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Small Intestinal Absorption of Methylene Blue in Rats, Guinea Pigs and Rabbits[1]

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Small intestinal tracts of rats, guinea pigs, and rabbits were recirculated *in situ* with methylene blue (MB), and the average absorption ratios in 120 min were 54, 36, and 47%, respectively. Therefore the great species difference in MB absorption capacity was not observed in these three experimental animals. At the same time 7.7, 46, and 30% of the dose were found as leucomethylene blue (LMB) besides the unchanged methylene blue in the perfusate for rats, guinea pigs, and rabbits, respectively.

In situ perfusion experiments revealed that the concentration of total MB (MB+LMB) in bile at 150 min was five times greater than the initial MB concentration in the perfusate, and that 8 and 12.8% of the total MB absorbed were excreted in bile and urine, respectively. The total excreted MB contained 96.8% LMB in bile, and 94.1% in urine.

MB was reduced to LMB under anaerobic condition by incubation with everted sacs of the small intestines of guinea pigs and rats, and the reduction was not reversible in the incubation medium under the same condition in the sense of net reaction.

In vitro absorption experiment employing the everted sacs of the small intestines of rats, guinea pigs, and rabbits suggested that LMB besides MB would be also absorbable from the small intestines of the experimental animals *in situ*.

Keywords——methylene blue; leucomethylene blue; drug absorption; drug excretion; *in situ* perfusion; small intestine; rat; guinea pig; rabbit; species difference

Methylene blue is one of highly ionized drugs in the area of physiological pH, and DiSanto and Wagner[3] showed that the drug is well absorbed in man and poorly absorbed in the dog after oral administration. This suggests that the similar species difference in gastrointestianl absorption of methylene blue may be observed in other experimental aminals than the dog.

The primary purpose of this work is to determine the extent of small intestinal absorption of methylene blue in rats, guinea pigs, and rabbits by employing *in situ* recirculation method,[4] which may evaluate the absorption characteristics more directly than urinary recovery after oral administration.

Methylene blue was absorbed from small intestinal tracts of rats, guinea pigs, and rabbits with *in situ* recirculation experiments, and leucomethylene blue was found in the perfusate for each kind of animals. Leucomethylene blue was assumed to be absorbable in these experimental animals. In rabbits methylene blue and leucomethylene blue were excreted into bile, though the recovery from bile was less than that from urine.

Experimental

Materials——Methylene blue (MB) was purchased from Merck and Co., Inc. and purity was verifed by JP VIII test. MB content in the product was 99.6% after drying. Macrogol 4000 JP was purchased from Maruishi-seiyaku Co., Inc. All other chemicals and solvents were commercially available and of special grade.

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3) A.R. DiSanto and J.G. Wagner, *J. Pharm. Sci.*, **61**, 1086 (1972).

4) K. Iwamoto, N. Ozawa, Y. Hayashi, T. Tsukamoto, and J. Watanabe, *Chem. Pharm. Bull.* (Tokyo), **24**, 2021 (1976).

MB solutions for recirculation in rats and guinea pigs were prepared to be 2.4×10^{-5}M by dissolving appropriate amounts of MB in isotonic phosphate buffer solutions of pH 7.4. For perfusates in rabbits 0.15% macrogol 4000 (MG) as an indicator for water transport[5] was dissolved in the isotonic phosphate buffer solution which contained 2.4×10^{-5}M MB.

Animals——Male Wistar rats weighing 185 to 240 g, male Hartley guinea pigs weighing 300 to 350 g, and male albino rabbits weighing 1.9 to 2.8 kg were fasted for about 17 hr prior to the absorption experiments, but water was allowed *ad libitum*.

Experimental Procedures——(1) Absorption from small intestine of rats and guinea pigs: After anesthetization with pentobarbital sodium, the small intestine was cannulated for *in situ* recirculation, following the method described in the preceding paper.[4] Cannulation was applied to the tracts between upper portion of duodenum and ileocecal valve. The small intestinal tract was first perfused with 100 ml of isotonic phosphate buffer solution, and then the tubings attached to the inflow and outflow cannulae were connected to a flask containing 100 ml of the drug solution which was bubbled with nitrogen gas and followed to be continuously circulated through the tract for a programmed time at 37° by means of circulation apparatus adjusted to flow at 7.7, 8.3, 11, 14.8, and 15.3 ml/min, though most absorption experiments in rats and guinea pigs were carried out under the condition of 11 ml/min flow rate. After sacrifice of the animal by injection of 5 ml air into the heart the small intestinal tract was washed with 50 ml of drug free buffer solution, and the washings were added to the recirculated drug solution. The mixed solution was used for analytical determination of MB and total MB [MB+LMB (leucomethylene blue)]. In order to measure the inside surface area of the intestinal tract used, the small intestine was excised and filled with buffer solution at 50 cm hydrostatic pressure, and the length and volume of contents of the intestinal sac were determined. The inside surface area was calculated by assuming the sac as a simple cylinder. The intestinal tissue with the buffer solution, the volume of which had been measured, was then homogenized and added with 0.02N HCl to be 200 ml homogenate. The homogenate was analyzed for MB and total MB accumulation in the intestinal tissue. (2) Absorption from small intestine of rabbits: After cannulation was applied to the tracts between upper portion of duodenum and ileocecal valve according to the similar method described in (1) for rats and guinea pigs, the tracts were washed with about 700 ml of buffer solution and with about 500 ml of drug solution in succession. Then, 500 ml of drug solution was recirculated under anaerobic condition. Six ml of the sample solution was withdrawn periodically for 3 hr after 15 min lag of pre-recirculation. The concentration of the drug and indicator (macrogol 4000) was determined. (3) Urinary and biliary excretion in rabbits: During absorption experiments *in situ*, urine was drained and the bladder was washed with 10 ml of warmed 0.9% NaCl through a catheter at an interval of 30 min. Washings and water were added to urine to make the volume of 100 ml. The diluted urine sample solution was analyzed for MB and total MB. A bile fistula was made using a polyethylene tubing prior to recirculation of drug solution through the small intestine *in situ*, and bile was also collected periodically during absorption experiments. Bile sample solution was diluted to 25 ml with water, and the concentration of MB and total MB in the diluted bile solution was determined. (4) LMB formation by everted sacs: After anesthetization with pentobarbital sodium, the small intestines of rats and guinea pigs were excised, and everted sacs were made using 10 cm of these small intestine. The mucosal surface of the everted sac was blotted with filter paper and incubated at 37° in 1.6×10^{-5}M MB phosphate buffer solution which was bubbled with nitrogen gas during incubation. Aliquot samples of the medium were withdrawn at an interval of 30 min and the concentration of total MB was determined. (5) Penetration of LMB through the small intestine *in vitro*: Everted sacs of small intestines of rats, guinea pigs, and rabbits were prepared following the method described in (4). An everted sac was connected to a tubing which was attached to an injector. Serosal fluid used was 3 ml of isotonic phosphate buffer solution in rats and guinea pigs, and 7 ml of the buffer solution in rabbits. Then, the everted sac was incubated at 37° in 50 ml of LMB buffer solution which had been prepared by incubating other everted sacs of respective species in 1.6×10^{-5}M MB buffer solution for 90 min. *In vitro* penetration experiments were carried out under anaerobic condition with bubbling of nitrogen gas into mucosal medium. After incubating for a programmed time, both serosal and mucosal fluids were withdrawn respectively, and analyzed for LMB.

Determination of Methylene Blue, Total Methylene Blue, and Macrogol 4000——The concentration of leucomethylene blue (LMB) was calculated by substracting methylene blue (MB) from total methylene blue (total MB) concentration in the same sample solution. (1) MB and total MB in perfusate: Four ml aliquots were added with 1.2 g NaCl and 4 ml of 1,2-dichloroethane (DCE) in a centrifuging tube, which was covered with aluminum foil. The mixture was shaken for 15 min, and centrifuged for 10 min at 3000 rpm. Removing the aqueous layer, the optical density of MB in DCE layer was measured at 657 nm. For determination of total MB, 4 ml aliquots were added with 1.2 g of NaCl and 0.1 ml of 5N HCl, heated for 3 min on a boiling water bath, and 4 ml of DCE was added to the mixture after cooling. Succeeding procedure was just the same as described for MB determination. (2) MB and total MB in intestinal tissue: Analytical procedure was the same as mentioned for MB and total MB in perfusate except one condition that allowed adding 0.5 g NaCl instead of 1.2 g into 4 ml of the homogenate. (3) MB and total MB in urine: MB in urine was deter-

5) B. Borgström, A. Dahlquist, G. Lundh, J. Sjövall, *J. Clin. Invest.* **36**, 1521 (1957).

mined following the same method as employed for MB in perfusate. For total MB determination, 4 ml of diluted urine was added with 1.2 g NaCl and 0.1 ml of 0.5N HCl, and heated for 15 min on a boiling water bath. Succeeding procedure was just the same as described for determination of MB in perfusate. (4) MB and total MB in bile: Ten ml of diluted bile sample solution was added with 0.5 ml of 10% $Pb(CH_3COO)_2$ solution, and centrifuged for 10 min at 3000 rpm after shaking. Four ml of supernatant fluid was analyzed for MB following the same method as for MB in perfusate. Total MB in bile was determined using 4 ml of diluted bile sample solution by the same method as described for total MB in urine. (5) Total MB and LMB in incubation medium: Four ml of medium was used for total MB determination following the same method as for total MB in perfusate, and the total MB concentration was assumed to be due to LMB when the medium was completely colorless. (6) Macrogol 4000 (MG) in perfusate for rabbits: Two ml of the perfusion sample was used for the analysis of macrogol 4000 according to the method of Hyden.[6]

Results and Discussion

Effect of Recirculation Flow Rate on Intestinal Absorption

Koizumi, *et al.* reported that faster flow rate resulted in greaterabsorption rate of sulfamerazine *in situ* recirculation experiments using rats.[7] Figure 1 indicates that the similar tendency was observed in MB absorption experiments with slower flow rate than 11.0 ml/min in rats. As for experiments in guinea pigs and rabbits, however, the absorption ratio was not affected distinctly by the flow rate examined. Although higher flow rate was desirable to avoid or reduce the influence of flow rate on absorption ratio of MB, the mucosal epithelium of intestine was seriously injured with faster flow rate than 15 ml/min. Therefore the subsequent *in situ* absorption experiments were undertaken with the flow rate of 11.0 ml/min for rats and guinea pigs, and with 12.0 ml/min for rabbits.

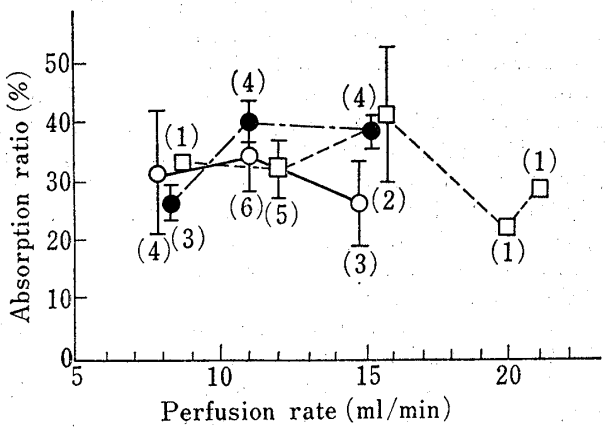

Fig. 1. Effect of Recirculation Flow Rate on Intestinal Absorption of MB in 60 min

The figure in parentheses represents the number of experiments, and the vertical bar indicates standard deviation.
—●—: rat, —○—: guinea pig, ------□------: rabbit

Intestinal Absorption in Rats, Guinea Pigs, and Rabbits

(1) Tissue accumulation of MB and LMB in small intestine: After *in situ* perfusion the amounts of MB and LMB in the whole small intestinal tissue used were determined. The results are shown in Table I. In rats and guinea pigs the ratio of total MB in the tissue to the initial amount of MB in the perfusate reached to the level of 10% approximately in 30 min. On the other hand, the ratio in rabbits remained only at 0.2% after 180 min perfusion, when total MB in perfusate was still more than 35%. Consequently, absorved amount of MB was defined as the difference between initial and residual amount of total MB in the intestinal tract, where residual amount was the sum of total MB in perfusate and total MB in the intestinal tissue of rats and guinea pigs. As for rabbits, only total MB in perfusate was assumed to be the residual amount unabsorbed, since the amount of total MB in the intestinal tissue of rabbits was negligibly small. (2) MB absorption and LMB formation *in situ*: After *in situ* recirculation for 120 min, remaining percentages of total MB in rats, guinea pigs, and rabbits were 46.4, 64.3, and 52.6%, respectively, as depicted in Fig. 2. Therefore, absorbed amounts of MB in 120 min using rats, guinea pigs, and rabbits were regarded as 54, 36, 47%,

6) S. Hyden, *Kgl. Lantbruks-Hogskel. Annlr.*, **22**, 139 (1956).
7) T. Koizumi, T. Arita, and K. Kakemi, *Chem. Pharm. Bull.* (Tokyo), **12**, 421 (1964).

TABLE I. Tissue Accumulation of MB and LMB in Whole Small Intestine after *in Situ* Perfusion

Species	Time (min)	% accumulated[a] ± SD[b] MB	% accumulated[a] ± SD[b] LMB	Total MB[c]
Guinea pig	20	3.40±1.25	5.37±2.13	8.77±2.95
	40	7.12±2.71	6.09±0.59	13.20±3.21
	60	4.08±0.64	3.45±0.44	7.53±0.81
	90	6.71±2.07	2.99±0.71	9.70±2.41
	120	7.47±3.26	8.42±0.18	15.89±3.09
	mean	5.75	5.26	11.02
Rat	15	4.51±1.39	2.52±0.89	7.03±1.21
	30	7.19±2.03	3.24±1.29	10.44±2.66
	45	5.86±0.74	2.37±0.98	8.24±1.40
	60	9.21±1.16	3.32±2.19	12.53±2.58
	120	8.73±1.21	3.03±0.21	11.75±1.42
	mean	7.11	2.90	10.00
Rabbit	180	0.09	0.28	0.37

a) % accumulated: percentages to initial amount of MB
b) SD: standard deviation
c) total MB=MB+LMB

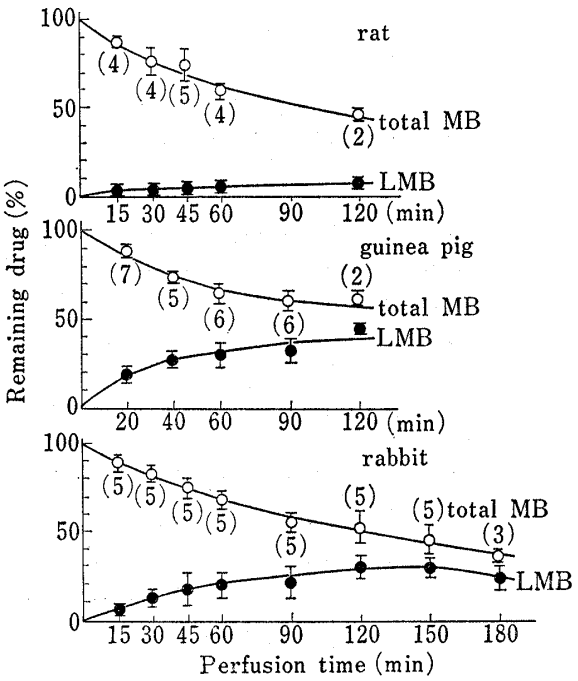

Fig. 2. Remaining Drug in Small Intestinal Perfusate and the Intestinal Tissue

The figure in parentheses represents the number of experiments, and the vertical bar indicates standard deviation.
○: total MB=MB+LMB, ●: MB

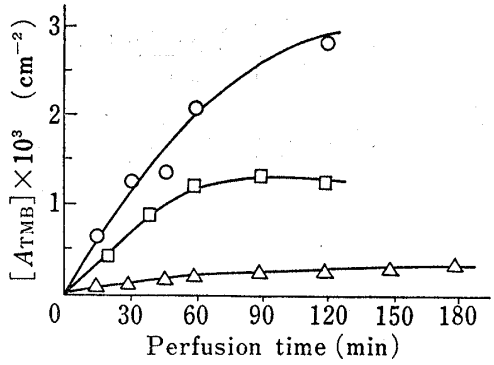

Fig. 3. MB Absorption Ratio per Unit Area of Small Intestine

○: rat, □: guinea pig, △: rabbit
As for $[A_{TMB}]$, see the text.

respectively. Since the whole small intestines were used in these recirculation experiments, the inside surface area (S) of the intestine of each species was measured by assuming the intestinal sac as a simple cilinder in order to compare the MB absorption ratio per unit area among these animals. The average areas and the standard deviations were 189±7.3 cm^2 for rats, 291±24.9 cm^2 for guinea pigs, and 1915±181 cm^2 for rabbits, and significant differences were observed among these areas. Then, the total MB absorption ratio per unit surface area ($[A_{TMB}]$) was calculated according to the following equations:

$$[A_{TMB}] = \left(1-\frac{T_{M+L}}{D_0}\right)/S \qquad \text{in rats and guinea pigs} \qquad (1)$$

$$[A_{TMB}] = \left(1-\frac{R_{M+L}\cdot MG_0}{C_0\cdot MG}\right)/S \qquad \text{in rabbits} \qquad (2)$$

where D_0 is the dose, T_{M+L} the sum of MB and LMB amounts in the perfusate and intestinal tissue, R_{M+L} the concentration of total MB in the perfusate, C_0 the initial concentration of MB, MG_0 the initial concentration of MG, and MG the concentration of MG at sampling time. As shown in Fig. 3, the absorption ratio per unit area of the small intestine for rabbit took the smallest value in these three experimental animals, though the absorption ratio for the whole small intestine of rabbit was larger than that for guinea pig. Therefore after 120 min recirculation the absorption ratio of rabbit: guinea pig: rat for unit surface area is 1: 5: 12, and that for the whole small intestine is 1: 0.77: 1.2. According to either calculation it should be assumed that there was species difference in absorption of MB from the small intestines examined. However, the differences were not so much as that seen in the article[3] which showed the urinary excretion of total MB was 2.4% in dog, and 74% in human after oral administration, *i.e.*, dog: human=1: 31.

In the recirculation experiments *in situ*, the considerable amount of LMB was observed in the perfusate for three species and in the intestinal tissue of rat and guinea pig. Remaining amounts of LMB, which had been formed from MB and unabsorbed, increased with time as shown in Fig. 2. After 120 min recirculation the percentages of LMB in intestinal tract to the initial amount of MB were 7.7% in rat, 46% in guinea pig, and 30% in rabbit. Remaining ratio of LMB per unit surface area of the intestine ($[R_{LMB}]$) was also calculated according to the following equations:

$$[R_{LMB}] = \left\{\frac{(T_{M+L}-T_M)}{D_0}\right\}/S \qquad \text{in rats and guinea pigs} \qquad (3)$$

$$[R_{LMB}] = \left\{\frac{(R_{M+L}-R_M)MG_0}{(C_0\cdot MG)}\right\}/S \qquad \text{in rabbits} \qquad (4)$$

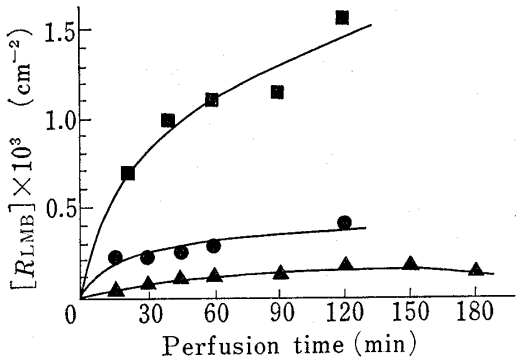

Fig. 4. Remaining Ratio of LMB per Unit Area of Small Intestine

●: rat, ■: guinea pig, ▲: rabbit
As for $[R_{LMB}]$, see the text.

where T_M is the sum of MB amounts in the perfusate and intestinal tissue, and R_M the concentration of remaining MB in the perfusate at sampling time. T_{M+L}, D_0, S, R_{M+L}, C_0, MG_0, and MG have the same meaning as those in equations 1 and 2. As illustrated in Fig. 4, the remaining ratio of LMB per unit surface area increased as follows: rabbit<rat<guinea pig. The ratio for rabbit was smallest, though the remaining ratio for the whole small intestine in rat took the least value in these three species as shown in Fig. 2. After 120 min recirculation the remaining ratio of LMB per unit surface area in rabbit: rat: guinea pig is 1: 2.6: 10 from Fig. 4, and the ratio for the whole small intestine is 1: 0.25: 1.5 in the same order of species. Then, species difference was observed also in remaining amounts of LMB in the intestinal tracts of these animals.

Urinary and Biliary Excretion in Rabbits

During absorption experiments *in situ*, urinary and biliary excretion of total MB were determined in rabbits. The cumulative amounts excreted were shown in Tables II and III. MB was excreted into urine and bile mostly as LMB in rabbits. Since the estimate of absorbed amount of MB was about 4.27 mg in 180 min, the percentage excreted were 12.8% in urine and 8% in bile. The sum of the both excretory ratios was only 20.8%. However, the excretion in the both routes appeared to continue further to some extent. Because these ex-

Table II. Urinary Excretion of MB and LMB during *in Situ* Perfusion Experiments in Rabbits

Time (min)	Cumulative amount (μg) ± SD[a]			Time (min)	Mean excretion rate (μg/min) Total MB
	MB	LMB	Total MB[b]		
0— 40	0.4± 0.5 (14.3)[c]	2.4± 3.4 (85.7)	2.8± 3.4 (100)	20	0.07
— 70	4.4± 3.8 (9.4)	42.2±18.8 (90.6)	46.6± 21.7 (100)	55	1.46
—100	12.8± 9.5 (7.2)	164.3±53.1 (92.8)	177.1± 60.8 (100)	85	4.35
—130	19.4±11.3 (6.0)	303.8±73.5 (94.0)	323.1± 82.3 (100)	115	4.87
—160	25.9±11.5 (5.8)	422.1±78.7 (94.2)	448.0± 87.1 (100)	145	4.16
—180[d]	32.4±12.4 (5.9)	515.2±98.2 (94.1)	547.6±105.7 (100)	175	4.92
—190	35.1±12.8 (5.9)	560.5±96.0 (93.9)	595.5±103.4 (100)		

a) SD: standard deviation ($n=4$).
b) total MB MB+LMB
c) Figures in parentheses represent percentages to total MB.
d) Data at 180 min were calculated by interpolation.

Table III. Biliary Excretion of MB and LMB during *in Situ* Perfusion Experiments in Rabbits

Time (min)	Cumulative amount (μg) + SD[a]			Time (min)	Mean excretion rate (μg/min) Total MB
	MB	LMB	Total MB[b]		
0— 50	0.8±1.6 (3.2)[c]	24.1± 33.0 (96.8)	24.9± 34.6 (100)	25	0.50
—100	4.5±5.4 (3.6)	121.6±110.3 (96.4)	126.1±115.1 (100)	75	2.02
—120	6.9±6.9 (3.7)	178.3±139.5 (96.3)	185.2±144.8 (100)	110	2.96
—150	9.1±7.9 (3.4)	260.8±172.4 (96.6)	269.9±178.3 (100)	135	2.73
—180	10.8±8.8 (3.2)	331.8±198.3 (96.8)	342.6±204.6 (100)	165	2.52

a) SD: standerd deviation ($n=4$)
b) total MB MB+LMB
c) Figures in parentheses represent percentages to total MB.

cretion rates took considerably high values at about 180 min yet, which was indicated on the right columns in Tables II and III. The concentration of total MB in bile increased to the same level as the initial MB concentration in perfusate at about 60 min, and at 150 min the concentration was five times greater than the initial MB level in perfusate as shown in Fig. 5. On the basis of these observations it may be assumed that urine and bile are the major routes of excretion after MB absorption in rabbits.

Formation and Penetration of LMB *in Vitro*

During absorption experiments *in situ* it was observed that the considerable amount of MB was reduced to LMB and it was found in the perfusate for these three species and in the intestinal tissue of rats and guinea pigs. In order to clarify the ability of MB reduction of the small intestinal tissue, the everted sacs of rats and guinea pigs were incubated with MB solution. Table IV indicates that MB in the mucosal fluid was reduced to LMB and that almost all MB disappeared in about 90 min in rats and guinea pigs. Since total MB in the

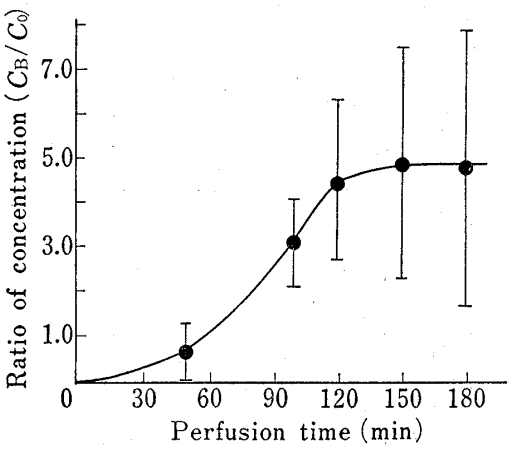

Fig. 5. Total MB Concentration in Bile of Rabbits

C_0: The initial concentration of MB in perfusion solution, C_B: The concentration of total MB in bile. The vertical bars indicate standard deviations (n=4).

colorless fluid was LMB itself, the small intestinal tissue should be assumed to have the ability to reduce MB under anaerobic condition. When the colorless fluid containing LMB was incubated with another fresh everted sac at 37° under anaerobic condition, no MB was observed in the medium. Therefore, the reduction of MB with the small intestine should be thought as irreversible one in the sense of net reaction.

The colorless fluid containing LMB was used as mucosal fluid in penetration experiments *in vitro* employing everted sacs of small intestine of rats, guinea pigs, and rabbits. The results were shown in Table V, in which each value represents an experiment with one everted sac. LMB was detected in the serosal fluid and gradually increased with time. It appeared that LMB was able to penetrate through the small intestinal barrier *in vitro* in each species examined. As

Table IV. Formation of LMB by Everted Sacs of Small Intestine

Species	Time (min)	Remaining total MB[a] (%) in mucosal medium	Color of mucosal medium
Rat	0	100	blue
	30	91.7±3 .6[b]	blue
	60	87.2±1 .8	blue
	150	73.4±7 .7	colorless[c]
	180	59.6±6 .4	colorless
	270	27.9±5 .0	colorless
Guinea pig	0	100	blue
	30	85.8± 4.0	blue
	60	60.7±10.5	blue
	90	37.9± 5.3	colorless
	150	30.9± 4.7	colorless

a) total MB=MB+LMB
b) standard deviation (n=3)
c) When the mucosal medium was colorless, it contained only LMB.

Table V. Penetration of LMB through the Small Intestinal Barrier *in Vitro*

Time (min)	$[C_s/C_m]$[a]		
	Rat	Guinea pig	Rabbit
15	0.056	—	0.059
30	0.230	0.163	0.396
45	0.413	0.127	—
60	0.458	0.110	0.407
90	0.446	0.312	0.842

a) C_s: concentration of LMB in serosal side
C_m: concentration of LMB in mucosal side

these experiments were done under anaerobic condition, *i.e.*, non-physiological condition, it should not be assumed that these results in Tables IV and V represent completely the quantitative characteristics of LMB absorption *in vivo*. However, these observations suggested partially that LMB besides MB would be also absorbable from the *in situ* small intestine of the experimental animals tested.

Acknowledgement The authors thank Miss Jinko Matsuyama for her technical assistance.