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Role of Membrane Components, Glycocalyx and Lipid in Absorption of Water-soluble Dyes from the Rat Small Intestine

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Role of membrane components in the absorption from the rat small intestine of four water-soluble dyes, methylene blue (MB), bromthymol blue (BTB), bromphenol blue (BPB), and phenol red (PR) was investigated.

Uptake by the isolated epithelial cells, binding to the brush borders, and uptake by the everted sac of BTB and BPB, anionic dyes, were significantly reduced by digestion with papain, but in PR no effect was found. Binding of the dyes to the components released by the cellulose papain complex digestion from the brush borders was examined using the method of equilibrium dialysis. More MB and BTB bound to the components than BPB and PR, poorly absorbable dyes. These results suggested that membrane component contributing to the dye binding to the brush borders may be glycocalyx (surface coat).

Lipids, other major membrane components, were extracted from rat intestinal mucosa and separated to neutral lipid (NL), glycolipid (GL), phosphatidylcholine (PC), and phosphatidylethanolamine (PE) fractions. Each fraction was dissolved in chloroform and partitioning behavior of the dyes was examined using a non-emulsifying system. Extent of transfer of the dyes from aqueous phase into chloroform containing GL and total lipid (TL) was well correlated to the extent of net absorption and tissue accumulation respectively.

In the previous report,[2] it was suggested that the binding to the mucosa, especially to the brush borders (microvilli), is important as the first step in the absorptive process of four water-soluble dyes, methylene blue (MB), bromthymol blue (BTB), bromphenol blue (BPB), and phenol red (PR).

The intestinal cell surface is covered with a carbohydrate-rich glycocalyx.[3] From autoradiographic studies using labelled precursors, this glycocalyx appears to be an integral part of the brush border membrane.[4] Forstner, *et al.*[5] have shown that the rat intestinal glycocalyx (surface coat) consists of glycoprotein and may be involved in the pre-absorptive binding or filtering of intestinal solutes. They may form a protective mucosal barrier against the external environment.

It is widely accepted, also, that lipid is one of the other major components of plasma membrane. Despite the extensive body of literature describing the importance of lipids in the biological transfer, past work on the role of mucosal lipids in the intestinal absorptive process has been relatively sparse. Thus, through the understanding of the role of glycocalyx and lipids in the absorption of four water-soluble dyes from the rat small intestine and the identification of the components to which these dyes bind at the brush borders should be of general interest, since their absorptive characteristics do not obey so-called lipoid theory and the mode of intestinal absorption of this type of compounds has been a subject of speculation.

1) Location: *Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto.*
2) J. Nakamura, Y. Yoshizaki, M. Yasuhara, T. Kimura, S. Muranishi, and H. Sezaki, *Chem. Pharm. Bull.* (Tokyo), **24**, 683 (1976).
3) S. Ito, *J. Cell. Biol.*, **27**, 475 (1965).
4) A. Rambourg, M. Neutra, and G.P. Leblond, *Anat. Rec.*, **154**, 41 (1966).
5) G.G. Forstner, *Amer. J. Med. Sci.*, **258**, 172 (1969).

Experimental

Materials——Dyes used in this paper were of reagent grade. All other reagents used in these experiments were of the finest grade available.

Analytical Methods——The same spectrophotometric methods were used as described previously.[2]

Preparation of Brush Borders and Isolated Epithelial Cells——Methods of preparation of brush borders and isolated epithelial cells were the same as described previously.[2]

Digestion of Everted Sac by Papain——Male Wistar albino rats weighing 150—200 g were used. Animals were anesthetized with pentobarbital, given intraperitoneally, and the small intestine, from Treitz ligament to the distal end of the ileum, was washed with ice-cold pH 6.5 buffer solution and isolated. The isolated intestine was cut into two segment: 30 cm from the Treitz ligament, and 30 cm from the distal end of the ileum. Each segment was everted with a wire and ligated at both ends. Each segment was incubated for 30 min with 30 ml of the incubation mixture at 37°. The incubation mixture contained (per ml) 1 mg of papain, 3 μmole of cysteine, and 12.0 μmole of potassium phosphate buffer, pH 7.0. At the end of each digestion period the everted sac was washed with 50 ml of ice-cold pH 6.5 buffer for 3 min and then placed in a centrifuge tube containing 10 ml of buffered dye solution (0.1 mM, pH 6.5). At the end of incubation for 5 min at 37°, the everted sac was washed with 50 ml of ice-cold pH 6.5 buffer solution. After weighing, the everted sac was homogenized and determined as mentioned elsewhere.

Determination of Hexose and Hexosamine——After digestion of the everted sac by papain, the incubation mixture was centrifuged at 3000 rpm for 10 min. The supernatant was employed for determination of hexose and hexosamine. Hexose was determined with anthrone with D-glucose as standard.[6] Hexosamine was determined by the method as described by Allison.[7] D-Glucosamine was used as standard.

Protein Determination——Protein was estimated by the method of Lowry, *et al.*[8] using bovine serum albumin as standard.

Digestion of Brush Borders with Proteolytic Enzymes and Treatment with Organic Solvents and Detergents——Brush borders were incubated for 30 min at 37° with proteolytic enzymes (0.1 mg/ml of trypsin and pronase in pH 6.5 buffer solution), detergents [0.1, 1.0, and 5.0 mM of sodium lauryl sulfate (SLS) and 0.5% of Triton X-100] in pH 6.5 buffer solution and in ice-cold water with organic solvents (*n*-propanol and 10% water in acetone). Ten-fold of organic solvents its wet weight and 25-fold of proteolytic enzymes and detergents were employed. In the case of papain digestion, the incubation medium contained (per ml) 0.01 mg of papain, 3 μmole of cysteine, 3 μmole of ethylenediaminetetraacetic acid disodium salt (EDTA 2Na), brush borders (0.17 mg of protein) and 12.5 μmole of potassium phosphate buffer, pH 7.0. At the end of incubation period the mixture was centrifuged at 2500 rpm for 15 min. The pellets were washed twice with ice-cold pH 6.5 buffer solution and suspended in pH 6.5 buffer solution (33.5 ml per wet weight of brush borders). Binding experiment was done by the same method as described in the previous paper.[2]

Digestion of Isolated Epithelial Cells by Papain——Isolated epithelial cells from the rat small intestine, prepared by the method as described in the previous paper,[2] were incubated for 30 min with papain at 37°, being bubbled with oxygen gas (95% O_2, 5% CO_2). The incubation mixture contained 1 mg/ml of papain in the presence of 5 mM cysteine and 5 mM EDTA 2Na in pH 6.5 buffer solution. Total volume of the incubation mixture was 50-fold its wet weight. At the end of incubation period the mixture was cooled in ice-cold water and centrifuged at 2500 rpm for 5 min. The pellets were washed twice with ice-cold, oxygenated pH 6.5 buffer solution and suspended in 50-fold its wet weight of pH 6.5 buffer solution. Uptake experiments were the same as described in the previous paper.[2]

Binding to the Supernatant Fraction after Cellulose-Papain Complex Digestion of Brush Borders——Cellulose-papain complex was prepared as described by Eichholz.[9] Brush borders were incubated for 30 min with the cellulose-papain complex. The incubation mixture contained (per ml) 0.11 mg of cellulose-papain complex, 3 μmole of cysteine, 3 μmole of EDTA 2Na and 12.5 μmole of potassium phosphate buffer, pH 7.0. At the end of incubation period the mixture was cooled in ice-cold water and centrifuged at $13500 \times g$ for 30 min at 2°. The supernatant was dialysed against pH 6.5 buffer solution at 4° and then concentrated by ultrafiltration using Eastman Chemical HF35 membrane (mol. wt. 5000). Equilibrium dialysis method was adopted to estimate the binding. Dyes were dissolved in pH 6.5 buffer solution at the concentration of 0.1 mM. Four ml of the dye solution was placed in a 10 ml centrifuge tube as the outer fluid, cellulose tubing (Visking Co, #8/32) containing 2 ml of the supernatant was immersed in the centrifuge tube. After equilibration, the dye concentration in the outer fluid was spectrophotometrically determined as described in the previous paper.[2]

Extraction, Separation, and Estimation of Lipids——The intestinal mucosa was scraped off by the method as described previously.[2] Lipids were extracted from the mucosa as described by Folch, *et al.*[10] Low and

6) J.H. Roe, *J. Biol. Chem.*, **212**, 335 (1955).
7) D.J. Allison and Q.T. Smith, *Anal. Biochem.*, **13**, 510 (1965).
8) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
9) A. Eichholz, *Biochim. Biophys. Acta.*, **163**, 101 (1968).
10) J. Folch, M. Lees, and G.H. Sloane-Stanley, *J. Biol. Chem.*, **226**, 497 (1957).

upper phases were allowed to separate by standing overnight at 4°. The low phase was then evaporated to dryness under N_2 and stored in chloroform under N_2. (Total lipid) The total lipid dissolved in chloroform was applied to a column of silicic acid (100 mesh, Mallinckrodt), containing Hyflo-super-cel. The neutral lipid (NL) was eluted with chloroform, then glycolipid (GL) with acetone, phosphatidylethanolamine (PE) containing phosphatidylserine with chloroform–methanol (8: 2, v/v), and finally phosphatidylcholine (PC) containing sphingomyeline and lysophosphatidylcholine with chloroform–methanol (6: 4, v/v). The eluted fractions were resolved with thin-layer chromatography in Silicagel G with chloroform–methanol–H_2O (65: 25: 4).

Determination of Dye Partitioning——Dye partitioning was determined by the same methods as those reported in the paper from this laboratory[11] except that lipid was dissolved in chloroform at 1 mg/ml.

Results

Effect of papain digestion on the uptake of dyes by the everted sac of the rat small intestine was examined. After digestion with papain for 30 min, uptake by the everted sacs, 30 cm from Treize ligament (jejunum) and 30 cm from the distal end of the ileum (ileum) for 5 min were carried out. The results are given in Fig. 1. Uptake by the everted sac (jejunum+ ileum) of MB, BTB, and BPB by digestion with papain were reduced 14.0, 21.3, and 26.5%, respectively. No effect was found in PR. Forstner has recently reported that rat intestinal surface-membrane glycoprotein labelled with [1-^{14}C]-glucosamine released its radioactivity by brief digestion with papain.[12] In order to examine whether intestinal surface-membrane glycoprotein was released by digestion with papain in this study, sugar components were determined. As is evident from Table I, hexose and hexosamine were found in components released by digestion of the everted sac with papain. This result confirmed that papain digested glycocalyx (glycoprotein) of intestinal cell surface (brush borders).

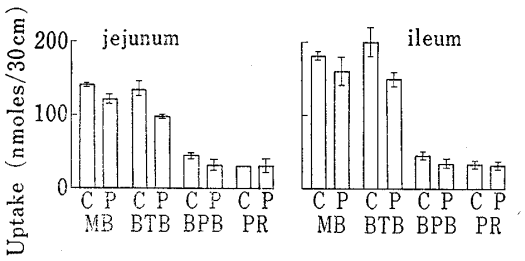

Fig. 1. Effect of Papain Digestion on the Uptake of Dyes by the Everted Sac

c: control
p: papain digestion
jejunum: 30 cm from Treitz ligament
ileum: 30 cm from the dital end of the ileum
Each segment was incubated for 30 min with the incubation mixture at 37°. Dyes uptake was expressed as nmoles/each segment (30 cm)/5 min.

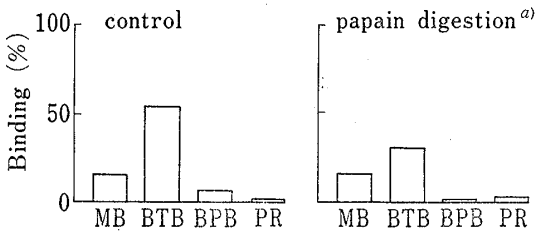

Fig. 2. Effect of Papain Digestion on the Binding to Brush Borders

a) Brush borders were incubated for 30 min at 37° with the incubation mixture (papain).
Four ml of dye solution (0.1 mM) was added to 2 ml of the brush border suspension and incubated for 60 min at 37°. After centrifugation, the supernatant was determined as described elsewhere.

Table I. Hexose and Hexosamine Released by Digestion of the Everted Sac with Papain

	Hexosamine (μg/ml)	Hexose (μg/ml)
Control	6.4	3.9
Papain digestion	19.0	42.6

Hexosamine was determined with D-glucosamine as standard.
Hexose was determined with anthrone with D-glucose as standard.

11) S. Furusawa, K. Okumura, and H. Sezaki, *J. Pharm. Pharmacol.*, **24**, 272 (1971).
12) G.G. Forstner, *Biochem. J.*, **121**, 781 (1971).

In the previous report, it was suggested that the binding to the mucosa, especially to the brush borders (microvilli) at the first step of intestinal absorption, is an important absorptive process of these water-soluble dyes, MB, BTB, BPB, and PR. Effect of papain digestion on the binding to brush borders that dyes seem to contact at the first step of intestinal absorption, was examined. The results are shown in Fig. 2. It was found that the binding to the brush borders of BPB and BTB were significantly reduced. Although the uptake by the everted sac of MB after digestion with papain was slightly reduced, no effect was observed in the case of the binding to the brush borders. It is of interest to note that BPB could not bind to the brush borders after digestion with papain. In order to clarify the role of glycocalyx in the intestinal absorption of these dyes, effect of papain digestion on the uptake by the isolated epithelial cells was examined. As is evident from Fig. 3, it was found that the uptake by the isolated epithelial cells of BTB and BPB were reduced like the binding to the brush borders. Slight reduction was found in MB and no effect in PR. As was noted in the case of the binding to the brush borders, the uptake by the isolated epithelial cells of BPB after digestion with papain could not be found. In addition to the binding to the brush borders and the uptake by the isolated epithelial cells after digestion with papain, the binding to the components released was examined. The brush borders were digested with papain complexed with cellulose for 30 min at 37° and then centrifuged at 13500×*g* for 30 min to remove the brush borders and the complexed papain. After dialysis and concentration, the binding to supernatants

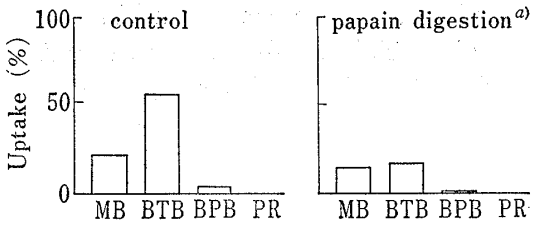

Fig. 3. Effect of Papain Digestion on the Uptake by Isolated Epithelial Cells

a) Isolated epithelial cells were incubated for 30 min at 37° with the incubation mixture (papain), being bubbled with oxygen gas (95% O_2, 5% CO_2).
Forty ml of dye solution (0.1 mM) and 20 ml of the cell suspension were placed in a 100 ml beaker. The beaker was stirred with a magnetic stirrer for 60 min at 37°. After centrifugation. The supernatant was determined as described elsewhere.

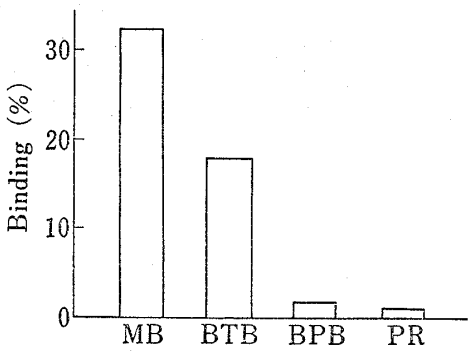

Fig. 4. Binding to the Supernatant Fraction after Cellulose-Papain Complex Digestion of Brush Borders

Brush borders were incubated for 30 min at 37° with the incubation mixture (cellulose-papain complex). Equilibrium dialysis method was adopted to estimate the binding.

was examined using equilibrium dialysis method. As shown in Fig. 4, the binding to supernatants of MB and BTB was greater than that of BPB and PR, poorly absorbable dyes.

Recently, it has been demonstrated that intestinal hydrolases have been released by proteolytic enzymes and detergents in addition to papain. Louvard, *et al.*[13] reported that the material solubilized by Triton X-100 was obviously different from that released by papain and papain digestion released 50% of the total sugar and glycolipid of the isolated brush border membrane vesicles without disruption of the unit membrane, but not trace of phospholipids and cholesterol. Therefore, proteolytic enzymes, organic solvents, and detergents in addition to papain were employed to examine the role of membrane components in the intestinal absorption of these dyes. Effect of proteolytic enzymes digestion, organic solvents and detergents treatments on the binding to the brush borders is shown in Table II.

The binding to the brush borders of BTB and BPB was significantly reduced by digestion with trypsin and pronase as well as papain. Ten percentages water in acetone and *n*-propanol

13) D. Louvard, S. Maroux, Ch. Vannier, and P. Desnuelle, *Biochim. Biophys. Acta.*, 375, 236 (1975).

Table II. Effect of Proteolytic Enzymes Digestion, Organic Solvents and Detergents Treatments on the Binding to Brush Borders

Treatment and digestion	Binding %			
	MB	BTB	BPB	PR
Control	15.5	54.0	5.8	1.2
Pronase	12.9	12.0	0.5	0.3
Trypsin	10.1	17.2	0.7	0.2
10% water in acetone	19.3	72.5	28.9	1.1
n-Propanol	22.6	70.2	19.9	1.3
SLS 0.1 mM	16.2	51.5	5.1	0.4
1.0 mM	8.4	45.0	6.2	0.9
5.0 mM	2.6	22.7	2.4	0.2
Triton X-100	13.0	39.1	8.7	0.6

Brush borders were incubated for 30 min at 37° with proteolytic enzymes, detergents and in ice-cold water with organic solvents. Four ml of dye solution (0.1 mM) was added to 2 ml of the brush border suspension and incubated for 60 min at 37°. After centrifugation, the supernatant was determined as described elsewhere.

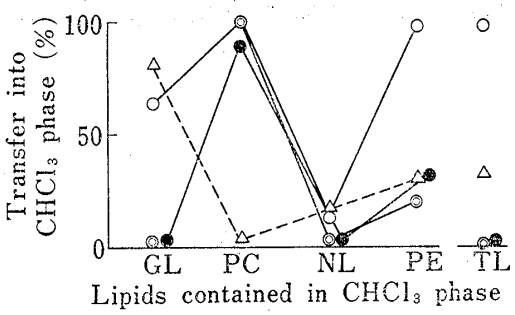

Fig. 5. Effect of Lipids on the Transfer of the Dyes from Water into Chloroform

GL: glycolipid, PC: phosphatidylcholine, NL: neutral lipid, PE: phosphatidylethanolamine, TL: total lipid
△: MB ○: BTB ◎: BPB ●: PR
concentration of dyes=0.1 mM, pH 6.5
Lipid was dissolved in chloroform at 1 mg/ml.

were employed to extract lipid from the brush borders. Enhancement effect of the binding to the brush borders of BTB, MB, and BPB was observed by treatment with 10% water in acetone and with *n*-propanol. In PR, no enhancement effect of the binding to the brush borders was found. As detergents, SLS and Triton X-100 were selected. It is of interest to note that the binding to the brush borders of MB, compared with BTB and BPB was reduced by treatment with SLS (1.0 mM). By treatment with Triton X-100, the binding to the brush borders of BTB was significantly reduced. Detergent can solubilize membrane components. However, in this study, it is not clear that which component is preferentially solubilized by detergents, protein or lipid. In the case of pronase digestion, SLS, and 10% water in acetone treatment, similar results were also obtained with the uptake by the isolated epithelial cells.

It is well accepted that lipid is one of the major component of plasma membrane. Recently, there appears a few reports concerning the role of phospholipid in the intestinal absorption of drug. The enhancement of the intestinal absorption of sulfaguanidine, poorly absorbable drug, by bile salts was reported by Kakemi, *et al.*[14] It is well known that bile salts have some effects on the micellar organization of membrane phospholipids. In addition, Sezaki, *et al.*[11] have investigated the absorption mechanism of the ionized form of acidic drugs by examining drug partitioning between water and chloroform containing lecithin and suggested a role for phospholipids in the intestinal absorption of these drugs. However, there are few reports concerning the role of other lipids in the intestinal absorption.

Role of lipid in the intestinal absorption of water-soluble dyes was examined. Total lipid (TL) was extracted from the intestinal mucosa by the method of Folch, *et al.*[10] and then seperated to neutral lipid (NL), glycolipid (GL), phosphatidylcholine (PC), and phosphatidylethanolamine (PE). The transfer of the dyes from water into chloroform containing various lipids has been examined using a non-emulsifying system.[11] As is evident from Fig. 5, BTB, BPB, and PR, anionic dyes had similar pattern of the transfer from water at pH 6.5 to chloroform containing lipids, but the transfer pattern of MB, cationic one, was different from that of anionic ones. Relationship between the transfer from water to chloroform containing lipids and absorption characteristics of these dyes from the rat small intestine was examined at various pH. As shown in Fig. 6 and 7, the degree of the transfer from water to chloroform

14) K. Kakemi, H. Sezaki, R. Konishi, T. Kimura, and A. Okita, *Chem. Pharm. Bull.* (Tokyo), 18, 1034 (1970).

containing TL and GL was correlated relatively well to the accumulation in the intestinal tissue and net absorption, respectively.

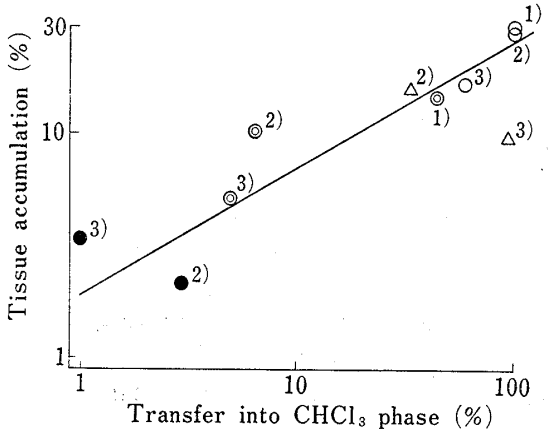

Fig. 6. Correlation between Tissue Accumulation and Transfer into Chloroform Containing Total Lipid

Symbols are the same as in Fig. 5.
concentration of dyes=0.1 mM, 1) pH 4.5, 2) pH 6.5, 3) pH 8.3
Lipid was dissolved in chloroform at 1 mg/ml.

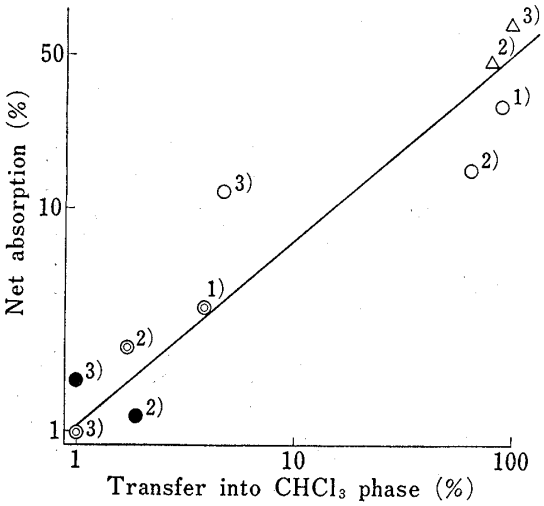

Fig. 7. Correlation between Net Absorption and Transfer into Chloroform Containing Glycolipid

Symbols are the same as in Fig. 5.
concentration of dyes=0.1 mM, 1) pH 4.5, 2) pH 6.5, 3) pH 8.3
Lipid was dissolved in chloroform at 1 mg/ml.

Discussion

Recent study demonstrated that the brush border consists of finger-like projections (microvilli) covered by a glycocalyx (surface coat).[3] Current interest in this glycocalyx (surface coat) derives from the fact that it lies at the cell-lumen interface of the intestine; a location peculiarly suited to protecting the intestinal mucosa, and modifying the intestinal surface functions of digestion and absorption. In order to clarify the mechanism of the absorption of four water-soluble dyes from the rat small intestine, the role of glycocalyx, be present on the brush border, was systematically investigated. In early report,[3] prolonged *in vitro* and *in vivo* exposure of intact absorptive cells to potent mucolytic and proteolytic substances such as N-acetyl cysteine, chymotrypsin, hyaluronidase, neuraminidase, trypsin, papain, and *Streptomyces griseus* protease failed to remove the surface coat so long as integrity of the epithelial cells is maintained. However, Forstner, *et al.*[3] have been recently showed that brief digestion with papain released a major fraction of the total membrane glycoprotein, accounting for almost half of the entire protein-bound hexosamine as well as similar fraction of the total radioactive label. Similarly, Louvard, *et al.*[13] reported that papain digestion of closed, right side out vesicles from pig, rat, and rabbit jejunum brush border induces the release of the hydrolases bound to the membrane without grossly affecting the lipid bilayer limiting the vesicles.

Uptake by the everted sac, uptake by the isolated epithelial cells, and binding to the brush borders of BTB and BPB, anionic dyes, were reduced by digestion with papain. Consequently it seems reasonable to assume that BTB and BPB could bind to glycocalyx as the first process of intestinal absorption. In BPB, it is worthy to note that the binding to the brush borders and the uptake by the isolated epithelial cells failed to find after papain digestion. In addition. uptake by the everted sac (jejunum+ileum) of BPB by digestion with papain was reduced 26.5%, the highest value in these dyes. The most part of the amount of BPB disappeared from lumen by *in situ* perfusion experiments was found in the intestinal mucosa. From the

results above described, it seems that the accumulation site of BPB is mainly the brush border. Although BPB is able to bind to the brush border, it seems that the following step, that is, the transport across lipid bilayer is main cause of the poor absorbability of BPB. This result is consistent with the report that the epithelial border was found to be a critical anatomical barrier to the *in vitro* and *in situ* intestinal absorption of poorly lipid-soluble compounds.[15] In the previous report,[2] it was suggested that the poor absorbability of PR is due to its very low affinity to the intestinal mucosa in addition to poor lipoid solubility. However, no effect of proteolytic enzymes, organic solvents, and detergents on the binding to the brush borders was observed. In MB, slight reduction of the uptake by the everted sac and by the isolated epithelial cells was found by digestion with papain, but no effect on the binding to the brush borders. It is reasonable to assume that the transport across the brush borders was reduced without the reduction of binding to the brush borders by digestion with papain. As shown in Fig. 4, MB can bind to glycocalyx, compared with other dyes. Although the binding to glycocalyx may be reduced by digestion with papain, it is reasonable to assume that apparent effect on the binding to the brush borders failed to find by the enhancement of the binding to other components of membrane. In BTB, uptake by the everted sac, uptake by the isolated epithelial cells, and binding to the brush borders by digestion with papain were significantly reduced. Consequently it is evident that glycocalyx is an important component in the intestinal absorption of BTB. Although the role of glycocalyx in the uptake by the mucosa is evident in this study, further investigation is in progress to elucidate the contribution of glycocalyx to entire absorptive process.

It is well established that lipid is essential to the structural integrity and physiological function of a biological membrane. Lipid composition of the isolated intestinal microvillus membrane was analysed in detail by Forstner, *et al.*[16] Several workers reported that the microvillus membranes of intestinal epithelial cells were relatively rich in cholesterol and glycolipids, but poor in phospholipids. Forstner, *et al.*[17] reported that a minimum of 19.1% of microvillus membrane lipid is made up by glycosphingolipid. This concentration is much higher than levels previously reported for extraneural mammalian plasma membrane. It was suggested that these lipids must have an important and specific role in intestinal membrane function.

The degree of the transfer of dyes from water to chloroform containing GL was related to net absorption. Although a good relationship between the accumulation in the intestinal tissue and the degree of the transfer of dyes from water to chloroform containing TL was found, it is considered from the results obtained by papain digestion that both lipid and protein (glycoprotein) made a contribution to the accumulation of dyes in the intestinal mucosa.

Recently the lipid compositions of the two different regions of the plasma membrane of mouse intestinal epithelial cells, *i.e.*, microvillus membrane and basolateral membrane, were analysed by Fujita, *et al.*[18] and it was reported that they showed very different compositions. Also an asymmetric distribution of phospholipids in the erythrocyte membrane has been demonstrated.[19] Since lipids examined in this paper were extracted from the intestinal mucosa, it is necessary to investigate the role of lipid in the intestinal absorption of drug in more detail.

15) M. Gibaldi and B. Grundhofer, *Proc. Soc. Exptl. Biol. Med.*, **141**, 564 (1972).
16) G.G. Forstner, K. Tanaka, and K.J. Isselbacher, *Biochem. J.*, **109**, 51 (1968).
17) G.G. Forstner and J.R. Wherrett, *Biochim. Biophys. Acta.*, **306**, 446 (1973).
18) K. Kawai, M. Fujita, and M. Nakao, *Biochim. Biophys. Acta.*, **369**, 222 (1974).
19) M.S. Bretscher, *J. Mol. Biol.*, **71**, 523 (1972); S.E. Gordesky, and G.V. Marinetti, *Biochem. Biophys. Res. Commun.*, **50**, 1027 (1973).