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# Mechanism of Transfer of Bleomycin into Lymphatics by Bifunctional Delivery System *via* Lumen of Small Intestine

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The mechanism of lymphatic transfer of bleomycin from the lumen of the small intestine of rats following the administration of a bifunctional delivery system was investigated. This system is a combination of macromolecular bleomycin·dextran sulfate complex as a lymphotropic carrier and lipid-surfactant mixed micelles as an absorption promoter. After administration of this bifunctional delivery system into the lumen of the small intestine, 56% of the complex was left intact in the lumen after 2 h, but 85, 95 and 97% of the absorbed bleomycin were detected as the free drug in the tissue of the small intestine, the lymph and the blood, respectively. The absorption percentage of bleomycin from the lumen of the small intestine at 3 h after the administration of the bifunctional delivery system was significantly different from that of dextran sulfate, and the total lymphatic transfer of dextran sulfate was greater than that of bleomycin. These findings suggest that the extent of the selective lymphatic transfer of bleomycin was smaller than that of dextran sulfate because of the dissociation of the bleomycin·dextran sulfate complex in the lumen and the tissue of the small intestine.

**Keywords**——bleomycin; lymphatic transfer mechanism; bifunctional delivery system; lipid-surfactant mixed micelles; dextran sulfate; macromolecular carrier; bleomycin·dextran sulfate complex; small intestinal absorption; blood-lymph barrier

A bifunctional delivery system for the selective transfer of a poorly absorbed drug into the lymphatics *via* the enteral route in rats was described previously.[1] In that work, we chose bleomycin (BLM), mol wt. 1500, a basic glycopeptide anticancer drug, for the intestinal absorption studies. It was found that BLM was absorbed only to a very small extent, and the BLM level achieved in the lymph was identical with that in the plasma. This result indicated that there was no specificity of lymphatic transfer of BLM. In an effort to obtain more selective uptake by the lymphatic system, a new delivery system was developed using the following two adjuvants: 1) monoolein–sodium taurocholate mixed micelles as an absorption promoter[2-5] and 2) macromolecular anionic dextran sulfate (DS), mean mol wt. 500000, as a lymphotropic carrier with the ability to form an ionic complex with BLM. Administration of the bifunctional delivery system into the lumen of the intestine produced a selective concentration rise of BLM in the lymph, and this effect was more pronounced after administration into the lumen of the large intestine. We have also reported that the formation of the BLM·DS complex and the maintenance of the complex in the tissue were essential to the selective lymphatic transfer of BLM in the large intestinal administration of the bifunctional delivery system.[6] In the present work, we investigated the mechanism of lymphatic transfer of BLM administered in the form of this bifunctional delivery system into the lumen of the small intestine. Stability and absorption studies of the BLM·DS complex clarified the mechanism of transfer of BLM into the lymphatics by the bifunctional delivery system.

## Experimental

**Materials**——BLM was supplied by Nippon Kayaku. DS was purchased from Nakarai Chemicals and fluorescein isothiocyanate was purchased from Sigma Chemicals. The monoolein used was of high purity grade (Nikko Chemicals). Sodium taurocholate was synthesized according to the method of Norman.[7]

The purity of sodium taurocholate was checked by thin-layer chromatography[8] and infrared spectroscopy. All other chemicals were of reagent grade.

**Synthesis of Fluorescein Isothiocyanate-labelled DS (FDS)**——FDS was synthesized according to the method of Belder and Granath.[9] DS (1.0 g) was dissolved in dimethyl sulfoxide (10 ml) containing a few drops of pyridine. Fluorescein isothiocyanate (0.1 g) was added, followed by dibutyltin dilaurate (20 mg), and the mixture was heated for 2 h at 95°C. After several precipitations in ethanol to remove most of the free dye, the precipitate was dissolved in distilled water, and chromatographed on a Sephadex G-50 column (3.5×40 cm), then the macromolecular fraction was dried *in vacuo* at 80°C.

**Preparation of Test Solution**——Solution of mixed micelles was prepared by dissolving monoolein (40 mM) and sodium taurocholate (40 mM) in distilled water followed by sonication at 37°C with an Ohtake sonicator, model 5202 (100 W, 4 min). Test solution of free BLM was prepared by dissolving BLM (1.0 mg/ml) in 40 mM mixed micellar solution. Test solution of the bifunctional delivery system (mixed micelles+BLM·FDS complex) was prepared by dissolving BLM and FDS in 40 mM mixed micellar solution (BLM, 1.0 mg/ml; FDS, 3.34 mg/ml). The formation of the BLM·FDS complex in the test solution was confirmed by gel filtration; the test solutions (BLM+FDS with 40 mM mixed micelles and free BLM with 40 mM mixed micelles) and solution of free FDS (3.34 mg/ml) with 40 mM mixed micelles, were chromatographed on a 1.8×20 cm column of Sephadex G-50 using distilled water as the eluent. Fractions (3 ml each) were automatically collected, and BLM and FDS were determined by ultraviolet and fluorescent spectroscopy, respectively (as detailed later in this section).

**Absorption Experiment**——Male Wistar albino rats weighing 200—250 g were anesthetized intraperitoneally with sodium pentobarbital (32 mg/kg of body weight). The small intestine was exposed by midline incision, and a closed loop of the entire small intestine (duodenum, jejunum and ileum) was prepared by ligation at the proximal and distal ends. Doses of 5 mg BLM (1.0 mg/ml) and 16.7 mg FDS (3.34 mg/ml) per rat, were used. Five ml of the test solution of the bifunctional delivery system (40 mM mixed micelles+BLM·FDS complex) was introduced into the loop of the small intestine. A polyethylene catheter (i.d. 0.5 mm, o.d. 0.8 mm, Dural Plastics, Australia) was placed in the carotid artery and blood samples were collected periodically. Plasma was separated by an Eppendorf centrifuge, model 3200 (at 15000 *g* for 2 min.) A modification of the method of Bollman *et al.*[10] was used for the collection of lymph from the thoracic duct. The thoracic duct was cannulated with a heparin-filled flexible vinyl catheter (i.d. 0.5 mm, o.d. 0.9 mm, Dural Plastics, Australia) and fixed with a drop of tissue cement (Aron Alpha, Sankyo). This cannula allowed continuous drainage of the lymph throughout the experiments. The plasma and the lymph samples were immediately immersed in an ice bath after collection. Disappearance of BLM and FDS from the lumen of the small intestine was studied at 15 min, 1 and 3 h after the administration of the test solutions of free BLM with 40 mM mixed micelles and the bifunctional delivery system. At the end of each experiment, the remaining test solution in the loop was thoroughly collected by infusion of saline solution for assay.

**Stability of BLM·FDS Complex in Lumen and Tissue of Small Intestine, Plasma and Lymph**——At 2 h after the administration of the test solution of the bifunctional delivery system into the lumen of the small intestine, the remaining solution in the lumen was thoroughly expelled and the entire small intestine was removed and homogenized with isotonic mannitol solution. This homogenate was centrifuged for 10 min at 15000 *g*. These samples (test solution from the lumen, the supernatant of the tissue homogenate, the plasma and the lymph) were fractionated by gel filtration on a 1.8×20 cm column of Sephadex G-50 using distilled water as the eluent. Fractions (3 ml each) were automatically collected, and the concentrations of BLM and FDS were determined.

**Analytical Methods for BLM and FDS**——An antimicrobiological activity assay was used for the determination of BLM in the remaining solution from the lumen, the tissue homogenate, the plasma and the lymph. The disc plate method using *Bacillus subtilis* PCI-219 as test microorganism was employed for BLM.[11] The detection limit with this method was 0.8 μg/ml. Mixed micelles did not affect the antimicrobiological activity. The *in vitro* formation of the BLM·FDS complex was tested by optical density measurement using an ultraviolet spectrophotometer (Hitachi model 200-20) at 290 nm for BLM. The fluorescence intensity of FDS was measured with a fluorescence spectrophotometer (Hitachi model 650-10s) at 520 nm using an excitation wavelength of 490 nm.

## Results

### Formation of BLM·FDS Complex

Our previous reports[1,12] have indicated the presence of an extensive interaction of BLM, a cationic molecule, and DS, a strongly anionic macromolecule. In order to provide a basis for understanding this phenomenon, DS was labelled with fluorescein isothiocyanate, and this labelled compound (FDS) was used as a tracer of DS. The elution position of the solution of BLM+FDS with 40 mM monoolein–sodium taurocholate mixed micelles on Sephadex G-50

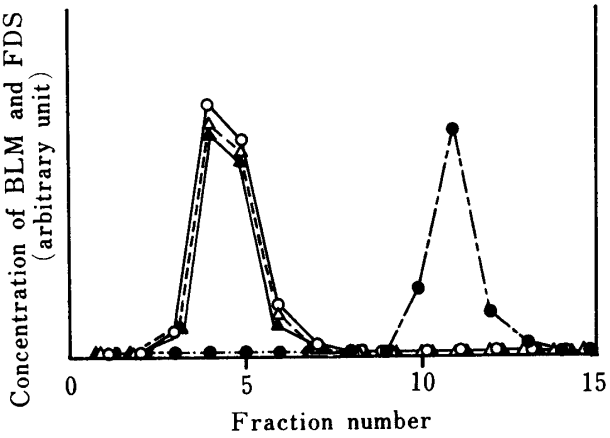


Fig. 1. Formation of BLM·FDS Complex

Gel filtration chromatogram (Sephadex G-50) of free BLM with 40 mM mixed micelles (●—·—·—●), BLM+FDS with 40 mM mixed micelles (analyzed for BLM, ○——○, and for FDS, △---△) and free FDS with 40 mM mixed micelles (▲——▲).

indicated its integrity as a macromolecular complex; free BLM was not detected (Fig. 1). The peak of free FDS coincided with the BLM and FDS peaks of the BLM·FDS complex. These results suggest that the BLM·FDS complex did not dissociate in the mixed micellar solution.

### Stability of BLM·FDS Complex in Lumen and Tissue of Small Intestine, Lymph and Plasma

The stability of the BLM·FDS complex in the body was analyzed in the lumen and the tissue of the small intestine, the lymph and the plasma, at 2 h after the administration of the bifunctional delivery system (mixed micelles+BLM·FDS complex) into the lumen of the small intestine (Fig. 2). In the lumen and the tissue of the small intestine, the lymph and the plasma, the labelled FDS remained stable as shown by the fluorescence detected in the macromolecular elution fraction (Fig. 1). In the lumen of the small intestine, 44% of the BLM·FDS complex dissociated to free BLM (Fig. 2(a)), and 85% of the BLM absorbed by mixed micelles in the tissue was detected as free BLM (Fig. 2(b)). In the analysis of the lymph and the plasma, 95 and 97% of the BLM was detected as free BLM, respectively (Fig. 2(c), (d)).

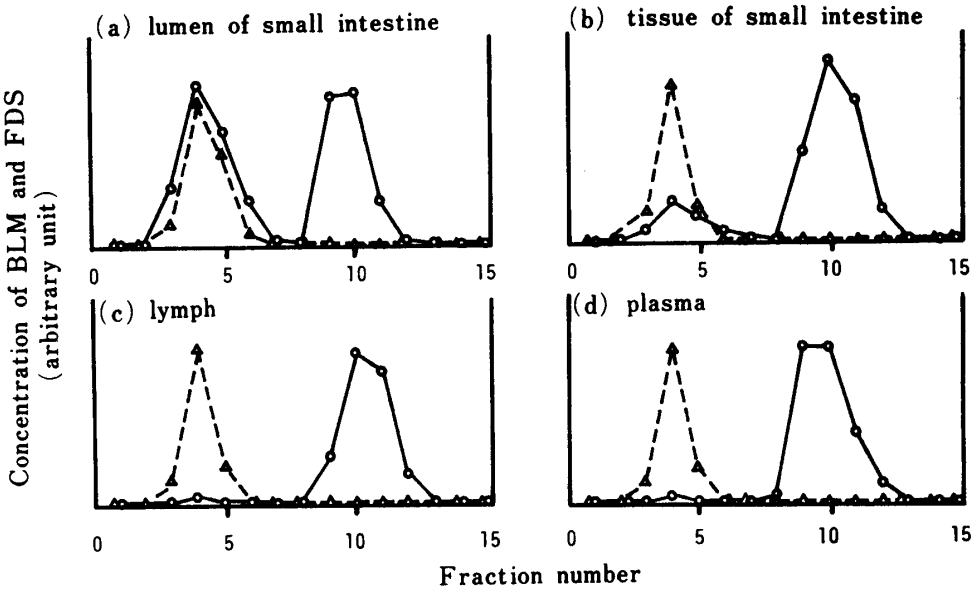


Fig. 2. Stability of BLM·FDS Complex in Various Tissues

Gel filtration chromatogram (Sephadex G-50) of (a) remaining solution in the small intestinal lumen (b) the supernatant of the tissue homogenate of the small intestine; (c) the lymph of the thoracic duct and (d) the plasma, at 2 h after administration of the bifunctional delivery system into the lumen of the small intestine (analyzed for BLM, ○——○, and for FDS, △---△).

### Absorption of BLM·FDS Complex from Lumen of Small Intestine into Blood and Lymph

Administration of the bifunctional delivery system (mixed micelles+BLM·FDS complex) into the lumen of the small intestine produced more selective transfer of the FDS into the lymphatics. The concentrations of BLM and FDS found in the plasma and the lymph are

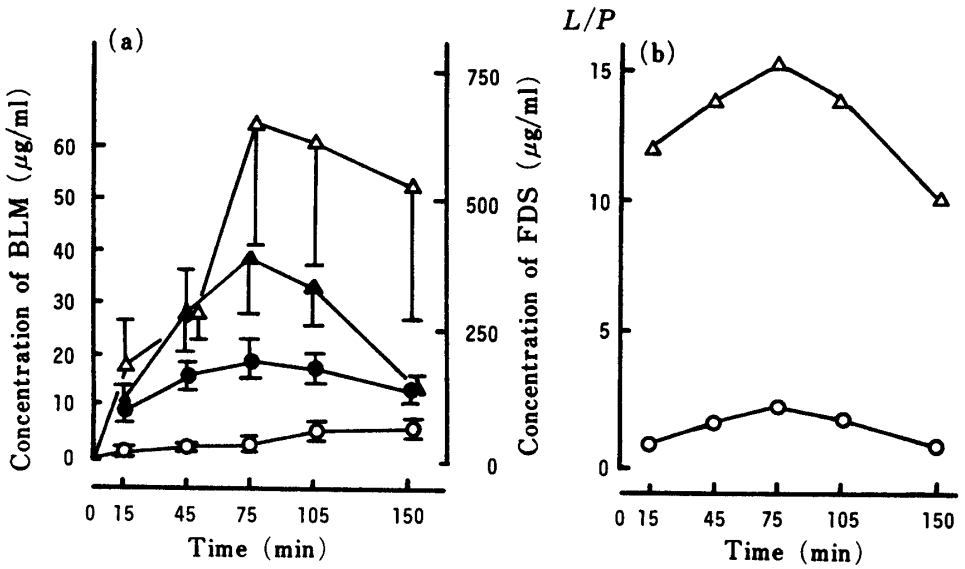


Fig. 3. Absorption of BLM·FDS Complex into Blood and Lymph from Lumen of Small Intestine

(a) Concentrations of BLM and FDS in the plasma and the lymph of the thoracic duct after administration of the bifunctional delivery system into the lumen of the small intestine. Key: open symbol (○, △), FDS; closed symbol (●, ▲), BLM. circle, plasma; triangle, lymph. Each value represents the mean ± S.E. of 4—8 experiments.
(b) Ratios of concentration in the lymph relative to that in the plasma (*L/P*) for BLM and FDS calculated from the data in Fig. 3 (a). ○, BLM; △, FDS.

shown in Fig. 3(a). BLM activity in the lymph was up to 2-fold higher than that in the plasma, while the FDS concentration in the lymph was 10-to 15-fold higher. The lymph/plasma concentration ratios (*L/P*) of BLM and FDS calculated from the data of Fig. 3(a) are shown in Fig. 3(b). Then, in order to investigate the absorption mechanism, the behavior of free BLM with 40 mM mixed micelles and the BLM·FDS complex of bifunctional delivery system in the lumen was studied. The time course study (Fig.4) indicated that the absorption rate of BLM was significantly greater than that of FDS ($p<0.05$) at 3 h after the administration of the bifunctional delivery system. At 3 h after the administration of the bifunctional delivery system, 67±4% as BLM and 85±7% as FDS could still be detected in the lumen, while upon administration of free BLM, 60±7% of BLM remained. The transfer rates and cumulative amounts of BLM and FDS from the small intestine in the thoracic duct lymph were monitored for 3 h following the administration of the bifunctional delivery system, as shown in Fig. 5. Comparative cumulative amounts of BLM and FDS in the lymph at 3 h post administration with the bifunctional delivery system are shown in Table I. The cumulative amount of BLM

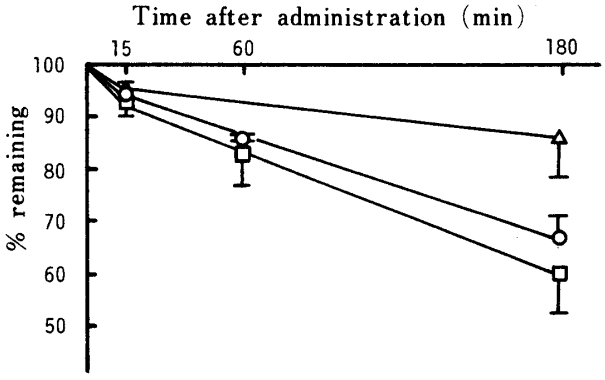


Fig. 4. Time Course of Absorption of BLM and FDS from Lumen of Small Intestine after Administration of Bifunctional Delivery System or Free BLM with 40 mM Mixed Micelles

○, BLM (administered as bifunctional delivery system);
△, FDS (administered as bifunctional delivery system);
□, BLM (administered as free BLM).
Each value represents the mean ± S.E. of 4—6 experiments.

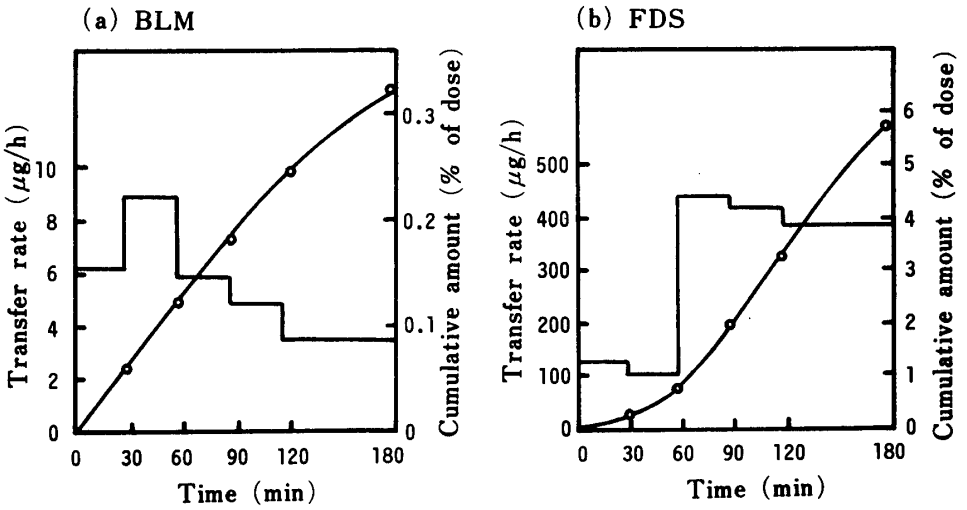


Fig. 5. Transfer and Cumulative Amounts of BLM and FDS into Thoracic Duct Lymph after Administration of Bifunctional Delivery System into Lumen of Small Intestine

(a) BLM, (b) FDS.
Bars are transfer rates and curves (○——○) are cumulative amounts of BLM and FDS (percentage of dose).
Each value represents the mean of 4 experiments.

TABLE I. Cumulative Amount of Drug in Lymph at 3 h after Administration of Bifunctional Delivery System into Lumen of Small Intestine

| Drug | BLM[b] | FDS[c] |
|---|---|---|
| % of dose[a] | 0.33 | 5.78 |
| % of absorbed quantity[a] | 0.87 | 33.47 |

*a*) Each value represents the mean of 4 experiments.
*b*) BLM; bleomycin.
*c*) FDS; fluorescein isothiocyanate-labelled dextran sulfate.

was 0.33% of the dose (0.87% of that absorbed from the lumen) and that of FDS was 5.78% of the dose (33.47% of that absorbed from the lumen). The ratio of total FDS to total BLM in the lymph (% of the absorbed quantity from the lumen) was estimated as 38: 1.

## Discussion

The selective transfer of BLM, a poorly absorbed anticancer agent, into the lymphatics *via* the enteral route has been achieved by the development of a bifunctional delivery system.[1] This system is a combination of macromolecular BLM·DS complex as a lymphotropic carrier[12] and lipid-surfactant mixed micelles as an absorption promoter.[2–5] The lymphotropic effect of this system was more pronounced after administration into the large intestine than into the small intestine.[1] We have also reported that the maintenance of the BLM·DS complex in the tissue is essential for selective lymphatic transfer of BLM in the large intestinal administration of the bifunctional delivery system.[6] In the present work, we investigated the mechanism of lymphatic transfer of BLM administered by use of the bifunctional delivery system into the lumen of the small intestine, and the reason for the less selective lymphotrophy of BLM in this system after small intestinal administration as compared with large intestinal administration. In order to trace the DS, we synthesized fluorescein isothiocyanate-labelled DS (FDS), which formed an ionic complex (BLM·FDS complex) with BLM in the mixed micellar

solution (Fig. 1). After administration of the bifunctional delivery system into the lumen of the small intestine, analysis showed that 56% of the BLM·FDS complex remained stable in the lumen for 2 h; however, analysis of the tissue of the small intestine, the lymph and the plasma indicated that 85, 95 and 97%, respectively, of the absorbed BLM existed as free BLM (Fig. 2). This is reasonable since the BLM·FDS complex is an ionic complex, and as such dissociates in the lumen and the tissue of the small intestine, and in the circulatory system. Studies on the absorption time course of the BLM·FDS complex upon administration of the bifunctional delivery system into the lumen of the small intestine showed that the BLM absorption curve had different characteristics from that of FDS, but resembled that observed when free BLM was administered (Fig. 4). This result supports the partial dissociation of the BLM·FDS complex in the lumen of the small intestine (Fig. 2(a)): about half of the BLM·FDS complex was dissociated in the lumen, and consequently the absorptions of BLM and FDS from the lumen after the administration of the bifunctional delivery system were not equal, probably because of the difference of molecular weight between BLM and FDS.

The selective lymphotrophy of the BLM·DS complex observed might be due to a molecular sieving mechanism of the blood–lymph barrier in the tissue of the intestine, since the pore radius of the blood capillaries in rat intestine has been reported to be under 40—50 Å[13] as opposed to the lymphatic capillary having a pore radius of 100—150 Å.[14] The average molecular radius of DS (mean mol wt. 500000) was estimated to be around 130 Å from the molecular size of dextran.[15] Therefore, once the BLM·DS complex is taken up intactly by the tissue of the intestine, it would be preferentially transferred into the lymph capillary rather than the blood capillary. Thus, after the administration of the bifunctional delivery system, the lymph/plasma ($L/P$) ratios for BLM and for FDS concentration reached values from 1 to 2 and from 10 to 15, respectively (Fig. 3(b)). The cumulative amounts of BLM and FDS transferred into the lymph (% of the absorbed quantity) were estimated to be about 0.9 and 33%, respectively (Table I). Thus, a large difference of transference of the absorbed BLM and FDS to the lymphatic system was detected. Our previous report[1] has shown that equal distribution of free BLM into the lymph and the blood can expected, but macromolecules such as DS or the intact BLM·DS complex are preferentially transferred into the lymph rather than into the blood. It was considered that the intact BLM·FDS complex (15%, Fig. 2(b)) and FDS in the tissue of the small intestine transferred selectively into the lymph, and free BLM (85%, Fig. 2(b)) transferred into the lymph and the blood at almost equal concentrations. Therefore, the total amount of BLM in the lymph (% of absorbed quantity) after administration of the bifunctional delivery system into the small intestine (0.87%, Table I), was a little larger than that after administration of free BLM with mixed micelles (0.58%[1]), but much smaller than that of FDS (33.5%, Table I). The percentage of the intact BLM·FDS complex was greater in the tissue of the large intestine (44%[6]) than in that of the small intestine (15%, Fig. 2(b)), and the extent of the selective lymphatic transfer of BLM by this system was therefore more pronounced after large intestinal administration (large intestine,[6] 3.9% of the absorbed quantity; small intestine, 0.87%; Table I). More selective lymphatic transfer of BLM as well as the FDS would be observed if the complex remained wholly intact in the lumen and the tissue of the small intestine. The ratio of total DS to total BLM in the lymph (% of absorbed quantity) from the small intestine was estimated to be 38: 1 (Table I), but the net ratio might be smaller because of the inactivation of BLM in the lymph; it has been reported that BLM is more strongly inactivated in the lymph than in other tissues,[16] and inactivated BLM can not be measured by the antimicrobiological assay method.

In conclusion, the following mechanism is proposed for the lymphatic transfer of BLM by the bifunctional delivery system (mixed micelles+BLM·DS complex) upon administration into the lumen of the small intestine (Fig. 6). About half of the BLM·DS complex remains intact in the lumen. Then, the complex and free BLM dissociated from the complex penetrate the epithelial barrier with the aid of the mixed micelles as an absorption promoter. Of the

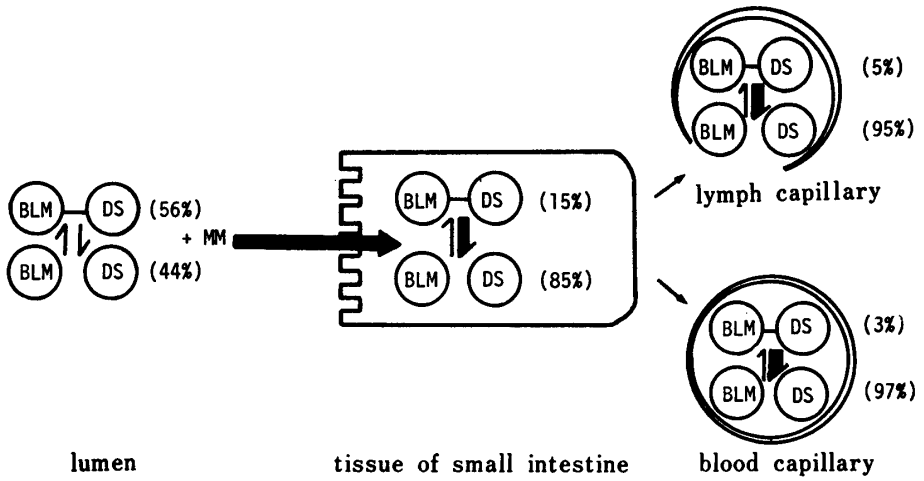


Fig. 6. Proposed Mechanism of Lymphatic Transfer of BLM from Lumen of Small Intestine by Administration of Bifunctional Delivery System

MM, monoolein-sodium taurocholate mixed micells as an absorption promoter.

total BLM absorbed from the lumen, 15% remains as an intact complex, while the remaining 85% existed as free BLM in the tissue of the small intestine (Fig. 2(b)). The free BLM generated from the dissociation of the BLM·DS complex transfers into the lymph and the blood in almost equal concentration, but the intact BLM·DS complex and the DS preferentially transfer into the lymph. The effectiveness of the selective lymphatic transfer of BLM by the bifunctional delivery system could be closely related to the stability of the absorbed complex in the tissue of the intestine. Therefore, the administration of the bifunctional delivery system into the small intestine showed less effective lymphatic transfer of BLM compared with that into the large intestine because of the lower stability of the complex in the small intestinal lumen and tissue. The above scheme provided a rational basis for understanding the lymphatic transfer of BLM from the small intestine using the bifunctional delivery system.

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