

## Effects of Aspirin and/or Salicylate on Hydrolysis and Glucuronidation of Indomethacin in Rat Erythrocytes and Hepatocytes

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**This study was conducted to explore the mechanism of the pharmacokinetic interaction between aspirin (ASP) and indomethacin (IND) using rat erythrocytes (RBCs) and hepatocytes. ASP was hydrolyzed to salicylic acid (SA) in both the RBCs and hepatocytes. Within RBCs, aspirin and/or salicylate (ASP/SA) increased the concentration of IND, accompanied by a constant hydrolysis of IND. In hepatocytes, a low dose of IND was subjected to glucuronidation rather than hydrolysis, and ASP/SA inhibited both the acylglucuronidation of IND and hydrolysis of IND glucuronide. A high dose of IND underwent hydrolysis with about double the glucuronidation, and ASP/SA decreased the ratio of hydrolysis to glucuronidation, accompanied by a loss of ASP, IND and their metabolites from the medium. Collectively, the results provide metabolic insight into the mechanism of drug-drug interaction between ASP/SA and IND in the hepatocytes and RBCs.**

**Key words** aspirin; indomethacin; des-*p*-chlorobenzoyl-indomethacin; ATP; salicylate; uridine 5'-diphosphate (UDP) glucuronosyltransferase

Indomethacin (IND) is a widely used nonsteroidal anti-inflammatory drug. Its clinical pharmacological effect is known to be diminished by co-administration of aspirin (ASP).<sup>1,2)</sup> Its pharmacokinetic interaction with ASP is generally not well understood, although it is reported that a single or multiple doses of ASP decreased the plasma concentration of IND, but not of ASP, in both healthy volunteers<sup>3)</sup> and rheumatic patients given both IND and ASP orally.<sup>2)</sup> Chronic treatment with ASP was found to decrease the gastrointestinal absorption, to enhance the biliary clearance and the enterohepatic circulation, and suppress the renal clearance of IND.<sup>3)</sup>

With regard to the serum concentration related to the interactions between ASP and IND, these drugs bind to the same site of human serum albumin, 85 and >90%, respectively.<sup>4–6)</sup> This indicates that IND is displaced by ASP competitively in sites I and II, so that the concentration of unbound IND becomes higher in the serum than when IND is administered alone. If the unbound IND were subjected to glucuronidation by uridine 5'-diphosphate (UDP)-glucuronosyltransferases (UGTs) and/or hydroxylation by cytochrome P450s (CYPs), followed by excretion into the feces and urine, the pharmacological activities of IND would be diminished. Therefore, it is necessary to examine the metabolic interaction of both drugs.

ASP was biotransformed to salicylic acid (SA) in the serum, liver and kidney, and the salicylate (SA) was further metabolized to the conjugates with glucuronic acid and glycine, and to the hydroxylated metabolites, 2,3-dihydroxy-, 2,5-dihydroxy- and 2,3,5-trihydroxybenzoic acids.<sup>7,8)</sup> The 5-hydroxylation of SA was performed by human-CYPs 2E1 and 3A4,<sup>9)</sup> and rat-CYP2E1.<sup>9,10)</sup> The 3-hydroxylation was due to rat CYP3A and CYP2B<sup>11)</sup> but was not CYP dependent in human,<sup>9)</sup> whereas IND was metabolized to its O-desmethyl- (DMIND) and N-des-*p*-chlorobenzoyl-IND (5-methoxy-2-methyl-3-indoleacetic acid; MMIAA) and their glucuronides.<sup>12)</sup> The O-demethylation of IND was catalyzed mainly by CYP2C9, and minimally by CYPs 1A2 and 2D6 in human liver microsomes.<sup>13)</sup> DMIND and IND were glu-

curonidated in the liver microsomes<sup>14,15)</sup> and kidney.<sup>16,17)</sup> Although these metabolites were also found in rat bile, feces and urine,<sup>18)</sup> a high percentage of IND and small amounts of DMIND and MMIAA were found in the bile.<sup>18)</sup> Taking these observations together, this metabolic evidence is not competitive and does not support a drug-drug interaction in which concomitant ASP can be claimed to reduce the pharmacological activity of IND. Therefore, crucial to the interaction is whether ASP/SA induce(s) a higher permeability of IND into the organs and tissues and/or a higher metabolism of IND.

### MATERIALS AND METHODS

**Chemicals** ASP, IND, des-*p*-chlorobenzoyl-IND MMIAA, SA, and uridine 5'-diphosphoglucuronic acid (UDPGA) were purchased from Sigma-Aldrich Com./Japan. Carboxylesterase (from hog liver) was from Boehringer Mannheim GmbH. Benzbromarone, bovine serum albumin (BSA), 5,5-dithio-bis (2-nitrobenzoic acid) (DTNB) and ketoprofen were from Wako Pure Chemicals (Tokyo). Other reagents and other assay kits were also from Wako Pure Chemicals (Tokyo).

**Experimental Animals** Male Wistar rats (250–300 g) were obtained from Charles River Laboratories, Inc. (Japan). The animals were housed in cages under controlled conditions of temperature and humidity, and were subjected to a 12 h light/dark cycle. The rats were fed an Oriental laboratory diet and water *ad libitum*. After acclimatization for one week, the animals were divided into two groups for experiments on rat red blood cells (RBCs) and hepatocytes.

**Incubation of IND and RBCs with/without the Existence of ASP** Blood samples (about 5 ml) were collected from the jugular vein and subjected to biochemical examination. Erythrocytes (RBCs) from the control rats were isolated from the plasma by centrifugation at 1000×*g* for 10 min and washed three times with saline (15 ml), respectively. A suspension (20 µl) of the washed RBCs (9.0×10<sup>6</sup>/µl) was incubated with the respective ASP and IND (each at 0, 6, 12, and 24 fmol/RBC) in 143 mM phosphate buffer (PB) which in-

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cluded 5.5 mM glucose (2 ml, pH 7.4) or 5.8 mM phosphate buffer (PBS) which included saline and 5.5 mM glucose (2 ml, pH 7.4) at 37 °C for 2 h.

Osmotic pressures of the drug in PB or PBS were adjusted at  $300.5 \pm 2.5$  mOsm/kg using a Crytomatic Osmometer Model 3CII (Advanced Instruments).

PB was used for low pKa drugs because of its higher buffer action and PBS was used for the transport of drugs because human and rat sera contain chloride ion of 99 to 109 meq/l, corresponding to saline.

The incubation mixture was centrifuged at  $1000 \times g$  for 10 min to give a sediment and supernatant fractions. The supernatants were subjected to measurement of hemoglobin (Hb) concentration. The sediment was lysed with distilled water (500  $\mu$ l) and gave a lysed solution and ghost pellets. The lysed solution was used to measure levels of ATP, glutathione (GSH) and Hb.

**Incubation of ASP or SA with RBCs** A suspension (20  $\mu$ l) of the washed RBCs ( $9.0 \times 10^6/\mu$ l) was incubated with ASP or SA (each at 25 fmol/RBC) in PB (2 ml) for 10, 30, 60, 90, and 120 min. The incubation mixture was treated as described above.

**Effects of ASP on Concentration of Benzbromarone in RBC** A suspension ( $100 \mu\text{l} \times 5$ ) of the washed RBCs described above was incubated with ASP (10 fmol/RBC) and benzbromarone (10 fmol/RBC) in PB ( $4 \text{ ml} \times 5$ ) at 37 °C for 2 h. After treatment as above, the sediment was lysed with distilled water (500  $\mu$ l) and separated from the ghost to give a lysed solution, which was then freeze-dried and extracted with methanol ( $1 \text{ ml} \times 3$ ). The methanol extract was chromatographed on a silicagel plate (Kieselgel F254) using benzene:methanol (8:2). The developed band was extracted with ethyl acetate and levels of the extracted drug were measured from the respective calibration curves at 230 nm for ASP, and 273 nm for benzbromarone, respectively.

**Incubation of IND with Rat Plasma or Carboxylesterase** The above plasma (0.2 ml) or carboxylesterase (13 U) and ASP or IND (10, 20 and 50  $\mu$ l of 0.1 mg IND/ml) in PB (2 ml) were incubated at 37 °C for 60 min. The reaction mixture was centrifuged using centricut (W-10) at 4400 rpm for 40 min to give a filtrate. The compounds in the filtrate were identified with authentic specimen on TLC and HPLC. TLC conditions: plate, silica gel 60-F-254; developing solvents, benzene:dioxane:acetic acid (30:5:1 v/v) and HPLC described below.

**Hematological Examination of the RBCs** The number of RBC and mean corpuscular volume (MCV) were measured using a Sysmex M-2000 equipped with a SysmexDA-1000 (Sysmex Co., Ltd., Japan).

**Measurement of ATP, GSH, and Hb** ATP assay: A lysed solution of the RBCs was centrifuged at  $960 \times g$  for 15 min followed by recentrifugation at  $8000 \times g$  for 10 min at 4 °C to give a supernatant. ATP levels in the supernatant were assayed using a TOA ATP Analyzer AF 100 (TOA Electronics, Ltd.) equipped with a TOA Auto Dispenser DF 10 (by the luciferin-luciferase method).

GSH assay: Each of samples (0.2 ml) was added to a solution (0.3 ml) of 1.67 g of metaphosphoric acid, 0.2 g of EDTA- $\text{Na}_2$  and 30 g of NaCl in 100 ml of water. The mixture was allowed to stand for 5 min and centrifuged at  $9000 \times g$  for 10 min at 4 °C. The supernatant (0.2 ml) was diluted with

0.5 M sodium phosphate buffer (0.6 ml, pH 9.15). A solution of 0.1% *o*-phthalic aldehyde in methanol (0.1 ml) was added to the diluted solution and the fluorescence was determined after 1 min at 420 nm (emission) and 350 nm (excitation) using a Hitachi F-3010 Fluorescence Spectrophotometer, according to two known methods.<sup>19,20)</sup>

Hb assay: The levels in Hb were determined using an assay kit, Hemoglobin B Test Wako (by sodium lauryl sulfate method, Wako Pure Chemicals, Japan), respectively.

**Preparation of Rat Liver Microsomes** The animals were killed by decapitation, and individual liver microsomes were prepared by the usual method: The liver was homogenized with 0.25 M sucrose (9 vol.) to give a supernatant by centrifugation at  $960 \times g$  for 10 min and then  $9000 \times g$  for 20 min, and the supernatant was centrifuged at  $105000 \times g$  for 60 min to give a pellet. The microsomal pellet was suspended in 0.1 M potassium phosphate buffer (pH 7.4) and stored at -30 °C until use.

**Microsomal Protein Assay** Microsomal protein concentration was determined by the method of Bradford<sup>21)</sup> with a Bio-Rad Protein Assay Kit. Absorbance was measured at 595 nm with a Hitachi Model U-2001 spectrophotometer.

**Determination of Total CYP Contents** Total microsomal CYP content was determined from the sodium di-thionite-reduced carbon monoxide difference spectrum using a molar extinction coefficient of between 450 nm and 490 nm:<sup>22)</sup> The liver microsomal pellet suspension was adjusted to 1.0 mg/ml by dilution with a solution of 20% glycerol, 1 mM EDTA, 0.1 M potassium phosphate buffer (pH 7.25) and 0.2% Emulgen 913 (Kao Co. Ltd., Tokyo) and the diluted suspension was divided into a sample and reference. The sample was subjected to carbon monoxide gas for 1 min, and the sample and reference both were treated with di-thionite to give a different spectrum  $\text{OD}_{450-490}$ .

**Incubation of IND or MMIAA with/without Addition of ASP in the Microsomes** In the NADPH-generating system; the reaction mixture contained 0.1 M potassium phosphate buffer (pH 7.4), IND (0, 0.25, 0.50, and 1  $\mu$ M), an NADPH-generating system (0.33 mM  $\beta$ -NADP<sup>+</sup>, 8 mM G-6-P, 0.4 unit/ml G-6-P dehydrogenase, and 6 mM  $\text{MgCl}_2$ ), and a 250  $\mu$ g microsomal protein (0.46 nmol CYP/mg protein) in a final volume of 250  $\mu$ l. The mixture was incubated at 37 °C for various periods (0, 10, 30, 60, 90, and 120 min) and the reaction was stopped by addition of methanol. Metabolites were extracted with ethyl acetate for examination by HPLC.

In the UDPGA system; the reaction mixture contained 100  $\mu$ M IND, or 100  $\mu$ M MMIAA, 50 mM Tris/HCl (pH 7.4), 10 mM  $\text{MgCl}_2$ , 0.05% Brij, 10 mM D-succharic 1,4-lactone monohydrate, and 250  $\mu$ g microsomal protein in a final volume of 250  $\mu$ l. The mixture was allowed to stand for 2 h on ice and then preincubated for 10 min at 37 °C. Three mM of UDPGA was added and the mixture was incubated for various periods (0, 30, 60, 90, and 120 min), before being stopped by the addition of methanol.

**HPLC Analysis of ASP, ASP-Glucuronide, IND, IND-Glucuronide, MMIAA, and SA** The above incubation mixture was diluted 1000-fold with water. To the mixture was added an internal standard, 10  $\mu$ l of 2.5 mM ketoprofen, and then methanol (1.0 ml) was mixed in. After centrifugation at  $960 \times g$  for 10 min, the methanol extracted material was dried under  $\text{N}_2$  gas, dissolved in a solution (200  $\mu$ l) of

methanol: water (2:1) and centrifuged at  $960\times g$  for 10 min to give a supernatant. The supernatant (100  $\mu$ l) was subjected to HPLC analysis on a Shimadzu HPLC system Model CLASS-VP equipped with LC-10AT pump, SPD-10AVP detector, SIL-10AxL autosampler, CTO10A open column and DGU-4A degasser. The following HPLC conditions were employed: the mobile phase was a solution of methanol: acetonitrile: acetic acid: water (55:35:10:1). The HPLC system consisted of an analytical TSK-gel ODS-80Ts column (particle size 5  $\mu$ m, 4.6 mm $\times$ 15.0 mm I.D., Tosoh, Japan). The flow rate was 1 ml/min, and the column eluate was monitored with a UV detector at 260 nm.

ASP and SA were measured using a mobile solution of acetic acid: methanol: water (2:50:48, pH 2.7) and column, ODS-120T.

**Preparation and Incubation of Hepatocytes** Hepatocytes were isolated from adult male Wistar rats (body weight 250–300 g), according to the procedure described by Shi-maoka *et al.*<sup>23)</sup>. Briefly, the liver was first perfused *in situ* through the portal vein with 200 ml (30 ml/min) of Ca<sup>2+</sup>-free Hanks' solution containing 5 mM EGTA at 37°C for a few minutes, followed by perfusion with 400 ml (30 ml/min) of 0.05% collagenase solution. The perfused liver was excised and dispersed in cold Eagle's minimum essential medium (MEM) and the resulting cell suspension was filtered through a double layer of gauze. The liver cells were separated into two fractions, parenchymal hepatocytes and nonparenchymal cells (NPC), by differential centrifugation at  $50\times g$  for 1 min. The precipitated cells were washed three times with MEM to use as hepatocytes. Cell viability was determined by a trypan blue dye exclusion. The cells were plated at a density of  $1\times 10^5$  cell/0.2 ml/cm<sup>2</sup> in wells of Falcon culture dishes (60 $\times$ 15 mm) coated with collagen (Nitta Gelatin Co., Osaka) in 1 ml of WE medium supplemented with 5% calf serum. After incubation for 3 h for cell attachment, the medium and dead cells were removed. To the attached cells were added 1 ml of fresh medium supplemented with  $10^{-9}$  M insulin,  $10^{-9}$  M dexamethasone, and 30 mg/l kanamycin, and the mixture was incubated for 12 h to use in the experiment. After removal of insulin, dexamethasone and kanamycin, and washing with WE medium, the cells were incubated with 50  $\mu$ M or 100  $\mu$ M IND with/without addition of 50  $\mu$ M ASP or BSA in WE (total volume 4 ml) at 37°C under 95% O<sub>2</sub>–5% CO<sub>2</sub> for 3, 6, 12 and 24 h. The incubation was stopped by addition of methanol. Part of the metabolites was extracted with ether, ethyl acetate, or methanol, and the extracts were examined by HPLC.

**Measurement of Lactate Dehydrogenase (LDH) in the Hepatocytes** LDH assay was performed using a LDH assay kit (LDH-UV test Wako, Wako Pure Chemicals) in the hepatocytes after incubation with a maximum concentration of each drug, (50  $\mu$ M) and concomitant drugs (each 50  $\mu$ M) with/without the co-existence of BSA (50  $\mu$ M) for 3, 6, 12 and 24 h.

**Hydrolysis of IND-Glucuronide with Carboxylesterase, or  $\beta$ -Glucuronidase** Hydrolysis of IND-glucuronide was carried out by the usual method using carboxylesterase or  $\beta$ -glucuronidase: 200  $\mu$ l of a HPLC fraction containing IND glucuronide or MMIAA glucuronide was incubated with 100  $\mu$ l of  $\beta$ -glucuronidase (or carboxylesterase 1.3 mU) and 200  $\mu$ l of 0.2 M PB at 37°C for 2 h, according to the method

by Vree *et al.*<sup>24)</sup>

**Statistical Analyses** Data are expressed as the mean $\pm$  S.D. ( $n=5$ ). Data for control and treatment groups were compared using the unpaired Student's *t*-test (two-tailed) or the Dunnett-type mean rank test.<sup>25)</sup>

## RESULTS

**Effects of ASP/SA on RBC in PB** ASP/SA did not affect the levels of ATP, GSH and Hb at 25 fmol/RBC,  $98.9\pm 8.72$  ( $98.7\pm 7.36$ ) amol/RBC,  $0.34\pm 0.02$  ( $0.30\pm 0.04$ ) fmol/RBC, and  $21.1\pm 2.01$  ( $20.9\pm 1.85$ ) pg/RBC, respectively. Hydrolysis of ASP was examined by the ratio of SA to ASP after ASP was incubated with RBCs ( $9.0\times 10^6/\mu$ l) at 25 fmol/RBC dose in PB. The average ratios were 1.45, 2.35, 4.20, 4.30, and 4.37 after incubation for 10, 30, 60, 90, and 120 min. The permeability of SA into RBC showed a maximum of 0.006% at 60 min, corresponding to 1.67 amol/RBC at 25 fmol SA/RBC. Other metabolites included acylated derivatives of SA along with ASP.

**Effects of IND and MMIAA on RBC in PB and PBS** Table 1 shows the effects of IND and MMIAA on levels of ATP, GSH, and Hb. IND and MMIAA in PB did not change the levels of ATP, GSH, and Hb when BSA was not present (Table 1). IND in PBS decreased levels of ATP and Hb at 12.3 and 24.6 fmol/RBC (Table 1), at which MMIAA levels within RBC were about 4.1 and 11.3 amol/RBC, respectively. The number of RBC did not change on before and after incubation. Release of Hb was not observed in the medium although ATP and Hb were depleted within RBCs at 12.3 and 24.6 fmol/RBC in PBS. Levels of IND were  $0.18\pm 0.02$  amol/RBC,  $0.37\pm 0.04$  and  $0.65\pm 0.09$  amol/RBC at 6.15, 12.3 and 24.6 fmol/RBC in PB, respectively, and about 2, 2.3 and 2.3 fold in PBS (Fig. 1).

**Effects of ASP/SA on Permeability of IND into RBC** Levels of IND in the RBC were examined with/without the addition of ASP and/or BSA (Figs. 1, 2). When the same equivalent of ASP was concomitantly added to RBCs with IND, ASP/SA led to an increase in the levels of IND in the RBC in a dose-dependent manner compared with IND alone, showing about 9-fold at a 6 fmol dose without the existence of BSA, and about 2-fold at 12 and 24 fmol/RBC in both PB and PBS, respectively (Fig. 1). The dose dependent increase of IND was also observed when BSA was present, at 1/10 of the levels without BSA (Fig. 2), but the addition of ASP showed a higher constant increase in IND (about 10- to 3-fold at 6 to 24 fmol/RBC) compared with the respective IND level (Fig. 2). The ratios of PBS to PB for the intracorporeal levels in IND were similar, irrespective of the addition of ASP.

**Effects of ASP on Concentration of Benzbromarone in RBC** Benzbromarone and salicylate were excreted from RBCs by a multidrug resistance protein-1 (MRP-1).<sup>26)</sup> To determine whether ASP inhibited the excretion of benzbromarone from RBC, RBC was incubated with ASP and benzbromarone, respectively, and their levels following incubation were  $1.61\pm 0.08$  fmol/RBC and  $0.57\pm 0.09$  fmol/RBC within RBC, respectively. The level of benzbromarone under the concomitance of ASP was  $0.84\pm 0.04$  fmol/RBC, accompanied with  $1.10\pm 0.40$  fmol/RBC of ASP. ASP increased the level of benzbromarone to 150%, accompanied by both a de-

Table 1. Effects of IND and MMIAA on Rat Erythrocytes (RBCs)

Dose (fmol/RBC)	Medium BSA	ATP (amol/RBC)	GSH (fmol/RBC)	Hb (pg/RBC)
IND				
6.15	PB <sup>a)</sup>	100.3±10.0 (117.8±22.5)	0.39±0.07 (0.31±0.02)	21.1±1.1 (20.9±1.5)
	PB <sup>b)</sup>	102.6±13.7 (112.8±14.6)	0.34±0.15 (0.29±0.09)	21.2±0.9 (20.9±1.0)
	PBS <sup>a)</sup>	102.7±17.3 (102.2±13.3)	0.23±0.13 (0.30±0.04)	16.4±1.4 (20.7±0.6)
	PBS <sup>b)</sup>	98.9±13.2 (102.1±7.3)	0.27±0.02 (0.28±0.12)	20.8±1.8 (19.6±0.2)
12.3	PB <sup>a)</sup>	113.1±0.1 (111.8±6.1)	0.26±0.04 (0.30±0.03)	21.2±1.2 (21.7±1.8)
	PB <sup>b)</sup>	116.1±4.1 (113.6±4.4)	0.28±0.03 (0.28±0.08)	21.1±0.9 (19.7±0.8)
	PBS <sup>a)</sup>	85.1±5.32 (114.3±11.5)*	0.31±0.11 (0.27±0.05)	18.4±1.4 (21.0±0.4)*
	PBS <sup>b)</sup>	116.5±11.2 (114.8±5.7)	0.24±0.14 (0.38±0.07)	11.2±1.3 (19.1±2.0)*
24.6	PB <sup>a)</sup>	118.6±5.2 (117.1±4.6)	0.29±0.07 (0.311±0.09)	19.6±0.5 (19.3±1.0)
	PB <sup>b)</sup>	113.6±14.9 (113.3±13.2)	0.27±0.04 (0.27±0.03)	23.1±0.7 (22.9±0.5)
	PBS <sup>a)</sup>	86.9±1.18 (100.5±7.1)*	0.29±0.02 (0.31±0.06)	7.3±0.8 (20.5±0.3)***
	PBS <sup>b)</sup>	103.7±18.8 (117.2±19.3)	0.27±0.05 (0.27±0.03)	11.1±0.4 (21.5±1.2)***
MMIAA				
6.15	PB <sup>a)</sup>	98.2±4.7 (98.6±4.5)	0.26±0.04 (0.35±0.55)	20.4±2.1 (20.4±1.1)
	PB <sup>b)</sup>	95.8±1.2 (93.6±4.9)	0.27±0.07 (0.29±0.03)	20.4±1.1 (20.4±1.1)
	PBS <sup>a)</sup>	107.5±13.6 (114.5±8.4)	0.26±0.05 (0.32±0.05)	19.5±2.2 (18.0±2.1)
	PBS <sup>b)</sup>	108.8±10.6 (97.9±10.0)	0.30±0.12 (0.27±0.04)	19.7±2.5 (19.9±1.2)
12.3	PB <sup>a)</sup>	113.8±5.8 (119.7±3.7)	0.26±0.05 (0.32±0.04)	10.3±2.3 (19.4±2.1)
	PB <sup>b)</sup>	102.5±7.3 (112.9±5.6)	0.31±0.12 (0.31±0.06)	22.6±2.7 (23.8±1.7)
	PBS <sup>a)</sup>	118.6±3.6 (114.4±8.4)	0.28±0.13 (0.32±0.05)	21.9±0.4 (19.8±1.9)
	PBS <sup>b)</sup>	101.2±5.4 (99.9±11.9)	0.31±0.09 (0.31±0.01)	17.3±1.1 (19.6±1.9)
24.6	PB <sup>a)</sup>	122.6±12.8 (123.2±12.3)	0.28±0.03 (0.29±0.18)	19.9±1.1 (19.6±1.1)
	PB <sup>b)</sup>	124.1±12.9 (124.1±12.2)	0.33±0.09 (0.30±0.18)	20.1±2.7 (20.1±1.5)
	PBS <sup>a)</sup>	114.5±1.84 (114.5±1.84)	0.24±0.10 (0.33±0.08)	18.7±0.5 (18.0±1.0)
	PBS <sup>b)</sup>	89.4±24.0 (115.8±12.03)	0.18±0.16 (0.14±0.08)	21.5±0.3 (20.3±0.8)

RBCs were incubated with IND or MMIAA at 6.15, 12.3 and 24.6 fmol/RBC, respectively. The results are means±S.D. (controls) of 5 rats. a) No coexistence of bovine serum albumin (BSA); b) coexistence of BSA. \* $p<0.05$ , \*\* $p<0.005$  and \*\*\* $p<0.0005$  compared with the respective controls; Dunnett type mean rank test.<sup>25)</sup>

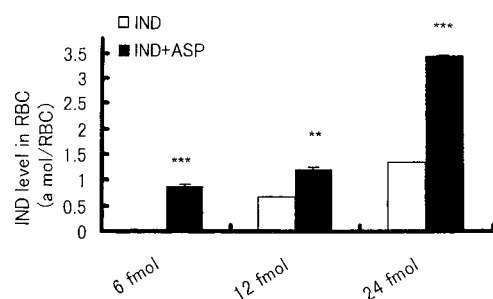


Fig. 1. Effect of ASP/SA on Levels of IND within rat RBC

Rat RBCs were incubated with IND at 6.15, 12.3, and 24.6 fmol/RBC in PBS at 37 °C for 2 h after addition of the respective equivalent of ASP (■) or under an ASP-free condition (□). A part of ASP was hydrolyzed to SA within 2 h under this condition. Concentrations of IND within RBCs were determined by HPLC and represented as amol/RBC. Results are mean±S.D. (RBCs from five rats were used). \*\* $p<0.01$  and \*\*\* $p<0.005$  compared with the respective controls without ASP/SA.

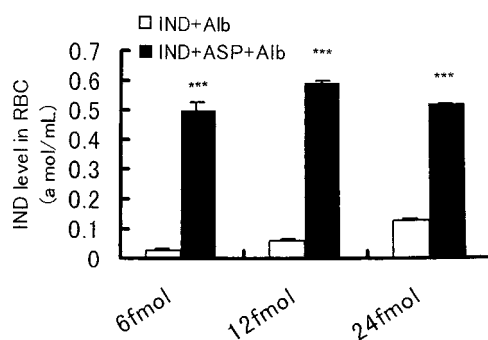


Fig. 2. Effects of ASP/SA on Levels of IND within Rat RBC in the Presence of BSA

Rat RBCs were incubated with IND at 6.15, 12.3, and 24.6 fmol/RBC in the presence of BSA (Alb) in PBS at 37 °C for 2 h after addition of the respective equivalent of ASP (■) or ASP-free BSA (Alb) (□). A part of ASP was hydrolyzed to SA within 2 h under this condition. Concentrations of IND within RBCs were determined by HPLC and represented as amol/RBC. Results are mean±S.D. (used RBCs from five rats). \*\*\* $p<0.005$  compared with the respective controls without ASP/SA.

crease of ASP to 68% and the existence of SA on TLC.

**Hydrolysis of IND in Rat RBC and the Plasma, and by Carboxylesterase** IND was hydrolyzed to MMIAA within the RBC and in the plasma, and by carboxylesterase, respectively. The hydrolysis of IND in RBCs was not affected by concomitance of ASP, although about 3.4 amol MMIAA/RBC was produced from IND within the RBC with no existence of BSA, respectively.

On incubation of MMIAA and RBCs in PB or PBS, irrespective of the existence of BSA, MMIAA was not observed in the cytoplasm, indicating that MMIAA did not penetrate into the RBC.

**Metabolism of IND by Rat Liver Microsomes and Identification of the Metabolites** Transformation of IND to DMIND by the microsomes was not observed in the presence

of an NADPH-generating system (the data not shown), but the existence of UDP-glucuronic acid induced a production of the two metabolites at a retention-time (RT) of 5.3 and 21 min on HPLC. Levels in the 21 min-metabolite showed linearly increased curves in an incubation-time dependent manner, but the 5.3 min-metabolite did not show this (Fig. 3). When the RTs of the metabolites were compared with those of IND at 50.6 min, and with MMIAA at 5.3 min on HPLC, respectively, the 5.3 min-metabolite was identified as MMIAA. The 21 min-metabolite was different from MMIAA-glucuronide (at 3.1 min) and was identified as an ester-type IND-glucuronide because its hydrolysis product by  $\beta$ -glucuronidase and carboxylesterase was identified to have

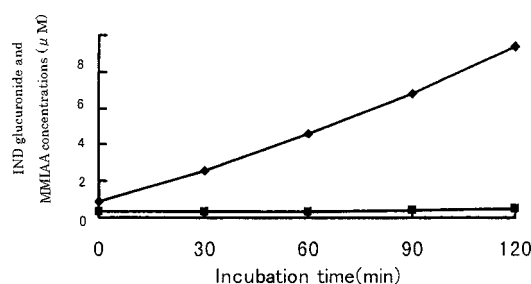


Fig. 3. Metabolism of IND by the Rat Liver Microsomes

The reaction mixture contained 100  $\mu\text{M}$  IND, 50 mM Tris/HCl (pH 7.4), 10 mM  $\text{MgCl}_2$ , 0.05% Brij, 10 mM D-succharic 1,4-lactone monohydrate, and 250  $\mu\text{g}$  microsomal protein. The mixture (250  $\mu\text{l}$ ) was allowed to stand for 2 h on ice and then preincubated for 10 min at 37  $^\circ\text{C}$ . 3 mM UDPGA was added and the mixture was incubated for various periods (0, 30, 60, 90, and 120 min). The levels of IND glucuronide (RT 21 min) and MMIAA (RT 5.3 min) were determined by HPLC and represented as their equivalent moles. IND glucuronide —◆—, MMIAA —■—.

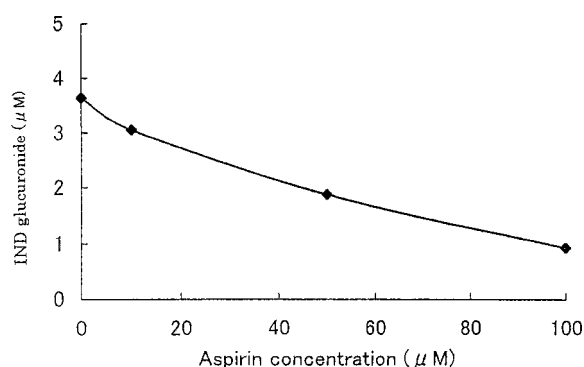


Fig. 4. Effect of ASP/SA on Transformation of IND to Its Glucuronide by Rat Liver Microsomes

The reaction mixture contained 100  $\mu\text{M}$  IND, ASP (0, 10, 50, and 100  $\mu\text{M}$ ), 50 mM Tris/HCl (pH 7.4), 10 mM  $\text{MgCl}_2$ , 0.05% Brij, 10 mM D-succharic 1,4-lactone monohydrate, and 250  $\mu\text{g}$  microsomal protein. The mixture (250  $\mu\text{l}$ ) was allowed to stand for 2 h on ice and then preincubated for 10 min at 37  $^\circ\text{C}$ . 3 mM UDPGA was added and the mixture was incubated for 90 min. A part of ASP was hydrolyzed to SA under this condition. The levels of IND glucuronide were determined by HPLC and represented as the equivalent moles.

a mixture of the authentic IND and MMIAA, and IND was partially hydrolyzed to MMIAA with a diluted activity (1/10) of  $\beta$ -glucuronidase for 3 min incubation.

**Effect of ASP/SA on Glucuronidation of IND in the Microsomes** Since the glucuronidation of IND showed increased curves in an incubation-time dependent manner (Fig. 3), the effects of ASP/SA on the glucuronidation were examined at the 90 min-incubation. The glucuronidation decreased to 84% ( $3.05 \pm 0.34 \mu\text{M}$ ), 51.4% ( $1.87 \pm 0.51 \mu\text{M}$ ,  $p < 0.001$ ), and 25.7% ( $0.94 \pm 0.34 \mu\text{M}$ ,  $p < 0.005$ ) at 1/10, 1/2, and 1 equivalent mol ratio between ASP and IND, respectively, compared with that of IND alone ( $3.64 \pm 0.54 \mu\text{M}$ ) (Fig. 4). This indicated that ASP/SA might inhibit the glucuronidation of IND.

**Effect of IND on Glucuronidation of Concomitant ASP in the Microsomes** The glucuronidation of ASP (0.1, 0.5 and 1 mM) by the coexistence of IND (1 mM) showed no significant rate of production of ASP-glucuronide:  $5.3 \pm 1.2\%$  at 0.1 mM ASP,  $6.4 \pm 3.9\%$  at 0.5 mM ASP and  $6.9 \pm 4.6\%$  at 1 mM ASP, respectively.

**Transformation of IND by Rat Hepatocytes and Effects on the Cells** Incubation of 50 and 100  $\mu\text{M}$  IND with the hepatocytes ( $5 \times 10^6$  cells) afforded decreases in IND levels and

did not show the existence of DMIND on HPLC when compared with the retention-time under the conditions reported by Nakajima *et al.*<sup>13)</sup> When the hepatocytes were incubated with 50  $\mu\text{M}$  IND for 3, 6, 12 and 24 h, the presence of two metabolites was observed at RTs 5.3 min and 21 min, and these were identified as MMIAA and IND-glucuronide, respectively. The IND levels showed a greater decrease at 3 h-incubation, and then an increase (Fig. 5a), being in inverse proportion between a decrease of IND and a production of IND-glucuronide (Figs. 5b, c), IND-glucuronide, in contract, showed a decrease from 3 to 6 h and constant production from 6 to 24 h without the addition of ASP, but the addition increased the levels of IND glucuronide from 3 to 24 h in an incubation time dependent manner, although the production of MMIAA showed no significant difference with or without ASP addition. Other metabolites than IND, IND-glucuronide, and MMIAA had retention-times ranging up to 3 min on HPLC, and their peaks were superimposed with solvent ones. Concentrations of the metabolites calculated by deduction of the solvent concentration were  $42.2 \pm 6.30 \mu\text{M}$  (corresponding to about 84% of the total IND, 50  $\mu\text{M}$ ) from 3 to 24 h without ASP, and  $72.4 \pm 5.69 \mu\text{M}$  (about 72% of the total drugs, 50  $\mu\text{M}$  ASP and 50  $\mu\text{M}$  IND). This suggested that the remainder, 16 and 28%, obtained by deduction of 84 and 72% from the total drug(s) concentrations might be lost from the medium into the hepatocytes, respectively.

When the hepatocytes were incubated with 100  $\mu\text{M}$  IND for 12 and 24 h, ASP/SA decreased the levels of IND to 80.7% ( $15.9 \pm 3.33 \mu\text{M}$ ,  $p < 0.05$ ) and ( $9.88 \pm 0.76 \mu\text{M}$ ) compared with the respective levels in IND ( $19.7 \pm 1.21 \mu\text{M}$  and  $11.3 \pm 0.31 \mu\text{M}$ ) without the addition of ASP. When the levels of IND glucuronide in the medium were measured as IND equivalence, the levels were  $3.78 \pm 0.88 \mu\text{M}$  (66.9%,  $p < 0.05$ ; versus  $5.78 \pm 0.53 \mu\text{M}$  without ASP) at 12 h and  $4.74 \pm 0.68 \mu\text{M}$  (vs.  $3.57 \pm 0.35 \mu\text{M}$  without ASP) at 24 h. The levels in MMIAA were  $2.73 \pm 0.59 \mu\text{M}$  (23.5%,  $p < 0.05$ ; vs.  $11.6 \pm 2.17 \mu\text{M}$  without ASP) and  $2.09 \pm 0.44 \mu\text{M}$  (34.7%,  $p < 0.01$ ; vs.  $6.03 \pm 0.62 \mu\text{M}$  without ASP) at 12 and 24 h, respectively. The levels in an unidentified metabolite at RT-6 min were  $3.32 \pm 0.64 \mu\text{M}$  (34.8%,  $p < 0.05$ ; vs.  $9.55 \pm 0.66 \mu\text{M}$  without ASP) and  $2.46 \pm 0.22 \mu\text{M}$  (63.2%,  $p < 0.05$ ; vs.  $3.89 \pm 0.18 \mu\text{M}$  without ASP), respectively. Other metabolites, including ASP-glucuronide, SA, and SA-glucuronide, were eluted more rapidly at RT than at 3 min on HPLC, and their peaks were superimposed with a solvent peak. The concentrations of the other metabolites were calculated as IND equivalent after deduction of the solvent levels. The other metabolites increased, but not significantly, from  $43.9 \pm 6.77 \mu\text{M}$  to  $49.8 \pm 3.19 \mu\text{M}$  (with ASP) at 12 h and from  $52.3 \pm 4.93 \mu\text{M}$  to  $62.7 \pm 5.33 \mu\text{M}$  (with ASP) at 24 h. The levels in ASP were  $10.6 \pm 0.59 \mu\text{M}$  without IND, and  $4.67 \pm 0.51 \mu\text{M}$  with IND at 24 h, respectively. The total levels of ASP, IND, IND-glucuronide, MMIAA, and other metabolites were  $81.6 \pm 10.4 \mu\text{M}$  (corresponding to about 82% of the total drug-concentration, 100  $\mu\text{M}$  IND) and  $77.1 \pm 8.32 \mu\text{M}$  (about 77%) at 12 h without the addition of ASP, and  $75.6 \pm 9.98 \mu\text{M}$  (about 50% of the total drug concentration, 150  $\mu\text{M}$  = 50  $\mu\text{M}$  ASP + 100  $\mu\text{M}$  IND) and  $81.9 \pm 8.79 \mu\text{M}$  (about 55%) at 24 h by addition of ASP. The results suggested that about 82% of the sum of IND and the metabolites was in the medium, indicating a loss of about 18%, perhaps into the hepatocytes, and

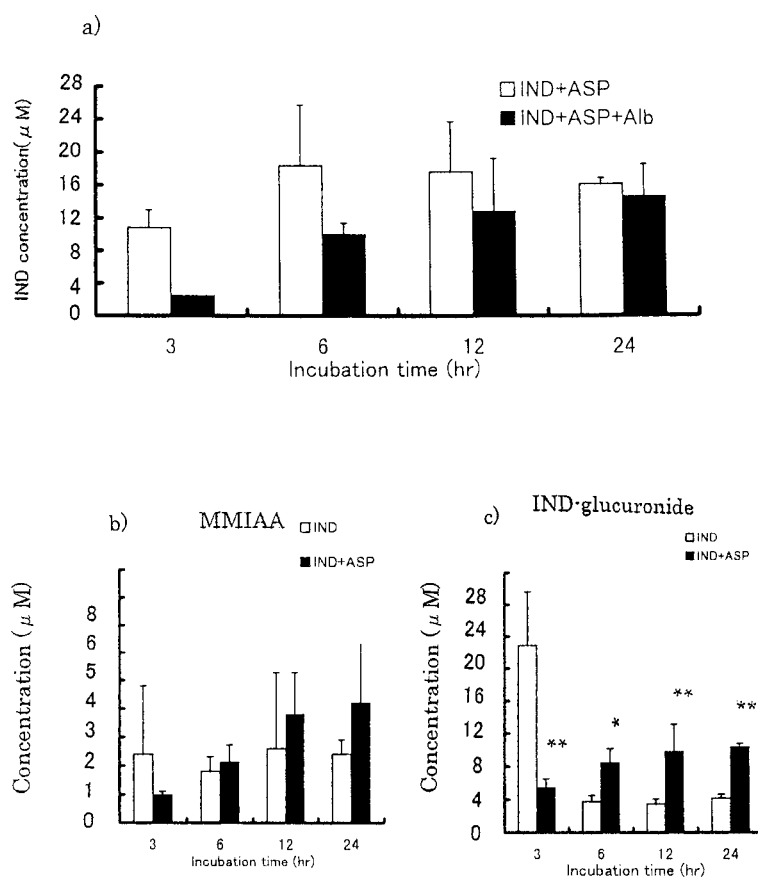


Fig. 5. Effects of ASP/SA on Metabolism of IND by the Rat Hepatocytes

The hepatocytes ( $1 \times 10^5$  cells) were incubated with  $50 \mu\text{M}$  IND in WE medium with the addition of  $50 \mu\text{M}$  ASP (■) or under ASP-free condition (□) under 95%  $\text{O}_2$ –5%  $\text{CO}_2$  for 3, 6, 12, and 24 h. A part of ASP was hydrolyzed to SA under this condition. Concentrations of a) IND, b) MMIAA, and c) IND glucuronide in the medium were determined by HPLC and represented as the respective equivalent mole. Results are mean  $\pm$  S.D. (the hepatocytes from five rats were used). \* $p=0.05$  and \*\* $p<0.01$  compared with the respective controls without ASP/SA.

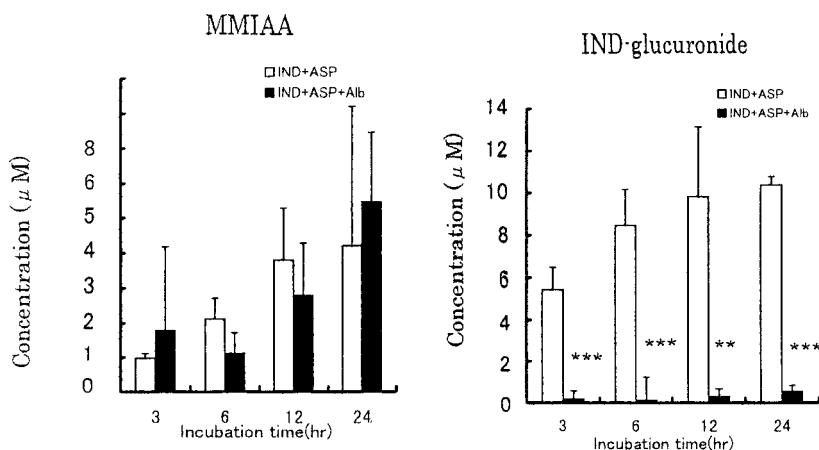


Fig. 6. Effects of BSA on Transformation of IND to MMIAA and IND Glucuronide by the Rat Hepatocytes

The hepatocytes ( $1 \times 10^5$  cells) were incubated with  $50 \mu\text{M}$  ASP and  $50 \mu\text{M}$  IND in WE medium in the presence of BSA (Alb) (■) or without BSA (Alb) (□) under 95%  $\text{O}_2$ –5%  $\text{CO}_2$  for 3, 6, 12, and 24 h. Concentrations of a) IND, b) MMIAA, and c) IND glucuronide in the medium were determined by HPLC and represented as the respective equivalent mole. Results are mean  $\pm$  S.D. (the hepatocytes from five rats were used). The levels of IND glucuronide and MMIAA were represented as their equivalent moles. \*\*\* $p<0.005$  compared with the respective controls without BSA(Alb).

ASP/SA increased the loss to about 50%, corresponding to  $(150-75.6)/150 \mu\text{M} \times 100$ .

Effects of BSA showed that the bound IND did not penetrate the hepatocytes (Fig. 6) and the unbound IND was metabolized to IND-glucuronide with significant difference, but MMIAA was not.

When the hepatocytes ( $5 \times 10^6$  cells) were incubated with 50 and  $100 \mu\text{M}$  IND for 3, 6, 12 and 24 h, LDH activities in the medium showed no change compared with the controls, irrespective of concomitant  $50 \mu\text{M}$  ASP and/or  $50 \mu\text{M}$  BSA.

## DISCUSSION

Concomitant administration of ASP is known<sup>1-3)</sup> to have induced a decrease in the plasma levels of IND for single or chronic IND treated patients, and that its clinically pharmacological effect was diminished. The pharmacokinetic interaction with ASP is generally not well understood, except for evidence that it decreases both the plasma concentration of IND and the gastrointestinal absorption, enhance the biliary clearance and enterohepatic circulation, and suppresses the renal clearance of IND.<sup>3)</sup> When ASP was administered orally, it underwent substantial hydrolysis to SA in the gut wall and the liver before entering the systemic circulation. Consequently, at the stage of circulation, since ASP/SA exhibited a competitive interaction for plasma protein binding, the unbound IND would become a higher serum concentration. The unbound IND would successively be subjected to metabolism, in part, to MMIAA in the plasma, RBCs and hepatocytes, and to IND-glucuronide in the hepatocytes, respectively.

Additional ASP apparently accelerated the permeability of IND into the RBCs, irrespective of coexistent BSA. The BSA-unbound IND seemed to penetrate into RBCs, due to higher levels of the IND released by ASP/SA from the BSA-bound IND, and then the penetrated IND was hydrolyzed in part to MMIAA, irrespective of the existence of BSA (Figs. 1, 2). Since MMIAA did not penetrate into the RBCs, it is tempting to suggest that the transportation of IND might be associated with depletions of ATP and Hb in the RBCs (Table 1). Namely, these results might lead to hypothetical mechanism(s) of whether the coexistence of ASP inhibited excretions of benzbromarone and IND from RBC and/or accelerated their higher absorption into RBC, because benzbromarone, IND, and SA were excreted from RBC by MRP-1.<sup>26,27)</sup> Considering that the levels of benzbromarone and IND within RBC were about 1.5 and 2–9 fold compared with the respective drug alone, the results from RBCs could expand to the hepatocytes. As IND was transported by MRPs,<sup>28)</sup> the IND penetrating into hepatocytes would remain in the cells as well as the RBC with the coexistence of ASP/SA. MRP-1 was localized to the lateral membrane domain<sup>28)</sup> and MRP-2 to the apical domains of hepatocytes.<sup>30,31)</sup> By whichever mechanism, absorption or excretion, the IND would be metabolized by CYPs and/or UGTs and the IND concentration would be diminished in the blood.

IND was metabolized to a major metabolite, IND-glucuronide, and minor metabolites, DMIND and MMIAA and their glucuronides, in human.<sup>24)</sup> The transformation to DMIND was not observed in the rat hepatocytes or liver microsomes, although the human microsomes gave DMIND.<sup>13)</sup> However, the metabolism of IND by the rat liver microsomes resulted in a constant level of MMIAA and an incubation-time-dependent increase of IND-glucuronide without the addition of ASP (Fig. 3). The effects of added ASP, therefore, were surveyed at 90 min-incubation for transformation to MMIAA and IND-glucuronide. The results showed that ASP/SA competitively inhibited the metabolism to IND-glucuronide by the rat microsomes in an ASP dose dependent manner (Fig. 4).

The metabolism of IND by the hepatocytes was determined from levels of both IND and its metabolites in the

medium, but not from their levels within the cells. The hydrolysis to MMIAA showed a tendency to increase in relation to incubation time, but there was no significant difference, whether or not ASP was added. The transformation to IND glucuronide reciprocally changed between the levels in IND and IND-glucuronide at various incubation-periods without the addition of ASP (Fig. 5), and was relatively higher (about 10-fold of the hydrolysis to MMIAA, Figs. 5b, c) at 3 h incubation, sequentially becoming lower and constant after 6 h incubation (Fig. 5c): 18% (4.2  $\mu$ M) at 6 h, when the production was compared at 3 h (23.8  $\mu$ M) without ASP/SA.

When ASP was equivalently added to IND, ASP itself was glucuronidated constantly irrespective of the incubation-time, but ASP induced changes in the ratio of IND glucuronidation with/without the addition of ASP at 3 h (24%), 6 h (230%), 12 h (330%), and 24 h (260%) (Fig. 5c). The results were believed to indicate that ASP/SA might inhibit the hepatic glucuronidation of IND at 3 h, being similar to the liver microsomes, and then might inhibit the hydrolysis of IND-glucuronide to IND from 6 h, accompanied by a decrease in ASP as if corresponding to an increase in other metabolites including SA on HPLC. In general, since the ester chemically existed with an equilibrium mixture of IND, acylglucuronide and glucuronic acid in the aqueous medium, the glucuronide might increase by inhibition of carboxylesterase activity, by which ASP was hydrolyzed to SA. This explanation could be expanded to cover the decrease in IND and increases in both IND glucuronide and MMIAA by ASP (Fig. 5).

As for the total concentration of the drug(s) (IND and/or ASP) and all metabolites (including IND-glucuronide, ASP-glucuronide, and MMIAA, SA and their glucuronides, and other metabolites), when the percent ratio of the non-additive ASP to the additional ASP at 50  $\mu$ M IND was compared to that at 100 IND, ASP/SA induced significant decreases in the sum of IND, ASP and all metabolites in the medium from 70% to 50%. This increased a loss of the drugs and all metabolites from 30% to 50%. Their loss from the medium could conceivably be interpreted as meaning that they remained in the hepatocytes, because it would be difficult for the drug(s) and all their metabolites to have been isolated intact from the hepatocytes. Therefore, the addition of ASP might enhance the remainder of drug(s) and/or their metabolites in the hepatocytes as well as the RBCs. These results, in the *in vivo* study, would appear to corroborate the report<sup>18)</sup> in which a high percentage of IND and small amounts of DMIND and MMIAA were found in the bile.

Moreover, the existence of BSA might suggest a possibility that the bound IND did not permeate into the hepatocytes (Fig. 6) but that ASP/SA displaced IND from BSA to the cells, in which the unbound IND was metabolized to IND-glucuronide, or that the IND-glucuronide might be bound to the albumin after excretion from the hepatocytes.

In conclusion, this study provides the hypothetical possibility that ASP/SA increased the concentration of IND in the hepatocytes and RBCs, and the IND was then, in part, metabolized to its glucuronide and/or MMIAA in the hepatocytes and/or RBCs, respectively; and further that ASP/SA inhibited both the acylglucuronidation of IND and hydrolysis of the IND glucuronide in the hepatocytes. However, there

remain questions of whether ASP/SA can competitively inhibit glucuronidation of IND by any family of UGT using human and rat microsomes, whether ASP/SA inhibits excretion of IND from the hepatocytes, or whether the unmetabolized IND is excreted to the bile from the liver.

Further work is in progress to find solutions to these questions.

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