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ENHANCED INTESTINAL PERMEABILITY TO MACROMOLECULES II. IMPROVEMENT OF THE LARGE INTESTINAL ABSORPTION OF HEPARIN BY LIPID–SURFACTANT MIXED MICELLES IN RAT *,**

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SUMMARY

The effect of lipid–surfactant mixed micelles on the intestinal absorption of heparin was investigated in the loop of large intestine of rats. It was observed that large intestinal absorption of heparin was essentially very small in the absence of adjuvants. Monoolein–taurocholate, oleic acid–taurocholate and oleic acid–HCO 60 mixed micellar solutions promoted a remarkable absorption of heparin, whereas the surfactant micellar solutions alone did not enhance its absorption. The promoting effect of monoolein–taurocholate mixed micelles in the large intestine was larger than that in the small intestine, and the minimum concentration of the mixed micelles to produce the potentiation of absorption was approximately one-fourth of that required in the small intestine. The effect of monoolein or oleic acid incorporated in micellar solution on the absorption inducement of heparin and the concomitant movement of these lipids suggest that lipids play a critical role in the improvement of absorption of macromolecules.

INTRODUCTION

The gastrointestinal tract of the mature mammal has long been considered as impermeable to large molecules; therefore, the parenteral route has mostly been the administration route for drugs such as heparin and insulin. Other routes of administration or some sophisticated drug delivery system have been greatly desired for the absorption studies of large molecular drugs intended for future clinical use (Higuchi, 1977). In our previous study (Muranishi et al., 1979), the effect of monoolein–bile salts mixed micelles on the intestinal permeability of heparin in the region of small intestine of rat has been reported, showing remarkable enhancement of its permeability.

* Part I of this series: Tokunaga et al. (1978).
** A preliminary report on this work has appeared previously (Muranishi et al., 1977).

Rectal administration is also one of the possible routes of administration for large molecular drugs since the suppository has been in use since well before our lifetime. Some studies concerning the rectal absorption of chymotrypsin (Kabacoff et al., 1963) or albumin (Dalmark, 1968; Warshaw et al., 1977) have been investigated. Large molecules' absorption in the large intestine following rectal administration has been remained an almost unexplored matter, although some recent study concerning absorption of bovine serum albumin reported that the fraction of the intact protein absorbed from the colon was significantly smaller than that absorbed from the small intestine (Warshaw et al., 1977.

In the present investigation, heparin, a large molecule, is chosen for basic studies on the absorption from the large intestine of rat. Safe delivery systems such as mixed micelles are examined. A remarkable absorption increase occurred as the impermeable drug was administered in a mixed micellar solution. This absorption rise occurred at even lower concentrations of the mixed micellar solution than that observed at the small intestinal area.

MATERIALS AND METHODS

Materials. Heparin sodium was purchased from Nakarai Chemicals. Radioactive [^{35}S]-labelled heparin sodium was purchased from New England Nuclear with a spec. act. of 12.4 μCi/mg. Monoolein used was of high purity grade (Nikko Chemicals). Sodium taurocholate was synthesized according to the method of Norman (Norman, 1955). The purity of the bile salt was checked by thin-layer chromatography and infrared spectroscopy. The non-ionic surfactant, polyoxyethylene derivative of hydrogenated castor oil (HCO 60), was supplied from Nikko Chemicals. All other chemicals used were of reagent grade quality.

Preparation of test solutions. Mixed micellar solutions were prepared by dissolving lipids in distilled water containing bile salt, synthetic surfactant and/or heparin. A clear solution was obtained upon sonicating the mixture at 37°C for 4 min with an Ohtake sonicator model 5202. The same sonicator was used for the preparation of oil in water (o/w) emulsions carried out for 5 min at 4°C. Components of each test solution are shown in Table 1.

Absorption studies. Male Wistar albino rats weighing 200–250 g were anesthetized with pentobarbital. The intestine was exposed through midline incision, and a closed loop of the entire large or small intestine was prepared by ligation at the proximal and distal ends. The test solution was introduced into the intestinal loop at a dose of 5 mg heparin/200 g body weight in a vol. of 2.0 ml/200 g body weight, or at a dose of 10 mg heparin/200 g body weight in a vol. of 4.0 ml/200 g body weight. Blood samples were collected by cardiac puncture using a 10 ml siliconized syringe 30 min after administration. Blood samples were placed in an ice bath immediately after collection. In the case of [^{35}S]-labeled heparin, blood samples were collected from the carotid artery using a polyethylene cannula 5, 15, 30, 45 and 60 min after administration. For the determination of monoolein or oleic acid, the luminal fluid was collected by washing out with saline at 5, 15 or 60 min, respectively, after administration.

TABLE 1

COMPOSITION OF TEST SOLUTIONS USED IN THIS INVESTIGATION

Test solution	Composition
Control	Heparin *
10 mM NaTC **	Heparin + 10 mM NaTC
10 mM monoolein–NaTC	Heparin + 10 mM NaTC + 10 mM monoolein
10 mM oleic acid–NaTC	Heparin + 10 mM NaTC + 10 mM oleic acid
20 mM NaTC	Heparin + 20 mM NaTC
20 mM monoolein–NaTC	Heparin + 20 mM NaTC + 20 mM monoolein
40 mM NaTC	Heparin + 40 mM NaTC
40 mM monoolein–NaTC	Heparin + 40 mM NaTC + 40 mM monoolein
PS 80 ***	Heparin + 0.2% PS 80
Oleic acid–PS 80	Heparin + 0.2% PS 80 + 10 mM oleic acid
HCO 60	Heparin + 0.2% HCO 60
Oleic acid–HCO 60	Heparin + 0.2% HCO 60 + 10 mM oleic acid
Oleic acid emulsion	Heparin + 0.2% HCO 60 + 4% oleic acid
Trioctanoin emulsion	Heparin + 0.2% HCO 60 + 4% Trioctanoin

* Concentration of heparin was 2.5 mg/ml.
** NaTC = sodium taurocholate.
*** PS 80 = polysorbate 80.

Analytical method

Plasma clearing factor activity (PCFA). Assay of PCFA using blood samples was carried out according to our previous paper (Tokunaga et al., 1978). The collected blood sample was immediately mixed with one-tenth volume of 3.8% sodium citrate solution, centrifuged in the refrigerated centrifuge, and the plasma samples were kept at 4°C until they were assayed. PCFA induced by heparin was determined via turbidimetric assay on aliquots of plasma (Korn, 1959). In a cuvette 1.2 ml of Tris (hydroxymethyl) aminomethane buffer (pH 8.5), 0.6 ml of 25% bovine serum albumin fraction V (pH 8.5), and 1.0 ml of the plasma sample were mixed. Substrate emulsion, prepared according to the method of Grossman, was added at zero time (Grossman and Colo, 1954) and the absorbance at 650 nm was immediately recorded. Then, the mixture was incubated at 27.5°C and absorbance readings were taken at 15 and 30 min. The results were reported in terms of decreased absorbance at the indicated times.

[^{35}S]Heparin. Plasma was separated by the Eppendorf centrifuge (Model 3200). A 0.2 ml aliquot of plasma sample and 0.1 ml of 1 N HCl were added to a 20 ml counting vial containing 15 ml of scintillation medium (500 ml of ethyleneglycol monoethylether and 5.0 g of 2,5-diphenyloxazole (PPO)/liter of toluene). Radioactivity was determined in a liquid scintillation counter, Beckmann Model LS-232.

Monoolein. Monoolein was determined by the modified method of van Handel (C. Naito et al., 1966). Chloroform extract containing monoolein was evaporated at 85°C and chloroform was completely removed under vacuum for 30 min. The residual monoolein was hydrolyzed to glycerol with potassium hydroxide and then oxidized to formaldehyde with sodium periodate. The latter was colored with chromotropic acid and

determined spectrophotometrically at 570 nm after the addition of thiourea to reduce the blank.

Oleic acid. Oleic acid was determined by the modified method of Itaya (Itaya and Ui, 1965). Copper triethanolamine solution was added to chloroform extract containing oleic acid, and the mixture, in a glass stoppered test tube, was shaken for 1 min. Separation of the chloroform phase was carried out and after filtration, two drops of sodium diethyldithiocarbamate solution were added for the spectrophotometric determination at 440 nm.

Histology. In another separate series of experiments, the intestinal loops of two animals were infused with each test solution (distilled water and 10 mM monoolein–taurocholate mixed micellar solution) for 30 min under the same conditions as the absorption experiment. After the animals were killed, the entire large intestine was placed in 10% formalin solution. Haematoxylin–eosine-stained slides were prepared and observed on light microscopy.

RESULTS

Absorption measured by PCFA. To test whether promotion of heparin absorption can occur in the large intestine as well as in the small intestine, determination of PCFA 30 min after the administration of heparin was taken as a criterion of its absorption. The addition of the following adjuvants which are relatively safe to the mucosa was tested in this study; surfactants such as sodium taurocholate (NaTC), polysorbate 80 (PS-80) and HCO-60; also lipids such as monoolein, oleic acid, trioctanoin and triolein.

Determinations of PCFA in the large intestine following the administration of various preparations are shown in Table 2. In the presence of aqueous heparin solution alone

TABLE 2

PLASMA CLEARING FACTOR ACTIVITY DETERMINED IN THE LARGE INTESTINAL LOOP AFTER THE ADMINISTRATION OF VARIOUS HEPARIN PREPARATIONS WITH ADDED ADJUVANTS

	Mean fall in OD_{650} ± S.E.M. *	
	At 15 min **	At 30 min **
Control	0.009 ± 0.006	0.026 ± 0.011
10 mM NaTC	0.017 ± 0.004	0.046 ± 0.011
10 mM monoolein–NaTC	0.214 ± 0.008	0.346 ± 0.017
20 mM NaTC	0.060 ± 0.009	0.125 ± 0.005
20 mM monoolein–NaTC	0.248 ± 0.021	0.398 ± 0.025
40 mM NaTC	0.205 ± 0.025	0.339 ± 0.031
40 mM monoolein–NaTC	0.349 ± 0.034	0.484 ± 0.035
Oleic acid emulsion	0.218 ± 0.013	0.023 ± 0.001
Trioctanoin emulsion	0.011 ± 0.004	0.023 ± 0.001

* Figures represent the mean ± S.E. of 4–5 animals.
** Incubation time.

(control), a slight PCFA indicated that the large intestine (area from the colon to the rectum) is impermeable to heparin. But, a significant increase in PCFA occurred when heparin was administered with any concentrations from 10 to 40 mM of monoolein–taurocholate mixed micelle solution. Although some increase in PCFA was also elicited by the presence of 20 or 40 mM sodium taurocholate micellar solutions, the value was obviously lower than that of the mixed micellar solutions. A remarkable difference of the 10 mM mixed micellar solution compared with the 10 or 20 mM micellar solution is noticeable. This fact suggests that monoolein is certainly a useful moiety for potential absorption of heparin. On the other hand, although an increased effect of oleic acid o/w emulsion was estimated, no effect of trioctanoin o/w emulsion was detected.

The enhancing effect of this mixed micellar solution on the absorption of heparin was comparatively studied in the small intestine and the large intestine, and the results are shown in Fig. 1. In the case of the small intestine the volume of test solution was 4 ml, twice the volume used in the large intestine (corresponding to the volume of intestinal lumen), and therefore the dose of heparin was twice that of the large intestine. In spite of this difference in volume the increase of PCFA by the mixed micellar solution in the large intestine was larger than that of the small intestine at any concentration tested. This result indicates that the large intestinal mucosa is more sensitive to the action of mixed micelles.

The effect of lipid addition in the mixed micellar solution was then evaluated. Solutions prepared with various concentrations of monoolein were used, and PCFA was measured after the administration of heparin as shown in Fig. 2. A sharp rise is observed over 2 mM, reaching a nearly equal effect over 2.5–10 mM. Some other adjuvant was also

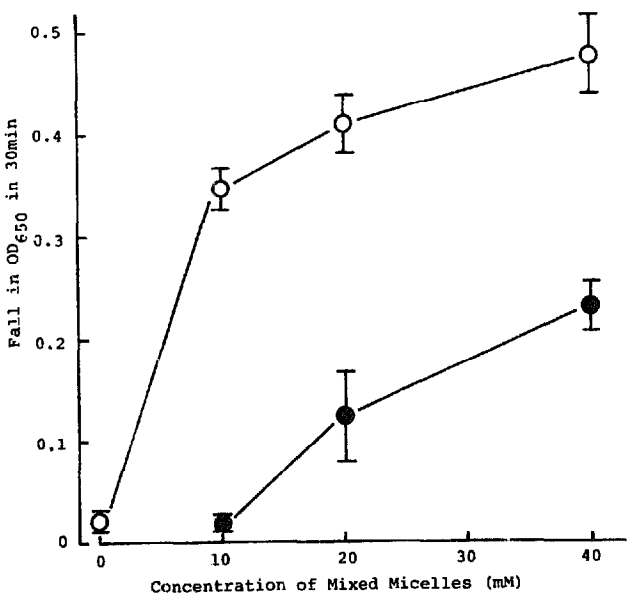

Fig. 1. Effect of mixed micelles concentration on plasma clearing factor activity after the administration of heparin. Comparative studies in the small intestine and the large intestine. Each value is the mean ± S.E.M. of 4–6 animals. •, small intestine; ○, large intestine.

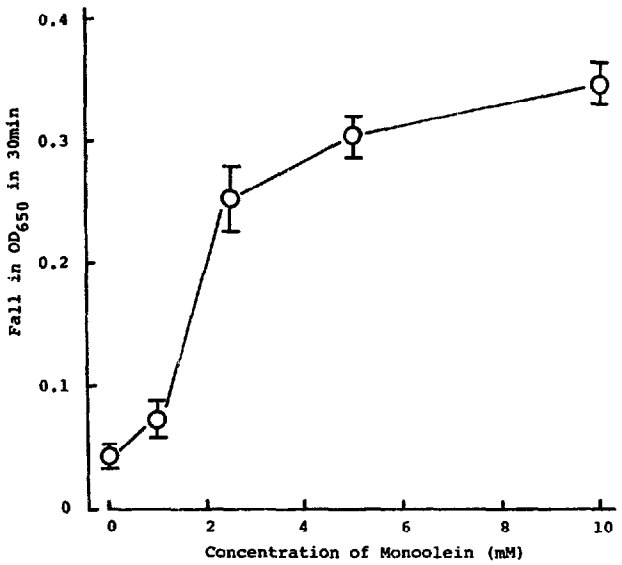

Fig. 2. Effect of monoolein concentration on plasma clearing factor activity (PCFA) after administration of heparin into the large intestine. Concentration of NaTC was kept constant at 10 mM in every experiment. Each value is the mean ± S.E.M. of 4–5 animals.

examined as a possible substitute for bile salts or monoolein in mixed micellar solution. Oleic acid could replace monoolein in taurocholate solution as shown in Fig. 3. Although the 0.2% polysorbate 80 or HCO 60 micellar solution alone did not increase the PCFA value, an obvious increase was found after the addition of oleic acid as a mixed micellar solution. If in this last solution monoolein was added instead, no such increment was observed.

Absorption measured by [^{35}S]-labeled heparin. Enhancement of heparin absorption by the large intestine was tested with [^{35}S]-labeled radioactive heparin. A time course of

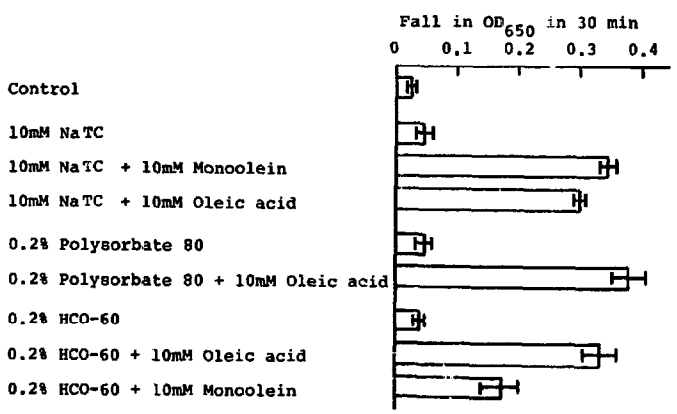

Fig. 3. Plasma clearing factor activity after the administration into the large intestine. Several types of mixed micelles containing oleic acid were tested. Each value is the mean ± S.E.M. of 4–5 animals.

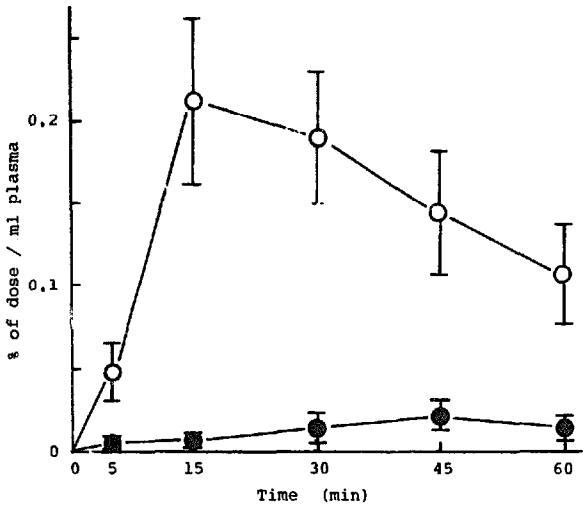

Fig. 4. Time course of plasma level radioactivity after the administration of [^{35}S]heparin into the large intestine. Each value is the mean ± S.E.M. of 4 animals. •, control solution; ○, 10 mM monoolein–NaTC mixed micellar solution.

the plasma level radioactivity following administration of 10 mM monoolein–taurocholate mixed micellar preparations with [^{35}S]heparin is comparatively shown in Fig. 4, with the control experiment carried out using an aqueous solution of [^{35}S]heparin. Very low plasma level radioactivity was observed in the control group. On the other hand, the plasma level of radioactivity after the administration of mixed micellar solution increased reaching a peak after 15 min and then followed by a gradual fall.

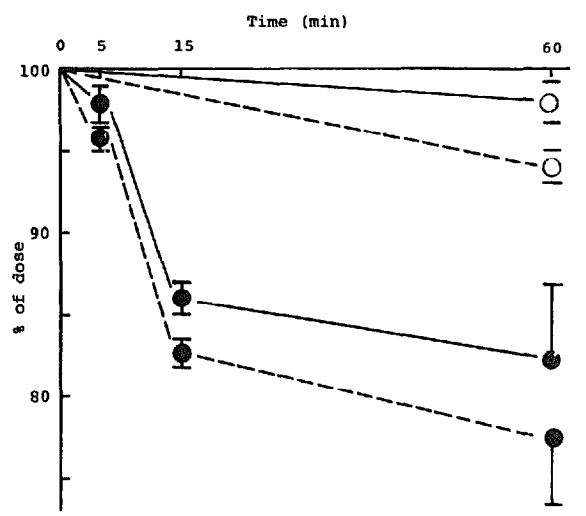

Fig. 5. Disappearance of radioactivity from the large intestine after the administration of [^{35}S]heparin. Each value is the mean ± S.E.M. of 4 animals. ○, control solution; •, 10 mM monoolein–NaTC mixed micellar solution. - - - - -, remained in the lumen; ———, remained in the lumen plus in the tissue.

[^{35}S]Heparin radioactivity clearance from the large intestinal lumen and tissue was also estimated, as shown in Fig. 5. In the case of the control solution, the disappearance of radioactivity was negligible even 60 min after administration. However, the administration of the mixed micellar solution enhanced the disappearance of activity from the intestinal lumen and about 15% of radioactivity had already disappeared from the large intestine 15 min after administration. After this period of sharp decrease a slight diminution was observed. These results suggest that the highest absorption of [^{35}S]heparin from the mixed micellar solution occurred within the first 15 min.

Behavior of lipids in mixed micellar solution. A knowledge of the absorption behavior of the lipid component of mixed micelles such as monoolein and oleic acid was more than desirable in the present study. Clearance of monoolein or oleic acid from the large intestinal lumen after its administration in the form of mixed micelles was determined. Fig. 6 shows the clearance of monoolein from taurocholate or HCO 60 mixed micellar solution and clearance of oleic acid from taurocholate mixed micellar solution. In the case of monoolein–taurocholate mixed micellar solution, monoolein disappeared rapidly from the lumen within 15 min, and after this period a smooth slope of disappearance was observed. Disappearance of oleic acid in the mixed micellar solution containing taurocholate registered a similar pattern to monoolein mentioned above. But, monoolein contained in HCO 60 mixed micellar solution disappeared at slower rate than that from taurocholate. So, better permeability of monoolein through the intestinal epithelium from the mixed micellar solution containing taurocholate rather than that containing HCO 60 can be predicted.

Histology studies. Using the light microscope, the observation of the mucosal surface in the large intestine exposed to 10 mM monoolein–taurocholate mixed micellar solution have shown that epithelial cells remained intact, but changes, like disorder of surface mucosa cells, were detected.

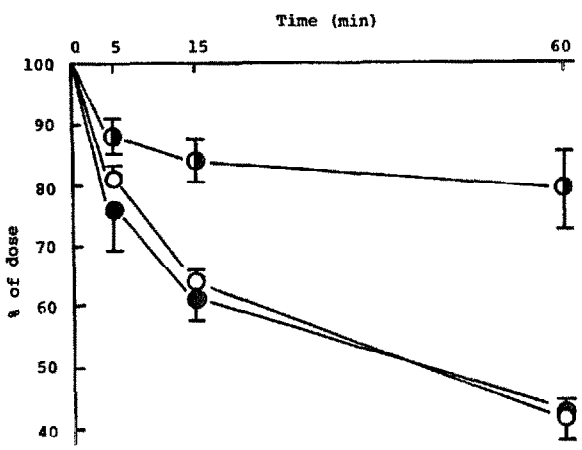

Fig. 6. Disappearance of monoolein or oleic acid from the lumen after the administration of 3 types of mixed micelles into the large intestine. Each value is the mean ± S.E.M. of 4–6 animals. ○, oleic acid–NaTC; ●, monoolein–NaTC; ⊙, monoolein–HCO 60.

DISCUSSION

Little is known about the absorption of macromolecules after its administration through rectal route into the large intestine. In a previous publication (Kabacoff et al., 1963), chymotrypsin appeared to be absorbed from the rectum of the rabbit in an enzymatically active form, though in small amount. On the other hand, Warshaw et al. reported that the fraction of bovine serum albumin absorbed from the colon was only 0.13%, which is significantly smaller than that absorbed from the small intestine (Warshaw et al., 1977). Our results showed that if heparin was instilled into the large intestine as a solution prepared in distilled water (control) only slight amounts reached the blood stream. Therefore, the normal colon and rectum were considered to be essentially impermeable to macromolecules.

In this investigation, polar lipid–surfactant mixed micelles were used as safe adjuvants to increase the absorption of heparin in the large intestine. Previous work (Tokunaga et al., 1978) has shown that a mixed micellar solution prepared in distilled water promoted the absorption of heparin more markedly than in its buffer solution, and so distilled water was chosen for dissolving either components in all experiments of the present study.

The adequate concentration of monoolein or oleic acid incorporated in mixed micelles to potentiate the absorption of heparin from the large intestine was in the range of 10 mM. This concentration represented one-fourth of the concentration needed in the absorption studies carried out in the small intestine. Similar results have been observed in the absorption of aminoglycosides (Muranishi et al., 1979). Furthermore, the enhancing effect estimated in the large intestine exceeded the phenomenon detected in the small intestine, over the range of 10–40 mM (Fig. 1). Thus, a strong evidence about the higher sensitivity of the rectum or colon rather than the upper gastrointestinal tract to affect absorption enhancement by the mixed micelles has appeared. Comparing the radioactivity maximum obtained in the plasma after the administration of [^{35}S]heparin into the large intestine, the peak concentration was reached in a shorter time than for the small intestine (Fig. 4) indicating that the enhancing effect of mixed micelles is faster in the large intestine.

Lipids certainly play an important role in the intestinal absorption of heparin. Although 10 mM taurocholate micellar solution did not cause a marked increase, an addition of more than 2.5 mM monoolein to that solution resulted in an induction of the absorption (Fig. 2). Monoolein and oleic acid caused a remarkable effect irrespective of the surfactant present, though some differences prevailed (Fig. 3). Another interesting finding was the ineffectiveness of triglycerides such as triolein and trioctanoin, so that the great efficacy of absorption of oleic acid plays an important role in the improvement of heparin absorption.

Furthermore, polar lipids administered as adjuvant can be also absorbed from the large intestine, penetrating through the epithelial cells. The disappearance of monoolein or oleic acid from the large intestinal lumen was remarkable within the initial 15 min (Fig. 6). This disappearance appears to be highly correlated with the absorption rate of heparin as is shown in Figs. 4 and 5. In addition, the absorption of heparin was higher from monoolein–taurocholate solution than from monoolein–HCO 60 solution, and con-

comitantly monoolein itself was absorbed in higher amounts from the taurocholate solution than from the HCO 60 solution. These findings suggest that some relationship exists between the penetration of polar lipids into the epithelial cell membrane and the delivery of heparin molecule into the cell. Also, a possible interaction of heparin with the mixed micelles might be an additional factor in the mechanism of absorption inducement described in the previous paper (Tokunaga et al., 1978).

Microscopic observations show that the exposure to 10 mM mixed micellar solution is unharmful. No severe damage, such as loss of epithelial cells or spreading of the intercellular spaces was detected. Further investigations, such as electron microscopy and/or effect of longer exposure to mixed micelles is needed before clinical application can be furnished.

In conclusion, rectal administration appears as an advantageous route for impermeable macromolecules like heparin if polar lipid–surfactant mixed micelles (absorption inducers) can be added in the drug formulation.

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