to 5 mM diethyl maleate or 10 mM oleate/HCO-60. Results were calculated as the concentration of protein-bound SH or non-protein SH per g wet weight of tissue and are represented as the mean of the number of determination in parentheses with standard error. Statistical comparison with the control was carried out using the Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$.

Treatment	Sulfhydryl concentration (μmol/g tissue)	
	protein-bound SH	non-protein SH
Control	8.89±0.68 (11)	1.45±0.07 (12)
	(51.73±1.72) (5)	
N-Ethylmaleimide		
1 mM	9.32±0.87 (5)	1.30±0.09 (7)
	(35.86±5.42) (4) *	
2 mM	7.58±0.89 (7)	0.61±0.18 (7) ***
	(26.28±3.82) (4) **	
5 mM	5.37±0.46 (9) ***	0.46±0.03 (9) ***
Diethylmaleate		
5 mM	9.40±0.60 (8)	0.52±0.05 (7) ***
Oleate		
10 mM	10.04±0.81 (7)	1.42±0.08 (7)

to that observed for the in vitro inhibitory effect. On the other hand, oleic acid did not affect either of the SH levels (Table II), although it markedly promoted the transmucosal permeability of carboxyfluorescein. At this point, a specific depleting agent of non-protein thiols, diethyl maleate [14], was applied in order to modify the SH levels for comparison with *N*-ethylmaleimide. 5 mM diethyl maleate significantly reduced the non-protein SH level, but not the protein-bound SH level (Table II). This was in contrast with the effects of *N*-ethylmaleimide. Furthermore, it was observed

during the in vitro permeation study that diethyl maleate did not affect the permeation enhancing effect of oleic acid, a result in contrast to that for *N*-ethylmaleimide (Table III). Therefore, these results exclude the possibility that *N*-ethylmaleimide can suppress the permeation-enhancing effect of oleic acid via a reduction of the non-protein SH level. Otherwise, it is likely that blocking either the protein-bound SH or both SH groups in the intestinal tissue may lead to inhibition of the permeability change. On the other hand, *N*-ethylmaleimide has also been shown to react with α-amino groups of proteins [13]. However, our experiments with DIDS and SITS (amino-group modifiers) did not show any pronounced effects on the oleic acid enhanced permeation. Furthermore, 2,4,5-trinitrobenzensulfonate (TNBS), an irreversible and potent amino-group modifier, was also shown to be ineffective (Table III).

TABLE III

EFFECT OF VARIOUS CHEMICAL MODIFIERS ON THE PERMEABILITY OF CARBOXYFLUORESCEIN

The effects of diethyl maleate and several amino-group modifiers on the transmucosal permeability of carboxyfluorescein in the presence of oleate (10 mM) were investigated. Pretreatment studies were performed as described in Table II. Abbreviations are described in the text. Data are expressed as the mean for two to four experiments.

	Reagent		Q_p (μg/90 min per 350 mm^2)
Control	–		7.09
Pretreatment			
	diethyl maleate	5 mM	8.01
	TNBS	1 mM	6.82
		5 mM	6.45
	SITS	1 mM	7.86
	DIDS	1 mM	6.71
Coadministration			
	SITS	10^{-6} M	6.00
		10^{-5} M	7.15
		10^{-4} M	6.49
		10^{-3} M	7.80
	DIDS	10^{-6} M	7.26
		10^{-5} M	5.98
		10^{-4} M	6.50
		10^{-3} M	7.50

TABLE IV

COMPARISON OF EFFECTS OF VARIOUS SULFHYDRYL MODIFIERS ON THE TRANSMUCOSAL PERMEABILITY OF CARBOXYFLUORESCEIN IN THE PRESENCE OF OLEIC ACID

Pretreatment studies were performed using various SH reagents as described in Table II and their inhibitory effects on the permeation enhancement of the colonic mucosa produced by oleic acid (10 mM) were compared. Data are expressed as the mean with S.E. for the number of animals. Abbreviations are seen in Materials. Statistical comparison with the control was carried out using Student's *t*-test: *, $P < 0.05$; **, $P < 0.01$; ***, $P < 0.001$; n.s., not significant.

Pretreatment		Q_p (μg/90 min per 350 mm^2)	*n*
None (control)	–	7.15 ± 0.552	8
PCMPS	5 mM	7.01 ± 0.668 n.s.	4
	10 mM	7.17 ± 0.454 n.s.	6
PCMB [a]	5 mM	4.36 ± 0.302 ***	6
$HgCl_2$	5 mM	4.64 ± 0.758 *	4
	10 mM	1.83 ± 0.298 ***	4
N-Ethylmaleimide	5 mM	1.85 ± 0.506 ***	4
	10 mM	0.76 ± 0.138 ***	3
Iodoacetamide	5 mM	5.01 ± 0.763 *	5
	10 mM	3.71 ± 0.467 ***	6

[a] PCMB was solubilized in the medium with a minimum amount of ethanol which showed no effect on the mucosal permeability.

Effects of the various sulfhydryl modifiers on the permeability-enhancing effect of oleic acid

On the basis of the results obtained in the above experiments, the effects of the other SH-modifying reagents were investigated. As shown in Table IV, PCMPS, a membrane-impermeable mercurial, did not shown any effect; however, PCMB and $HgCl_2$, membrane-permeable mercurials, reduced the permeability of carboxyfluorescein to about 20–80% of the control values. It was observed that the inhibitory effect of $HgCl_2$ was concentration dependent and comparable to *N*-ethylmaleimide with respect to intensity. Iodoacetamide also significantly suppressed the effect of oleic acid, although its inhibitory effect was weaker than that of *N*-ethylmaleimide. It appears that the intensities of the inhibitory effects of the SH reagents are related, at least in part, to their membrane permeability, since only PCMPS, an impermeable reagent, did not exhibit an inhibitory effect.

Discussion

Fatty acids may play an important role with respect to the barrier of biological membranes towards exogenous agents with high hydrophilicity. Several fatty acids had been clearly demonstrated to increase the in situ colonic absorption of carboxyfluorescein when administered in the micellar solutions of harmless surfactants or its own emulsion [2]. In the present paper, the in vitro everted perfused system was used to study the mechanism of absorption promotion by fatty acids. Transmucosal permeation from the in vitro rat colon (Table I) clearly showed that oleic acid-enhanced permeability was compatible with the previous results in situ. Similar results to those for linoleic acid were obtained (data not shown). The in vitro system dose not involve the removal of carboxyfluorescein from the intestinal mucosa into the blood and lymph circulations. This supports our claim that the improved absorption of poorly absorbable drugs observed with unsaturated fatty acids was not due to increased removal from the tissue via the capillary and lymphatic microcirculations, but rather to an enhancement of the intestinal mucosa permeability [5]. Hori et al. [15] have suggested that free fatty acids in the intestinal neutral lipid fraction, such as oleic acid and linoleic acid, may contribute to the increase in the permeability of water-soluble drugs. It has been pointed out that lipids having the ability to induce membrane fusion in vitro, i.e., fusogenic lipids [16], markedly enhance the intestinal absorption of water-soluble drugs [6]. With respect to the effect of fusogenic fatty acids, including oleic acid and linoleic acid, on the physiological activities of biological membranes, alteration of the lipid structural dynamics has been suggested to be a common mechanism [7]. As previously mentioned, in our physicochemical study on liposomal membranes, disorder of the lipid bilayer accounted for the enhanced absorption of these drugs [18]; however, their releasing properties from liposomes were not completely compatible with the in vivo observations.

Schaeffer et al. [18] suggested that an increase in the lipid fluidity of frog corneal epithelium, caused by unsaturated fatty acids, may promote the lateral diffusion of the membrane proteins leading to the accelerated ion transport. In addition to the lipid components of the membrane, the possibility that some peptide- or protein-like substances located in the mucosa may contribute to the enhancement of intestinal permeability can not be neglected. Thus, it is reasonable to assume that protein–lipid interactions may be connected with the enhancement in membrane permeability. In the present study, it is noteworthy that some SH-modifying reagents almost totally suppressed the enhancing effect of oleic acid. It has been suggested that monofunctional SH modifiers may protect the leak formation induced by oxidative stress or diamide, causing a reduction in the permeability coefficient to water in the human erythrocyte membrane [19,20]. Heller and coworkers [20] demonstrated that pretreatment of red cells with *N*-ethylmaleimide completely prevented the formation of protein aggregates. These reports suggest that intact SH groups are essential for the formation of protein aggregates within the membrane.

The present results indicated that *N*-ethylmaleimide was the most effective at promoting mucosal transport among the various SH-modifying reagents tested (Table IV) and that its inhibitory effect was concentration dependent (Fig. 3). Membrane-permeable SH modifiers such as $HgCl_2$, iodoacetamide and PCMB were also effective, whereas the membrane-impermeable reagent PCMPS was not (Table IV). Therefore, the ability of SH reagents to alter the inhibitory reactivity suggests that SH groups existing inside the membrane of the epithelium may play an important role in the transport process. Treatment with *N*-ethylmaleimide resulted in a reduction of both the non-protein SH and protein-bound SH contents in the mucosal epithelium (Table II). On the other hand, diethyl maleate, a depleting agent, which did not inhibit the in vitro permeation, reduced the SH level of non-protein SH, but not of protein-bound SH. A major non-protein SH in the tissue is glutathione, a compound which contributes to maintaining the integrity of the membrane. However, from our results, it is unlikely that non-protein SH compounds like glutathione are related to the inhibitory mechanism. It is possible that the protein-bound SH groups may be largely responsible for the increased transmucosal permeability with oleic acid.

Smyth et al. [13] reported that *N*-ethylmaleimide should be used below neutrality to avoid an excess of the reagent so as to obtain a high degree of specificity for thiol groups. Our results may also exclude an involvement of amino group modification with *N*-ethylmaleimide for the following reasons: (1) the tissues were exposed to *N*-ethylmaleimide at near neutrality (pH 7.4) for a shorter time (15 min) at relatively lower temperatures ($22 \pm 3°C$), (2) the positive effect of $HgCl_2$, a much more specific reagent for sulfhydryl modification, (3) the ineffectiveness of several aminogroup modifiers (Fig. 6) and (4) the good correlation between tissue SH contents (protein-bound SH rather than non-protein SH) and the extent of inhibition. These observation seem to suggest sulfhydryl modification.

In conclusion, on the basis of in vitro sulfhydryl inhibition, it is suggested that a specific interaction with membrane-associated proteins is involved in the mechanism of the enhancing effect of fusogenic lipids. However, the mechanistic relationship between the lipid bilayer and associated proteins, and the precise localization of these proteins remains unclear, suggesting the need for more detailed studies.

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References

1 Gibaldi, M. (1970) Fed. Proc. 29, 1343–1349.
2 Muranishi, S. (1985) Pharmaceut. Res. 2, 108–118.
3 Muranishi, S., Tokunaga, Y., Taniguchi, K. and Sezaki, H. (1977) Chem. Pharm. Bull. 25, 1159–1161.
4 Taniguchi, K., Muranishi, S. and Sezaki, H. (1980) Int. J. Pharmaceut. 4, 219–228.
5 Muranushi, N., Kinugawa, M., Nakajima, Y., Muranishi, S. and Sezaki, H. (1980) Int. J. Pharmaceut. 4, 271–279.
6 Muranushi, N., Takagi, N., Muranishi, S. and Sezaki, H. (1981) Chem. Phys. Lipids 28, 269–279.
7 Brenner, R.R. (1984) Proc. Lipid Res. 23, 69–96.
8 Murakami, M., Yoshikawa, H., Takada, K. and Muranishi, S. (1986) Pharmaceut. Res. 3, 35–40.
9 Murakami, M., Masuda, Y., Fukui, H., Yoshikawa, H., Takada, K. and Muranishi, S. (1985) J. Pharmacobio-Dyn. 8, s131 (Abstr.).
10 Sedlak, J. and Lindsay, R.H. (1968) Anal. Biochem. 25, 192–205.
11 Bensadoun, A. and Weinstein, D. (1976) Anal. Biochem. 70, 241–250.
12 Sakai, H. (1968) Anal. Biochem. 26, 269–276.
13 Smyth, D.G., Blumenfeld, O.O. and Konigsberg, W. (1964) Biochem. J. 91, 589–595.
14 Boyd, S.C., Sasame, H.A. and Boyd, M.R. (1979) Science 205, 1010–1012.
15 Hori, R., Kagimoto, Y., Kamiya, K. and Inui, K. (1978) Biochim. Biophys. Acta 509, 510–518.
16 Ahkong, Q.F., Fisher, D., Tampion, W. and Lucy, J.A. (1973) Biochem. J. 136, 147–155.
17 Muranushi, H., Nakajima, Y., Kinugawa, M., Muranishi, S. and Sezaki, H. (1980) Int. J. Pharmaceut. 4, 281–290.
18 Schaeffer, B.F. and Zadunaisky, T.A. (1979) Biochim. Biophys. Acta 556, 131–143.
19 Sha'afi, R.I. (1981) in Membrane Transport (Bonting, S.L. and De Pont, J.J.H.H.M., eds.), pp. 29–60, Elsevier/North-Holland, Amsterdam.
20 Heller, K.B., Poser, B., Haest, C.W.M. and Deuticke, B. (1984) Biochim. Biophys. Acta 777, 107–116.