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Mechanisms of the Absorption of Water-soluble Dyes from the Rat Small Intestine

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

Absorptive characteristics of these dyes, highly ionized compounds of very low lipoid solubilities at physiological pH range of the small intestine, varied widely. The percentage net absorption of MB, BTB, BPB, and PR were 46.0 ± 2.9, 14.7 ± 3.7, 2.4 ± 1.1, and 1.2 ± 0.5 %, respectively. The degree of binding to the intestinal mucosa preparation determined *in vitro* was correlated to the disappearance from the intestinal lumen and the accumulation in tissue rather than net absorption. The binding to brush borders, the first step of intestinal absorption, was paralleled to the tendency of uptake by isolated epithelial cells.

These results suggest that binding to the mucosa, especially to the brush borders (microvilli) as the first step, is important in the process of absorption of these water-soluble dyes from the rat small intestine. The poor absorbability of PR is due to its very low affinity to the intestinal mucosa in addition to poor lipoid solubility.

It is generally accepted that absorption behavior of the large majority of drugs is more consistent with the pH-partition theory developed by Brodie, *et al.*[2] Evidence has emerged, however, that organic electrolytes present in the intestinal lumen as poorly lipid-soluble molecules are rapidly absorbed. Attempts have been made to predict such absorptive behavior from their abilities to bind with the intestinal mucosal homogenate[3] and from their partition coefficients between water and organic solvents containing phospholipids.[4]

The present investigation was undertaken to clarify the absorption mechanism of water-soluble dyes which are highly ionized and have very small apparent chloroform/water partition coefficient at physiological pH of the small intestine. Three anionic dyes, bromthymol blue (BTB), bromphenol blue (BPB), and phenol red (PR) and a cationic dye, methylene blue (MB) were selected as model compounds.

Experimental

Materials——MB, BTB, BPB, and PR were of reagent grade and used without further purification. All other reagents used in these experiments were of the finest grade available.

Preparation of Drug Solutions——The compositions of isotonic buffer solutions used were citric acid–Na_2HPO_4 for pH 4.5, NaH_2PO_4–Na_2HPO_4 for pH 6.5, and $NaHCO_3$ for pH 8.3.

Apparent Partition Coefficient——Apparent partition coefficient was determined by the method described in the report from this laboratory.[5]

Analytical Methods——Spectrophotometric determination was applied to all dyes investigated.

1) Location: *Yoshida Shimoadachi-cho, Sakyo-ku, Kyoto.*
2) L.S. Shanker, D.J. Tocco, B.B. Brodie, and C.A.M. Hogben, *J. Pharmacol. Exptl. Therap.*, **123**, 81 (1958).
3) K. Kakemi, T. Arita, R. Hori, R. Konishi, and K. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **17**, 248 (1969); K. Kakemi, T. Arita, R. Hori, R. Konishi, K. Nishimura, H. Matsui, and T. Nishimura, *Chem. Pharm. Bull.* (Tokyo), **17**, 255 (1969).
4) S. Furusawa, K. Okumura, and H. Sezaki, *J. Pharm. Pharmacol.*, **24**, 272 (1972).
5) E. Suzuki, M. Tsukigi, S. Muranishi, H. Sezaki, and K. Kakemi, *J. Pharm. Pharmacol.*, **24**, 138 (1972).

MB in Perfused Solution: One g of NaCl and 8 ml of isoamyl alcohol were added to 2 ml sample solution. The mixture was shaken for 15 min and then centrifuged for 10 min at 2500 rpm. The optical density of the isoamyl alcohol layer was determined at 656 mμ.

MB in the Tissue: The small intestine was homogenized in three-fold its wet weight of 0.1N hydrochloric acid. Two g of NaCl and 7 ml of isoamyl alcohol were added to 3 ml of the homogenate. The mixture was shaken for 30 min and then centrifuged for 10 min at 2500 rpm. The optical density of the isoamyl alcohol layer was determined at 656 mμ.

BTB in Perfused Solution: Two ml sample solution was alkalinized with 5 ml of 1N NaOH and determined spectrophotometrically at 617 mμ.

BTB in the Tissue: The small intestine was homogenized in three-fold its wet weight of pH 6.5 buffer solution. A mixture of 5 ml of the homogenate and 5 ml acetone was shaken for 15 min and then centrifuged for 10 min at 2500 rpm. A 5 ml aliquot of the supernatant was alkalinized with 1 ml of 1N NaOH and determined spectrophotometrically at 621 mμ.

BPB in Perfused Solution: One ml sample solution was diluted with 9 ml of pH 6.5 buffer solution and determined spectrophotometrically at 591 mμ.

BPB in the Tissue: The small intestine was homogenized in twice its wet weight of pH 6.5 buffer solution. A mixture of 5 ml of homogenate and 5 ml acetone was shaken for 15 min and then centrifuged for 10 min at 3000 rpm. The supernatant was determined spectrophotometrically at 596 mμ.

PR in Perfused Solution: One ml sample solution was alkalinized with 5 ml of 1N NaOH and determined spectrophotometrically at 560 mμ after centrifugation.

PR in the Tissue: The small intestine was homogenized in three-fold its wet weight of pH 6.5 buffer solution. A mixture of 5 ml of homogenate and 5 ml acetone was shaken for 15 min and centrifuged for 10 min at 3000 rpm. An 1 ml aliquot of the supernatant was alkalinized with 5 ml of 1N NaOH and determined spectrophotometrically at 560 mμ after centrifugation.

Procedure of Absorption Experiments——Male Wistar albino rats weighing 150 —200 g were used. The procedure of *in situ* absorption experiment from the rat small intestine was the same as reported in the report from this laboratory.[6] The bile duct was ligated in all experiments. Rectal temperature was maintained at $37 \pm 1°$. Animals were anesthetized with pentobarbital, given intraperitoneally, and the samll intestine was cannulated for *in situ* recirculation. The entire length of the small intestine, from the proximal end of the duodenum to the distal end of the ileum, was used for the absorption experiments. Forty ml of dye solution, kept at 37°, were recirculated through the intestine at the rate of 5 ml/min. At the end of an absorption period, the perfused solution in the small intestine was withdrawn as completely as possible and washed with pH 6.5 buffer solution. The washings were combined to the perfused solution and made up to 100 ml with pH 6.5 buffer solution. The amount disappeared from the lumen was calculated by the difference in amount of a dye between the initial and the final solutions. Determination of the accumulation of a dye in the intestinal tissues was carried out as follows. Immediately after washing, the entire small intestine was isolated by tearing off the mesentery and the serosal surface was blotted by paper. After weighing, the small intestine was cut into small pieces and determined as mentioned above. The amount net absorbed was calculated by the difference in amount of a dye between the disappearance from the lumen and the tissue accumulation.

Binding to Mucosa of Rat Small Intestine——Mucosal preparation of rat small intestine was obtained by the modified method as those reported in the paper from this laboratory.[7] Rat small intestine, washed as in the absorption experiment, was isolated and placed on a chilled glass plate. After spliting open, the mucosa was scraped off with the edge of a microscope cover glass. The pooled mucosa was weighed and homogenized in ten-fold its wet weight to pH 6.5 buffer solution in a Potter-Elvehjem teflon homogenizer. Equilibrium dialysis method was adopted to estimate the binding. Dye 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 mucosal homogenates was immersed in a centrifuge tube, and equilibrated for 72 hr at 4°. Percentage of binding was calculated from the difference of dye concentration in the outer fluid in the presence of mucosal homogenate from that in the absence.

Preparation of Brush Borders and Isolated Epithelial Cells——Brush borders were prepared by the modified method of Forstner.[8] Rats were treated similarly as in the mucosal preparation. After scraping, the mucosa was then homogenized in a 50 ml glass homogenizer containing 50 volumes of 5.0 mM ethylenediaminetetraacetic acid (EDTA) (pH 7.4). Brush borders were prepared by centrifugation of the homogenate at $400 \times g$ for 30 min at 2°. The pellet was then suspended twice in 5.0 mM EDTA (pH 7.4) and collected by centrifugation at $400 \times g$ for 15 min at 2°. The pellet was then suspended in ice-cold isotonic buffer solution, and passed through four sheets of gauze. The brush border fraction was collected by centrifugation for 15 min at 2500 rpm.

6) K. Kakemi, T. Arita, and T. Koizumi, *Chem. Pharm. Bull.* (Tokyo), **12**, 421 (1964).
7) K. Kakemi, T. Arita, R. Hori, and R. Konishi, *Chem. Pharm. Bull.* (Tokyo), **15**, 1883 (1967).
8) G.G. Forstner, *J. Biol. Chem.*, **245**, 3584 (1970).

Alkaline phosphatase was determined with *p*-nitrophenyl phosphate as a substrate.[9] Protein was determined by the method of Lowry, *et al.*[10]

Isolated intestinal epithelial cells from the rat small intestine were prepared by the method of Reiser, *et al.*[11] The isolated epithelial cells were collected by centrifugation for 5 min at 3000 rpm.

Binding to Brush Borders and Uptake by Isolated Epithelial Cells——Brush border fraction was suspended in 33.5 ml of isotonic buffer solution per 1 g wet weight of the brush borders by shaking. Four ml of dye solution (0.1 mM) was added to 2 ml of the brush border suspension and incubated at 37°. At the end of an incubation period, the suspension was centrifuged for 15 min at 3000 rpm. The supernatant was determined as described above.

Similarly, the isolated epithelial cells were suspended in 50-fold its wet weight of isotonic buffer solution. Forty ml of dye solution (0.1 mM) and 20 ml of the cell suspension were placed in a 100 ml beaker. The beaker, being bubbled with oxygen gas (95% O_2, 5% CO_2), was maintained at 37° and stirred with a magnetic stirrer. Three ml of sample solution was pipetted and centrifuged for 5 min at 3000 rpm. The supernatant was determined as described above.

***In Vitro* Uptake by the Everted Sacs of the Small Intestine**——Rats were treated similarly as in the *in situ* absorption experiments. The small intestine was washed with pH 6.5 buffer solution, maintained at 37°, and isolated. The isolated intestine was cut into two segments: 30 cm from the proximal end of the duodenum, and 30 cm from the distal end of the ileum. Each segment was everted with a wire so that the mucosal surface is on the outside, and ligated at both ends without filling any medium in the serosal lumen. Immediately after ligation, the everted sacs were placed in a centrifuge tube containing 20 ml of an isotonic buffered dye solution (0.1 mM, pH 6.5) and incubated at 37° for 5 min. At the end of an incubation period, an aliquot of the medium was taken and centrifuged for 10 min at 2500 rpm. The supernatant was determined spectrophotometrically as mentioned elsewhere.

Results

Chemical structures of the dyes used in this report are presented in Chart 1. MB is a cationic dye and BTB, BPB, and PR are anionic ones. The p$K_{a'}$ molecular weight, and apparent partition coefficient to chloroform/water at pH 6.5 are summarized in Table I. Absorption experiments in one hour from the rat small intestine were carried out at pH 6.5 using the *in situ* perfusion technique and the results are summarized in Table II. Despite the fact that four dyes are almost completely ionized and their apparent partition coefficients to chloroform/water are extremely small at pH 6.5, their absorption characteristics from

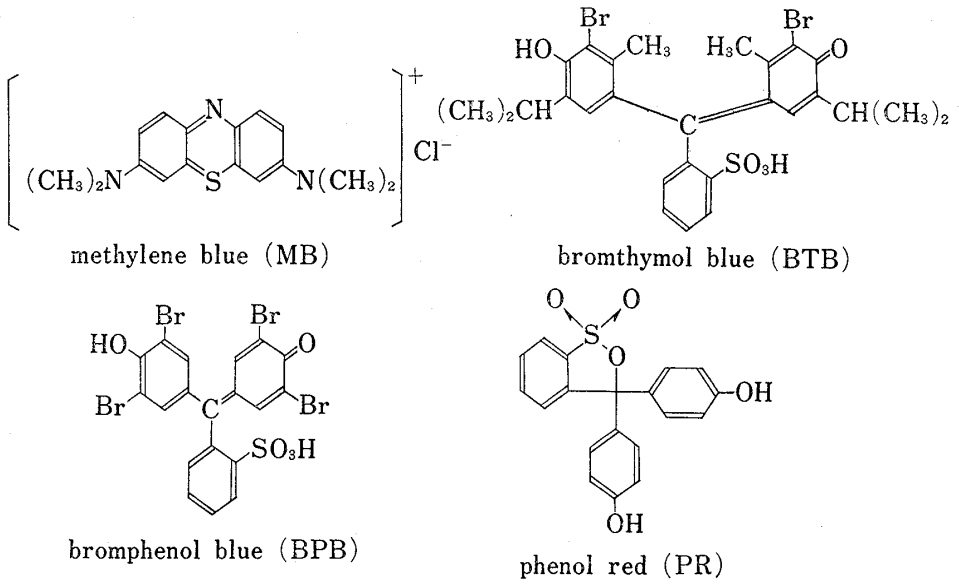

Chart 1. Chemical Structures of Four Dyes

9) M.M. Weiser, *J. Biol. Chem.*, **248**, 2536 (1973).
10) O.H. Lowry, N.J. Rosebrough, A.L. Farr, and R.J. Randall, *J. Biol. Chem.*, **193**, 265 (1951).
11) S. Reiser and P.A. Christiansen, *Biochim. Biophys. Acta*, **225**, 123 (1971).

Table I. Physico-Chemical Properties of Four Dyes

	pK_a	MW[a]	PC[b]
MB	strong <2	373.9	0.06
BTB	strong 7.0	624.4	0.11
BPB	strong 4.0	670.0	0.02
PR	strong 7.9	354.4	0.01

a) MW: molecular weight
b) PC: apparent partition coefficient of four dyes to chloroform at pH 6.5, 37°

Table II. Transfer of Four Dyes from Rat Small Intestine

	Disappearance (%)	Tissue accumulation (%)	Net absorption (%)[a]
MB	61.5±3.1	15.6±3.5	46.0±2.9(5)
BTB	42.5±6.7	27.8±3.4	14.7±3.7(4)
BPB	12.4±1.7	10.0±2.3	2.4±1.1(7)
PR	3.3±0.7	2.1±0.2	1.2±0.5(3)

concentration of dyes=0.1 mM, pH 6.5, *in situ* perfusion for one hour (flow rate 5 ml/min, 37°)
Numbers in parentheses represent number of experiments. Results are expressed as the mean±S.D.
a) The amount net absorbed was calculated by the difference in amount of a dye between the disappearance from the lumen and accumulation in the tissue.

0.1 mM solutions varied widely each other. The percentage net absorption of MB, BTB, BPB, and PR were 46.0±2.9, 14.7±3.7, 2.4±1.1, and 1.2±0.5%, respectively. It is of interest to note that the percentage accumulation values of MB and BTB in the tissue were 15.6±3.5 and 27.8±3.4%, respectively, although the order of the percentage disappeared from the lumen was reverse. Consequently, the amount absorbed of MB is more than that of BTB and larger fraction of the latter compound was found in the intestinal tissue. Wagner, *et al.*[12] have recently shown that MB was well absorbed in man and poorly in the dog after oral administration. As is evident from Table II, the dye was well absorbed from the rat small intestine. Phenol red, often used as a volume change indicator, was hardly absorbed at all. Poor absorption was also observed in the case of BPB. However, larger accumulation in the intestinal tissue was noted than PR. In Fig. 1, the time course of the intestinal transfer are shown. The disappearance from the lumen, the accumulation in the tissue, and the net absorption of MB and BTB tended to increase with time except that the net absorption of BTB remained constant after 30—45 min. Despite of slight increase of the disappearance from the lumen and the accumulation in the tissue of BPB after 30—45 min, the net absorption was very small.

In early reports from this laboratory,[3] it has been pointed out that intestinal absorption of barbituric acid derivatives as well as many other anionic and cationic drugs are related not to their lipoid/water partition coefficient but rather to their binding tendency to the intestinal mucosal preparations. Similarly, in the case of ion-pair complex, enhancement of the absorption of cationic compounds could better be related to the binding behavior of those drugs to the rectal mucosal preparations than their apparent chloroform or benzene/water partition coefficients.[13] Degree of binding to mucosal homogenates was determined *in vitro*

12) A.R. Disanto and J.G. Wagner, *J. Pharm. Sci.*, **61**, 1086 (1972).
13) K. Kakemi, H. Sezaki, S. Muranishi, and Y. Tsujimura, *Chem. Pharm. Bull.* (Tokyo), **17**, 1641 (1969).

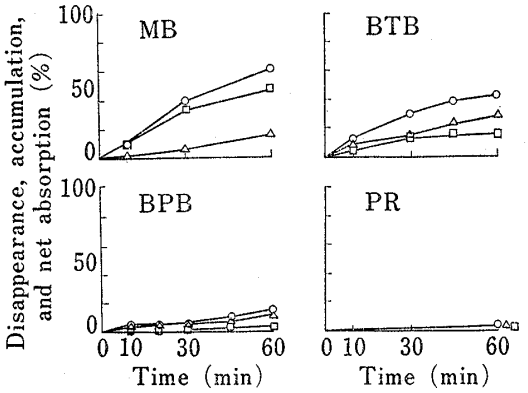

Fig. 1. Time Course of the Intestinal Transfer of Four Dyes

concentration of dyes=0.1 mM, pH 6.5, *in situ* perfusion (flow rate 5 ml/min, 37°)
—○— : disappearance from lumen
—△— : tissue accumulation
—□— : net absorption
Results are expressed as the mean in at least 3 animals.

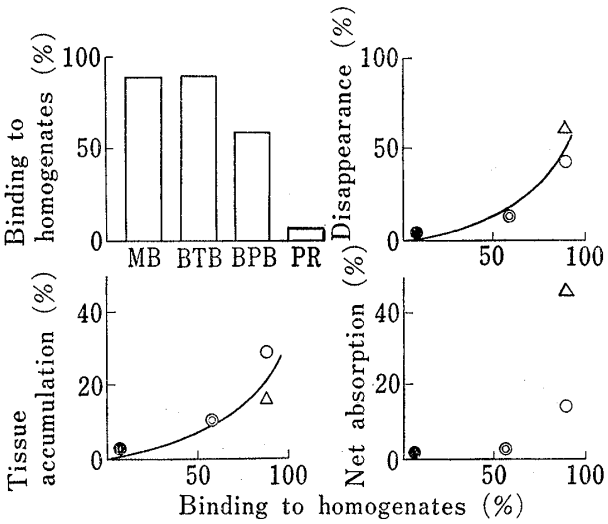

Fig. 2. Binding to Mucosal Homogenates and Its Correlation with Disappearance from Lumen, Tissue Accumulation, and Net Absorption

△ : MB ○ : BTB ◎ : BPB ● : PR
Equilibrium dialysis method was adopted to estimate the binding to mucosal homogenates.

Table III. Alkaline Phosphatase Activity

Fraction	Specific activity[a)]	Purification
Homogenate of intestinal mucosa	0.17	1.0
Brush borders	4.00	24.0

a) Micromoles of *p*-nitrophenyl phosphate split per min per mg of protein at 37° and pH 9.4

for these dyes and the result is shown in Fig. 2. As is evident from the figure, binding tendencies seem to be better correlated to the percentage disappearance from the lumen and accumulation in the tissue rather than net absorption. Intestinal absorption of drugs involves at least following four steps, (1) transport across the brush borders (microvilli), (2) intracellular transport, (3) transport across the basolateral membrane, and (4) transport into portal vein and lymphatic. Therefore binding to mucosal homogenates under present as well as previous experimental conditions includes not only the binding to the brush borders, but to the cytoplasmic protein, mitochondria, nuclei, basolateral membrane, and other cellular components. Therefore as the first step of the intestinal absorption, binding to brush borders and uptake by isolated epithelial cells were examined. As shown in Table III, the purity of brush borders was followed by measuring the activity of the marker enzyme, alkaline phosphatase. In Fig. 3, time course of binding to brush borders and uptake by isolated epithelial cells of the dyes are shown. As is evident from the figure, binding to brush borders of MB, BTB, and BPB reached maximum at 10 min incubation whereas the uptake by isolated epithelial cells of MB and BTB increased gradually. In Fig. 4 is shown the binding to brush borders and the uptake by isolated epithelial cells at pH 4.5, 6.5, and 8.3. The results of the binding to brush borders and the uptake by isolated epithelial cells of MB at pH 4.5 were omitted because of possible formation of ion-pair complex between MB and citric acid of the buffer component at pH 4.5. Although the apparent partition coefficient to chloroform/water of BTB at pH 4.5 was high, binding to the brush borders was paralleled to the tendency of uptake by the isolated epithelial cells. These values are represented in terms of pH-profiles of the binding to brush borders and the uptake by isolated epithelial cells in Fig. 5. Good correlation is noted between the binding to brush borders and the uptake by isolated epithelial cells. However, uptake by

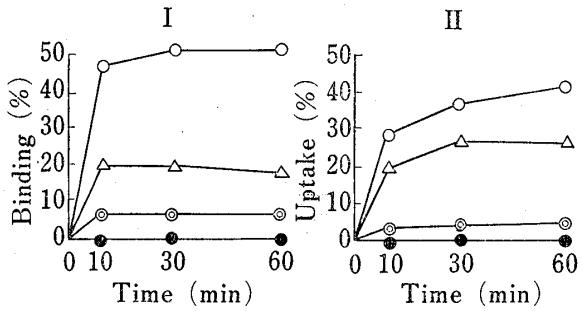

Fig. 3. Time Course of the Binding to Brush Borders (I) and the Uptake by Isolated Epithelial Cells (II) at pH 6.5

Symbols are the same as in Fig. 2.

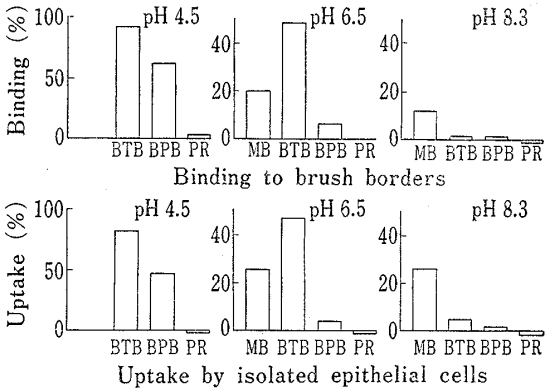

Fig. 4. The Binding to Brush Borders and the Uptake by Isolated Epithelial Cells for 60 min at pH 4.5, 6.5, and 8.3

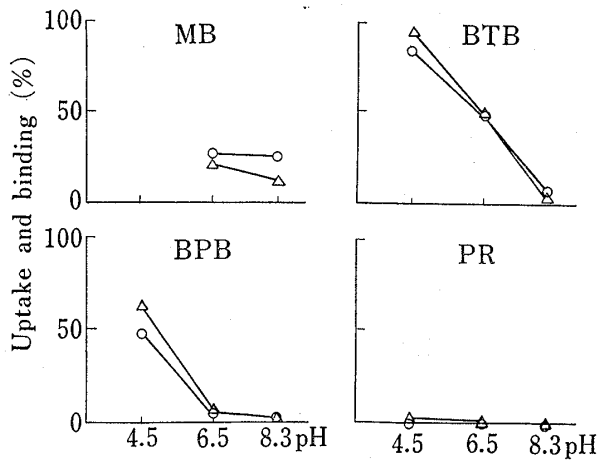

Fig. 5. pH-Profiles of the Binding to Brush Borders and the Uptake by Isolated Epithelial Cells

—○— : uptake by isolated epithelial cells for 60 min
—△— : binding to brush borders for 60 min

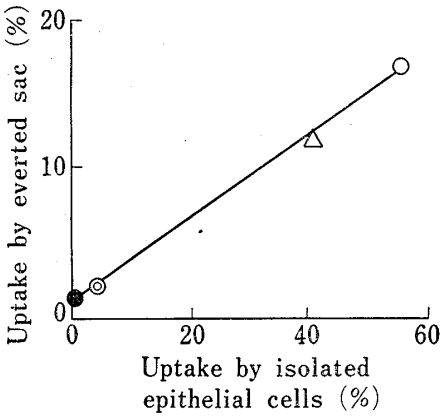

Fig. 6. Relationship between the Uptake by Isolated Epithelial Cells for 60 min and the Uptake by Everted Sac for 5 min

Everted sac of 30 cm from the distal end of the ileum was used.
Symbols are the same as in Fig. 2.
Similar relationship was obtained in the case of 30 cm from the proximal end of the duodenum.

isolated epithelial cells could occur from not only brush borders but basolateral membrane. To exclude the possibility of the transport across basolateral membrane, uptake by everted sac for 5 min was carried out. As shown in Fig. 6, a linear correlation exists between the uptake by isolated epithelial cells and the uptake by everted sac within 5 min. This indicates that uptake by isolated epithelial cells reflects normal absorptive process from the small intestine and the transport across the brush borders.

Discussion

According to the pH-partition theory, it would be expected that all of the dyes employed in the present investigation and having such physico-chemical properties mentioned elsewhere would be poorly absorbed. However, as is evident from Table II, their absorptive characteristics from the rat small intestine varied widely. MB was well absorbed as the percentage disappearance and net absorption values were 61.5±3.1 and 46.0±2.9%, respectively. Although the percentage accumulation in the intestinal mucosa was 27.8±3.4%, 14.7±3.7% of the total amount of BTB was absorbed. On the other hand, the percentage net absorption values of BPB and PR were 2.4±1.1 and 1.2±0.5%, respectively.

This observation is consistent with the results of Shanker, *et al.*[2] that BPB and PR were poorly absorbed from the rat small intestine. It is of interest to note that 10.0±2.3% of the total amount of BPB was accumulated in the intestinal mucosa. As the absorptive characteristics of these dyes could not be explained solely in terms of physico-chemical properties of the dyes in the intraluminal phase, in order to clarify the absorptive characteristics of four dyes, the binding to the intestinal mucosa that demonstrated some qualitative discrepancies of the pH-partition theory with barbituric acid derivatives previously, was examined. As shown in Fig. 2, the degree of binding to the intestinal mucosa preparation *in vitro* was correlated to the disappearance from the intestinal lumen and the accumulation in the tissue rather than net absorption. The binding to the intestinal mucosa preparation implies that to the brush borders, intracellular components, basolateral membrane, and other cellular components. As the first step of the intestinal absorption, the binding to brush borders and the transport across the brush borders are considered to be important steps. Figures. 3, 4, and 5 gave these results of the binding to brush borders and the uptake by isolated epithelial cells. From these results, there seems to be a good correlation exists between the binding to brush borders and the uptake by isolated epithelial cells. As can be seen from Fig. 6, the uptake by isolated epithelial cells reflects normal absorptive process from the small intestine and the transport across the brush borders. Shanker, *et al.*[14] have shown that phenol red was absorbed mainly by simple diffusion through lipoid regions of the intestinal boundary. On the other hand, two investigations have suggested that phenol red is absorbed from the rat small intestine in part by an active transport process of low capacity.[15] The results of Kakemi, *et al.*[16] support this view in that the percentage absorption of phenol red from the perfused rat intestine was found to decrease as the phenol red concentration was increased. In the case of PR in this study, binding to mucosal homogenates, binding to brush borders, and uptake by isolated epithelial cells were negligible, comparing with other three dyes. It is conceivable, therefore, that the poor absorbability of PR is due to its very low affinity to the intestinal mucosa in addition to its poor lipoid solubility. In contrast to PR, 10.0±2.3% of BPB was accumulated in tissue.

This observation is consistent with more binding to the mucosal homogenates, binding to the brush borders, and uptake by the isolated epithelial cells of BPB than PR. Consequently, it seems that the transport step from the intestinal epithelial cell to blood vessel is an absorption barrier of BPB from the rat small intestine. Rank order correlationship exists between the degree of the binding to brush borders or the uptake by isolated epithelial cells of MB and BTB and the tissue accumulation but not in the disappearance from lumen or net absorp-

Table IV. Transfer of MB and BTB from Rat Small Intestine for 10 min

	Disappearance (%)	Tissue accumulation (%)	Net absorption (%)[a]
MB	9.2±1.8	1.2±0.4	8.0±2.1(4)
BTB	12.4±1.2	8.9±2.2	3.5±1.0(3)

concentration of dyes=0.1 mM, pH 6.5, *in situ* perfusion (flow rate 5 ml/min, 37°)
Numbers in parentheses represent number of experiments.
Results are expressed as the mean±S.D.
a) The amount net absorbed was calculated by the difference in amount of a dye between the disappearance from the lumen and the accumulation in the tissue.

14) R.C. Lanman, C.E. Stremsterfer, and L.S. Shanker, *Xenobiotica.*, **1**, 613 (1971).
15) H. Kunze and W. Vogt, *Arch. Exptl. Pathol. Pharmakol.*, **256**, 139 (1967); H. Kunze, *Arch. Exptl. Pathol. Pharmakol.*, **259**, 260 (1968).
16) K. Kakemi, H. Sezaki, R. Konishi, T. Kimura, and M. Murakami, *Chem. Pharm. Bull.* (Tokyo), **18**, 275 (1970).

tion. The results of the absorption experiments in 10 min are summarized in Table IV. In MB, the most part of the amount disappeared from lumen was absorbed, but in BTB, it was found in the intestinal mucosa. It is worthy to note that the order of the amount disappeared from lumen was BTB>MB, in contrast to the results of absorption experiments in one hour. This suggests that the uptake by the intestinal mucosa of BTB is superior to that of MB at early step. These results are in good agreement with the order of the uptake by the everted sac as shown in Fig. 6.

In the case of *in situ* perfusion, the transport from the epithelial cell to the blood vessel is an important process of absorption. Uptake by the everted sac which can exclude the process of transport to blood vessel was correlated to the uptake by isolated epithelial cells as shown in Fig. 6. The order of the uptake by the intestinal mucosa is BTB>MB. As MB disappeared from lumen is liable to be transported to the blood vessel, the order of the amount disappeared from lumen in one hour was MB>BTB. From the foregoing results, it seems reasonable to assume that binding to the intestinal mucosa, especially to the brush borders (microvilli) as the first step, is important in the process of absorption of four water-soluble dyes from the rat small intestine. A high concentration of a dye at the membrane surface could be advantageous to the absorption across the mucosal membrane.