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Notes

**Effect of Albumin on the Absorption of Phenol Red, Bromphenol Blue and Bromosulphonphthalein as Model Drugs from the Liver Surface Membrane in Rats**

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The effect of bovine serum albumin (BSA) on drug absorption from the liver surface in rats was examined by using three organic anions (phenol red, bromphenol blue and bromosulphonphthalein) as model drugs which have a high affinity for albumin. The binding ratio of the model drugs (3 mg/ml in phosphate buffer) to BSA varied widely at a BSA concentration of 0.1 - 10 % (W/V). The model drugs (3 mg/ml X 0.1 ml) with or without BSA were applied to the rat liver surface *in vivo* employing a cylindrical glass cell (i.d. 9 mm, area 0.64 cm<sup>2</sup>). The absorption ratios of the model drugs from the rat liver surface at 6 h, calculated from the amount recovered from the glass cell, decreased with an increase in BSA concentration. A similar trend was observed with biliary recovery of the model drugs. A marked reduction in the absorption ratio was seen with bromosulphonphthalein, which has the highest binding activity to BSA among the three organic anions. Accordingly, protein binding appears to be a significant factor with respect to drug absorption from the liver surface.

**Key words** protein binding; absorption; liver surface; rat; organic anion; bovine serum albumin

Previously,<sup>1)</sup> we reported the possibility of drug absorption from the liver surface in rats and demonstrated that direct application to the liver surface can be useful for drug targeting to the desired site in the liver. Furthermore, we suggested that a specialized transport process might not exist for drug absorption from the rat liver surface.<sup>2)</sup>

Protein binding is one of the most important factors in determining drug disposition in the body, and it is assumed that the unbound fraction is involved mainly in the drug transport. The serous fluid in the peritoneal cavity has a total protein concentration of ca. 2 % (W/V), and it is well known that albumin concentration changes extensively in serious disease. Accordingly, the effect of protein binding on drug absorption from the liver surface needs to be elucidated in order to attain effective and safe drug delivery. Although there have been some reports concerning the intraperitoneal transport of proteins,<sup>3-5)</sup> the absorption characteristics of protein from a specific organ remains to be clarified.

In a series of investigations on elucidation of the absorption mechanism from the liver surface, we examined the effect of bovine serum albumin (BSA) on drug absorption from the liver surface in rats. In the present study, phenol red (PR), bromphenol blue (BPB) and bromsulphonphthalein (BSP) were selected as model drugs with a relatively high affinity for BSA, because their *in vivo* absorbability from the rat liver surface had been previously determined.<sup>1,2)</sup>

## MATERIALS AND METHODS

**Chemicals** PR and BPB were purchased from Nacalai Tesque, Inc. (Kyoto, Japan). BSA (fraction V) and BSP were obtained from Sigma Chemical Co. (St. Louis, MO, U.S.A.). All other chemicals were reagent grade products.

**Determination of Binding Ratio of the Model Drugs to BSA** The binding of the model drugs to BSA in an isotonic phosphate buffer (pH 7.4) at 37°C was studied with the chamber cells for equilibrium dialysis by the method of Takada *et al.*<sup>6)</sup> after slight modification. The two chambers were separated into donor and receiver sides using a Visking tube membrane (Mw cutoff, 12000 - 14000). BSA was dissolved in the buffer solution to give a concentration of 0.2 mM. The drug concentrations varied from 0.01 to 10 mM. Three ml of the mixture of the model drug and BSA and the buffer solution were added to the donor and receiver sides, respectively. After dialysis at 37°C for 72 h, the solutions in the donor and receiver sides were removed, then the concentrations of unbound and bound model drug were measured. Adsorption of the model drugs onto the dialysis membrane surface was negligible.

**In Vivo Experiment** All animal procedures in the present study conformed to the Guidelines for Animal Experimentation in Nagasaki University.

Male Wistar rats (230-250 g) were anesthetized with sodium pentobarbital (50 mg/kg, i.p.) and the left femoral artery was cannulated with a polyethylene tube (i.d. 0.5 mm, o.d. 0.8 mm; Dural Plastics, Dural, Australia). After the middle abdomen was cut open about 3 cm, the common bile duct was cannulated with a polyethylene tube (i.d. 0.28 mm, o.d. 0.61 mm; Becton Dickinson & Co., Parsippany, NJ, U.S.A.). A cylindrical glass cell (i.d. 9 mm, area 0.64 cm<sup>2</sup>) was attached to the rat liver surface at the area of the left lobe with Aron Alpha (Sankyo Co., Ltd, Tokyo, Japan). The body temperature of the rats was kept at 37°C by a

heat lamp during the experiment. The test solution was prepared in an isotonic phosphate buffer (pH 7.4) to yield a drug concentration of 0.3 mg/0.1 ml containing 0, 0.1, 1, 5 and 10 % (W/V) BSA, and added to the glass cell directly. The top of the glass cell was sealed with a piece of aluminum foil to prevent evaporation of the applied solution. Blood samples (200  $\mu$ l) were collected at selected times after dosing from the heparinized cannula inserted into the femoral artery over a 6-h period, and were centrifuged at 15,000 rpm for 5 min. Bile samples were collected at appropriate time intervals for 6 h. At 6 h after the application, urine was collected directly from the bladder with a syringe, and the solution remaining in the glass cell was withdrawn by more than five washings with saline.

**Analytical Method** The concentrations of the model drugs in the plasma, bile, urine and remaining solution in the glass cell were determined as follows.

PR: The concentration of free PR was determined spectrophotometrically at 560 nm after dilution with 1 N NaOH solution. The total concentration of free PR and its metabolite was measured in the same manner after they were subjected to acid hydrolysis (1 N HCl at 100°C for 30 min).<sup>7)</sup>

BPB: The concentration of BPB was determined spectrophotometrically at 591 nm after dilution with an isotonic phosphate buffer (pH 7.4).<sup>6)</sup>

BSP: The total concentration of free BSP and its metabolite was determined spectrophotometrically at 580 nm after dilution with 0.1 N NaOH solution.<sup>8)</sup>

**Statistical Analysis** Statistical analysis was performed by applying the unpaired Student's *t*-test.  $p < 0.05$  was considered to be statistically significant from the control. All results were expressed as the mean value  $\pm$  standard error of at least four experiments.

## RESULTS

***In Vitro* Binding of the Model Drugs to BSA** Figure 1 illustrates the Scatchard plots for *in vitro* binding data of the model drugs in an isotonic phosphate buffer (pH 7.4). In Fig. 1,  $r$  is the bound molar ratio of the model drugs ( $r = [D_b] / [P_t]$ ), where  $[D_b]$  and  $[P_t]$  are the molar concentration of bound drug and BSA, respectively, and  $[D_u]$  is the molar concentration of the unbound drug. Since Fig. 1 was close to the two-phase patterns for each model drug, the Scatchard plot can be explained as follows,

$$r = \frac{n_1 \cdot K_1 \cdot [D_u]}{1 + K_1 \cdot [D_u]} + \frac{n_2 \cdot K_2 \cdot [D_u]}{1 + K_2 \cdot [D_u]} \quad (1)$$

where  $K$  ( $K_1, K_2$ ) and  $n$  ( $n_1, n_2$ ) are the association constant and number of binding sites for each site, respectively.

The best fit of Eq. (1) was obtained by using the non-linear regression program MULTI<sup>9)</sup> (Fig. 1), based on the assumption of two types of binding sites with different affinities and capacities. As shown in Table 1, the binding parameters for the model drugs differed greatly. The extent of BSP binding to BSA was the highest among the three model drugs. The bound fraction (%) in the presence of 0.1, 1, 5 and 10 % (W/V) BSA was also estimated from these parameters (Table 1). The binding ratio of the model drugs to BSA increased according to BSA concentration. In particular, more than 90 % of BSP and BPB existed in the bound form above 5 % BSA.

**Effect of BSA on the Appearance of Model Drugs in the Plasma and Bile after Application to the Rat Liver Surface** PR was found in the plasma after its application to the rat liver surface in the presence or absence of BSA in the drug solution (Fig. 2). For BPB and BSP, their plasma concentrations were below the limit of detection (0.087  $\mu\text{g/ml}$  for BPB; 0.10  $\mu\text{g/ml}$  for BSP), probably because of rapid hepatic uptake. The PR plasma

concentration decreased with an increase in the concentration of BSA for up to 6 h, suggesting a decreased absorption rate from the rat liver surface. The maximum concentration of PR in the presence of 10 % BSA was about one-fourth of that in the control.

Every model drug absorbed from the rat liver surface appeared into the bile (excretion patterns not shown). The biliary excretion rates of the model drugs were decreased up to 6 h, according to the BSA concentration, similar to the plasma concentration of PR. This finding might be due to the suppression of the absorption rate of the model drugs from the rat liver surface.

**Recovery of the Model Drugs in the Bile, Urine and Solution Remaining in the Glass Cell** Table 2 summarizes the recovery of the model drugs in the bile, urine and solution remaining in the glass cell after application to the rat liver surface in the presence or absence of BSA. The absorption ratio of the model drugs at 6 h, calculated from the amount remaining in the glass cell, decreased considerably, with an increase in the BSA concentration. In the presence of 10 % BSA, the absorption ratio at 6 h for PR, BPB and BSP was 38, 22 and 10 % of the control, respectively. This order closely correlated with the tightness of their binding to BSA.

PR was excreted into the bile and urine, whereas BPB and BSP were predominantly excreted in the bile. The urinary recovery of PR and the biliary recovery of the model drugs were decreased in proportion to the decrease in the absorption ratio (Table 2). A marked reduction was observed with the biliary recovery of BPB and BSP at a BSA concentration above 5 %.

## DISCUSSION

In the present study, protein binding was found to be an important factor in controlling the drug absorption from the liver surface. The decreased absorption rate of the model drugs in the presence of BSA might be associated with an apparent increase in molecular weight according to protein binding. Accordingly, the absorbability of a drug with high protein binding activity or a macromolecular drug such as bioactive peptide and protein is considered to be extremely low. But, BPB and BSP were absorbed from the rat liver surface to some extent (18.9 and 6.6 % of dose in 6 h, respectively) in the presence of 10 % BSA (Table 2), although more than 99 % of them bound to BSA (Table 1).

The hepatic uptake of the three organic anions as models with a high affinity for BSA from the blood (sinusoid) space to the liver cell is assumed to be inhibited by plasma proteins such as albumin. On the other hand, several investigators have reported that the hepatic uptake rate is not proportional to the unbound fraction, and proposed the existence of albumin-mediated transport.<sup>10-12)</sup> However, the effect of albumin on the hepatic uptake of albumin-bound substances remains controversial, judging from the series of investigations of this phenomenon.<sup>13-18)</sup>

Previously,<sup>2)</sup> we suggested that the specific transport mechanism, such as active transport, might not involve the drug absorption from the rat liver surface membrane, which is constructed mainly of collagen and fibronectin. It is of physiological interest that absorption from the rat liver surface membrane proceeds such that almost all of the drug binds to albumin, although the precise mechanism could not be analyzed in the present experimental system.

Consequently, we clarified that protein binding tended to suppress drug absorption from the rat liver surface. The effect of molecular weight on drug absorbability from the

liver surface remains to be elucidated in the future.

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Figure titles and legends

Fig. 1. Scatchard Plots of Binding to BSA of PR ( ), BPB ( ) and BSP ( ) in pH 7.4 Phosphate Buffer at 37°C

Inset shows the same plot of PR for the small concentration range. Curves show the simulated functions obtained based on the binding parameters shown in Table 1.

Fig. 2. Plasma Concentration Profiles of PR after Application to Rat Liver Surface at a Dose of 0.3 mg in the Presence or Absence of BSA

BSA concentration is 0 ( ), 0.1 ( ), 1 ( ), 5 ( ) and 10 % ( ). The result of the control (without BSA) was reported previously.<sup>2)</sup> Each point represents the mean  $\pm$  S.E. of at least four experiments.

Table 1. Binding Parameters and Binding Ratio to BSA of PR, BPB and BSP (3 mg/ml) in pH 7.4 Phosphate Buffer at 37°C

	Binding parameters				Binding ratio (%)			
	$K_1$ ( $\text{mM}^{-1}$ )	$n_1$	$K_2$ ( $\text{mM}^{-1}$ )	$n_2$	+ BSA (%)			
					0.1	1	5	10
PR	34.6	1.4	0.4	8.8	1.5	14.3	59.0	83.3
BPB	158.5	3.6	0.3	14.8	4.0	35.4	90.0	99.5
BSP	209.2	6.6	0.8	29.5	9.8	71.9	99.5	99.9

Table 2. Recovery (% of dose) of PR, BPB and BSP after Application to Rat Liver Surface at a Dose of 0.3 mg in the Presence or Absence of BSA

+ BSA (%)	PR			BPB		BSP	
	Glass cell	Bile	Urine	Glass cell	Bile	Glass cell	Bile
Control	8.9 <sup>a)</sup> ±2.1	47.4 <sup>a)</sup> ±4.4	38.7 <sup>a)</sup> ±6.5	14.3 ±1.1	69.1 ±1.7	34.3 ±2.7	60.9 ±3.6
0.1	11.3 ±1.4	47.0 ±7.1	34.0 ±9.0	16.8 ±0.1	72.9 ±2.3	41.0 ±1.3	56.9 ±4.3
1	25.2 <sup>**</sup> ±1.8	36.0 ±2.1	31.9 ±2.4	32.0 <sup>**</sup> ±2.1	50.2 <sup>**</sup> ±2.5	62.8 <sup>**</sup> ±0.9	40.1 <sup>**</sup> ±3.1
5	35.1 <sup>**</sup> ±2.8	27.7 <sup>**</sup> ±3.0	22.2 ±6.2	72.2 <sup>**</sup> ±0.6	14.3 <sup>**</sup> ±2.2	84.5 <sup>**</sup> ±3.8	12.4 <sup>**</sup> ±1.9
10	65.5 <sup>**</sup> ±1.4	16.7 <sup>**</sup> ±1.1	15.6 <sup>*</sup> ±3.5	81.1 <sup>**</sup> ±0.4	10.0 <sup>**</sup> ±1.4	93.4 <sup>**</sup> ±0.8	12.4 <sup>**</sup> ±0.4

Each value is the mean ± S.E. of at least four experiments. a) Results were reported previously.<sup>2)</sup> Statistical significance from control (\* $p < 0.05$ , \*\*  $p < 0.01$ ).