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PHARMACOKINETIC ASPECTS OF SULFOBROMOPHTHALEIN TRANSPORT IN CHRONICALLY CARBON TETRACHLORIDE-INTOXICATED RATS

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Abstract—In order to elucidate the factors affecting the hepatic transport of sulfobromophthalein (BSP) in a pathological condition, the kinetics of the disappearance of BSP from the blood and appearance in the bile were studied in rats chronically intoxicated with carbon tetrachloride, and the importance of factors that might affect the kinetics, i.e. binding activites of plasma and hepatic cytoplasmic proteins, and glutathione S-transferase activity of Y-fraction, was examined. During chronic intoxication, although plasma protein albumin concentration decreased, no qualitative difference was shown in the high affinity binding site (K_1). A significant decrease was shown in the cytoplasmic protein concentration for the Y- and Z-fractions in intoxicated rats; no significant difference was observed in the number of binding sites for both fractions, but their binding constants decreased. Glutathione S-transferase activity and glutathione (GSH) content were not altered, although a decrease in conjugating activity per rat was expected. The time course for the plasma disappearance and biliary excretion of BSP showed a remarkable delay after CCl_4 intoxication, while no difference was shown in the bile flow rate. It was concluded that hepatic blood flow might play a primary role in the initial plasma disappearance of BSP and that the decrease in the Y-fraction binding activity might explain the decrease in BSP uptake rate into the liver which was observed in intoxicated rats.

The apparent plasma disappearance rate of sulfobromophthalein (BSP) is widely used to evaluate hepatic function as measured by the plasma retention of this dye after intravenous injection. The decrease in the rate of removal of BSP in liver disease may be caused by many factors; (1) hepatic blood flow, (2) binding to plasma proteins, (3) permeability across the sinusoidal plasma membrane, (4) interaction with cytoplasmic binding proteins (X, Y and Z) [1], other macromolecules and organelles, (5) metabolism, (6) permeability across the bile canalicular membrane, and (7) bile flow and transport in the bile duct.

In the present paper, the probable factors altering the hepatic clearance of BSP in rats with cirrhotic livers produced by chronic carbon tetrachloride (CCl_4) administration are elucidated. Hepatic intoxication by CCl_4 is well known and widely used as a pathological model of liver disease. The mechanism of intoxication by CCl_4 has been widely studied [2–6]. The repeated intoxication by CCl_4 is often used as a model of chronic liver disease, and after 8–10 weeks of intoxication (sixteen to twenty doses), the liver shows typical histological changes of chronic liver disease [7], namely cirrhosis [8, 9]. Furthermore, this method is superior to other intoxicants in both the reproducibility and the relatively short period for the intoxication (about 2 months).

Recently, Giorgi and Segre [10] reported on the effect of acute CCl_4 intoxication on the blood and bile kinetics of BSP in rats by compartmental analysis. However, little has been reported on the effect of chronic intoxication with CCl_4 on the pharmacokinetics of BSP.

The purpose of the present investigation was: (1) to examine the blood and bile kinetics of BSP in rats chronically intoxicated with CCl_4, and (2) to determine the importance of the following probable factors on the kinetics: binding of BSP to plasma and hepatic cytoplasmic proteins, glutathione *S*-transferase activity, and hepatic blood flow reported in the previous paper [11].

METHODS

Animals. Adult male Donryu (SD strain) rats (Nihon Rat Co., Ltd., Japan) weighing 120–140 g were used. CCl_4-intoxicated rats were produced by repeated injection of CCl_4 in olive oil (3:4, v/v), 0.1 ml/100 g of body weight, under the skin of the back twice a week for 8–9 weeks [12]. Control rats were produced by repeated injection of olive oil.

Blood and bile sampling. Femoral artery cannulation and bile fistula were carried out to study the excretion of the dye into the bile and the removal from the blood. After the dye was administered through a femoral vein, bile and blood samples were taken at given times. Heparin was injected at the dose of 0.1 ml/100 g of body weight (100 units) through the femoral artery cannulae. Ether anesthesia was used for the operation and the sampling period. The body temperature was 32 ± 1°.

Materials. BSP was purchased from Daiichi Pure Chemicals Co. Ltd., Japan. Rat albumin (Pentex, Fraction V) was purchased from Research Products Division of Miles Lab., inc. (Kankakee, IL, U.S.A.). Glutathione (reduced type, GSH) was commercially

prepared by Böeringer Manheim GmbH, West Germany. All other reagents were commercially available and of special grade.

Analytical Methods. For the blood samples, 0.1-ml samples of plasma were diluted and alkalinized with 3 ml of 0.05 N NaOH and measured photometrically at two wave lengths, 578 and 414 nm, respectively, in a Hitachi 124 spectrophotometer. The regression formula of Richards *et al.* [13] was used to correct for hemolysis, viz. 1.02 × (reading at 578 nm) − 0.29 × (reading at 414 nm). For the bile samples, after 0.1 ml was diluted and alkalinized with 4 ml of 0.05 N NaOH, the samples were centrifuged at 4° for 10 min, at 12,000 rev/min (about 12,000 *g*), in a Kubota KRP-65 centrifuge. The optical density of the supernatant was measured photometrically at 578 nm in a Hitachi 124 spectrophotometer.

Binding to plasma protein. In the column chromatography method, the procedure reported by Kamisaka *et al.* [14] was used after the following modification. To an aliquot of 0.5 ml plasma, BSP (0.3 μmole) was added. After 30 min at room temperature, the mixture was applied to a descending column system (1.75 × 60 cm) packed with Sephadex G-200 (Pharmacia Fine Chemical Co. Ltd., Sweden). Elution was performed with 0.01 M phosphate buffer, pH 7.3, at the flow rate of 12.5 ml/hr at 4°. Fractions (3 ml) were collected. Concentrations of protein and BSP in the effluent were measured photometrically in a Hitachi 124 spectrophotometer at 280 and 578 nm, respectively, after the samples were alkalinized with 0.05 ml of 5 N NaOH. In the equilibrium dialysis method, the plasma samples were previously dialyzed against 0.05 M Tris–HCl buffer, pH 7.4, for 24 hr at 4°. The dialysis cell (Kokugogomu, Co. Ltd., Japan), which has two chambers, was divided with Visking membrane (type: 18/32, Visking Co., U.S.A.). One ml plasma was put in one chamber, and 1 ml of 0.05 M Tris–HCl buffer containing BSP, pH 7.4, was put in the other chamber. The concentration of BSP in the buffer solution ranged from 30 to 200 μM. After the chambers were gently shaken for 96 hr at 4°, the concentration of unbound BSP in the buffer solution was measured photometrically at 578 nm in a Hitachi 356 spectrophotometer. The binding ratio was calculated by correcting the blank value resulting from the binding to the Visking membrane. The concentration of protein in the sample was determined by the method of Lowry *et al.* [15]. The binding parameters were calculated from Scatchard plots with a non-linear iterative least squares method using a Hitachi 8700/8800 digital computer.

Determination of plasma albumin. The cellulose acetate electrophoresis method was employed using a Separax electrophoresis apparatus (Joki Sangyo Co. Ltd., Japan). After 6 μl plasma was applied to the cellulose acetate membrane (Separax, 6 × 4 cm) soaked in 0.06 M Veronal buffer (pH 8.6, $\mu = 0.006$), the electrophoresis was carried out for 1 hr (1.0 mA/cm) in the same buffer. The cellulose acetate membrane was stained with 0.8 (w/v) Ponceu 3R in 6 (w/v) CH_3COOH, the colored slit of albumin was cut off, and then the stained dye was extracted into 3 ml of 0.25 N NaOH with vigorous shaking for 10 min. The optical density at 505 nm was measured photometrically in a Hitachi 124 spectrophotometer. The concentration of albumin was determined using a standard curve prepared after similar analysis of the known concentration of rat albumin (1–4 g/dl).

Preparation of supernatant fractions. Under light ether anesthesia, rats were killed by bleeding from a carotid artery. Thereafter, the liver was perfused with ice-cold 0.9% saline through the portal vein and rapidly removed, and a 25% and/or 50% homogenate was prepared in 0.25 M sucrose–0.05 M Tris–HCl buffer, pH 7.4, using a motor-driven, Teflon pestle, glass homogenizer (Takashima Shoten Co. Ltd., Japan). The homogenate was centrifuged at 105,000 *g* for 120 min in a Hitachi 65p ultracentrifuge (Hitachi Koki Co. Ltd., Japan) at 4°. The supernatant fraction was removed and either used immediately or stored at −20°.

Binding to cytoplasmic fraction. For the column chromatography method, 1 ml of the 105,000 *g* supernatant from a 25% liver homogenate was mixed with 1 μmole BSP and placed on a Sephadex G-75 column (2.0 × 65 cm). Elution was performed with 0.05 M Tris–HCl buffer (pH 7.4) using a pump-driven downward flow (12 ml/hr) at 4°. Protein concentration was estimated by absorbance at 280 nm, and protein-bound BSP by absorbance after alkalinization with 0.1 ml of 5 N NaOH at 578 nm in a Hitachi 124 spectrophotometer respectively. For the equilibrium dialysis method, 5 ml of the 105,000 *g* supernatant from a 50% liver homogenate was placed on a Sephadex G-75 column (5.4 × 85 cm). Elution was performed with 0.05 M Tris–HCl buffer (45 ml/hr) at 4°. X-, Y- and Z-fractions were collected following the nomenclature of Levi *et al.* [1], and the binding property to Y- and Z-fractions was studied. Equilibrium dialysis was performed in the same manner as the plasma protein binding study described above in the dose range from 0.05 to 20.0 μM.

Glutathione S-transferase activity. The procedure for the spectro-photometric assay of BSP-glutathione-conjugation enzyme activity proposed by Goldstein and Combes [16] was used to determine the specific enzyme activity of the Y-fraction. The reaction mixtures contained 0.5 μmole BSP in 0.05 ml of pyrophosphate buffer, and 47 μmoles glutathione in 0.15 ml of pyrophosphate buffer (pH 7.8), 1 ml Y-fraction in a final volume of 2.2 ml. The specific enzyme activity was measured by monitoring the increase in absorbancy at 330 nm for 5 min in a Hitachi 124 spectrophotometer with a thermostated sample cell holder (37°). A blank lacking enzyme was carried out for each assay to correct for the non-enzymatic formation of BSP–glutathione (BSP–GSH). Net enzymatic activity was calculated after subtracting the non-enzymatic formation from the value of the enzymatic formation. The protein concentration in the Y-fraction was determined by the method of Lowry *et al.* [15]. The specific enzyme activity was expressed as μmoles BSP–GSH/mg of protein/min.

Reduced glutathione (GSH) concentration in the liver. The fluorometric method of Cohn and Lyle [17] was used to determine the reduced (GSH) concentration in the liver.

Pharmacokinetic analysis. Pharmacokinetic parameters in the plasma and bile kinetics were calculated with a non-linear iterative least squares method [18] by use of a Hitachi 8700/8800 digital computer.

Table 1. Patho-physiological changes after chronic CCl_4 intoxication*

	Control rats (n = 11)	Intoxicated rats (n = 24)
Body wt (g)	341 ± 13	278 ± 6†
Liver wet wt (g)	13.1 ± 0.7	14.8 ± 0.5
Liver wet wt/body wt (g)	3.6 ± 0.01	5.0 ± 0.14†
Plasma transaminase activity (Karman's unit)		
GOT	87 ± 10	478 ± 120†
GPT	52 ± 9	220 ± 20†
Plasma albumin concn (g/dl)	3.51 ± 0.14‡	2.55 ± 0.13†,§
Bile flow rate (10^{-3} ml/min)	9.0 ± 1.1‖	8.5 ± 1.0¶
Hepatic blood flow** (ml/min/g liver)	0.97 ± 0.03††	0.64 ± 0.08†,††

* Results are given as the mean ± S. E. Chronic CCl_4-intoxication was produced by repeated injections of CCl_4 in olive oil (3:4, v/v), 0.1 ml/100 g of body weight, s.c. into the back twice a week for 8–9 weeks [12]. Controls were produced by repeated injections of olive oil by the method described above.

† Significantly different ($P < 0.05$) from the control.

‡ n = 8. § n = 15. ‖ n = 9. ¶ n = 11.

** Data from the previous paper [11].

†† n = 3.

Statistical analysis. All means are presented with their standard error (mean ± S. E.). Student's *t*-test was utilized to determine a significant difference between the control and the intoxicated groups.

RESULTS

Patho-physiological consequences of chronic CCl_4 intoxication. Parameters of patho-physiological changes by chronic CCl_4 intoxication are shown in Table 1. After 8–9 weeks (sixteen to eighteen doses of intoxication), body weight and plasma albumin concentration were significantly decreased. On the other hand, plasma transaminase activity (GOT, GPT) and the ratio of the liver wet weight to the body weight showed a significant increase. However, no significant difference was shown in bile flow rate. As reported in a previous paper [11], hepatic blood flow was decreased by the intoxication.

Pharmacokinetic aspects. Plasma disappearance curves of BSP for 30 min after intravenous administration are shown in Fig. 1. A typical delay of plasma disappearance was observed in the intoxicated rats.

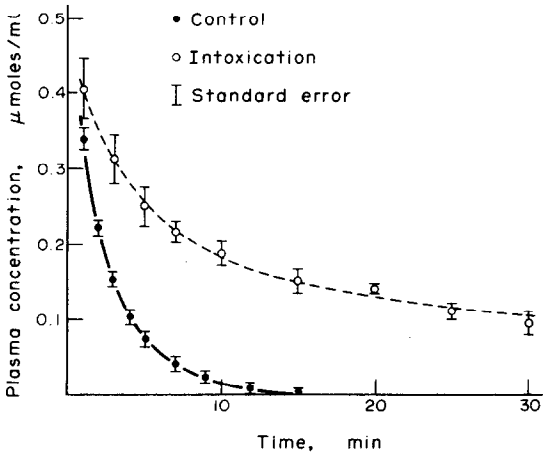
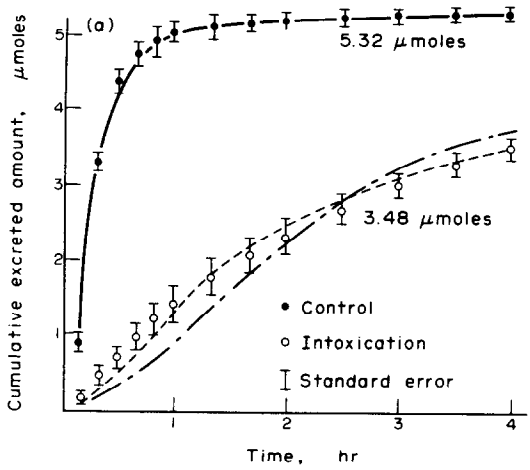


Fig. 1. Plasma disappearance curves of a 6-μmole dose of BSP after intravenous administration. Each point represents the mean ± S. E. of four rats. Curves were calculated by an iterative least squares method [18] using a digital computer. Key: (——) control; and (-----) intoxication.

The bile flow rate was not significantly altered by CCl_4 intoxication as shown in Table 1. BSP excreted into bile was identified by thin-layer chromatography on a Silica gel plate, the upper phase of a mixture of acetone–water–ammonia (81:15:4) being used as the developing solvent [19]. Authentic BSP and its metabolites were identified on the dried chromatograms by exposure to ammonia vapor. In the bile of both the control and the intoxicated rats, the main positive spots had the same R_f value (0.32) as the synthesized BSP–GSH [20]. On the other hand, the R_f value of authentic BSP was 0.76, and two slight pale tracer spots were observed at the same R_f value in both groups. These two unknown metabolites had R_f values of 0.46 and 0.62, respectively, and were considered to be metabolites B and C as reported by Combes [21]. From these results, the forms in which BSP is excreted into the bile of groups were mainly BSP–GSH and a small amount as free BSP and other metabolites. Cumulative biliary excretion curves are shown in Fig. 2a, and the amount excreted in 4 hr in control rats was 88 ± 1.7 per cent of the dose, while that of the intoxicated rats was 59 ± 2.5 per cent. Sigma minus plots of BSP excreted into the bile obtained from average excretion data points are shown in Fig. 2b. The control rats gave a typical high dose type [22] which consists of two rate constants, i.e. a fast rate constant, $k(I) = 7.20 \times 10^{-2}\ min^{-1}$ and a slow rate constant, $k(II) = 1.7 \times 10^{-2}\ min^{-1}$, while the intoxicated rats gave a low dose type [22] which has only a slow rate constant, $k(II) = 0.44 \times 10^{-2}\ min^{-1}$. A distinct change in the biliary excretion pattern was observed. In the intoxicated rats, the total recovery of dye in bile for 6 hr after administration was more than 75 per cent of the administered dose, and no significant increase in urinary excretion was observed.

The time course of the plasma disappearance and biliary excretion was found to be described by a four-compartment model (Fig. 3), since the plasma concentration time course was described by a two-compartment model and one more compartment was necess-



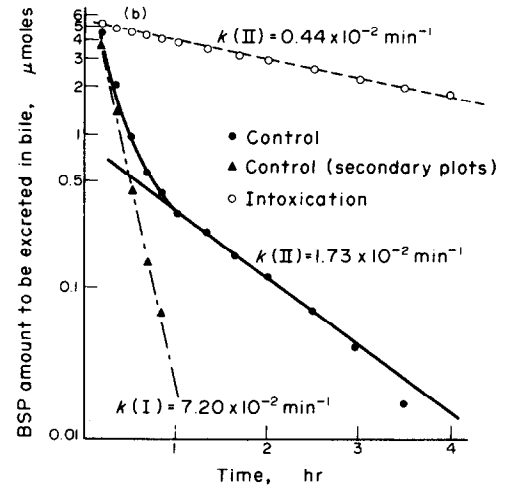


Fig. 2. (a) Cumulative biliary excretion curves of a 6-μmole dose of BSP after intravenous administration. Each point represents the mean ± S. E. of four rats. Curves were calculated by an iterative least squares method [18], using a digital computer. Key: (——) control, the ratio (R) of the total recovery of BSP from the bile to the administered dose was fixed at 0.90; (—·—) intoxication, $R = 0.75$; and (-----) intoxication, $R = 0.60$ (see text). (b) Sigma minus plots of BSP excreted in the bile after intravenous administration of a 6-μmole dose of BSP. Each point represents the mean value of four rats. Note that the curve of the control shows a typical high dose type [22] consistent with two constants, k(I) and k(II), while that of the intoxicated rats shows a low dose type consistent with only one rate constant, k(II).

ary to connect the plasma and bile data according to the precursor successor rule proposed by Beck and Recignio [23]. The pharmacokinetic parameters were computed by a non-linear iterative least squares method and are listed in Table 2. In the intoxicated rats, significant decreases were observed in k_{12}, k_{23}, k_{25} and k_{34}, while k_{21}, V_1 and V_2 were not altered.

Plasma protein binding. As shown in Fig. 4a, the chromatographic pattern of control plasma on Sephadex G-200 showed three peaks, namely I, II and III, according to the order of elution as proposed by Janecki and Krawcynski [24] and Kamisaka *et al.* [14]. Janecki and Krawcynski [24] reported that

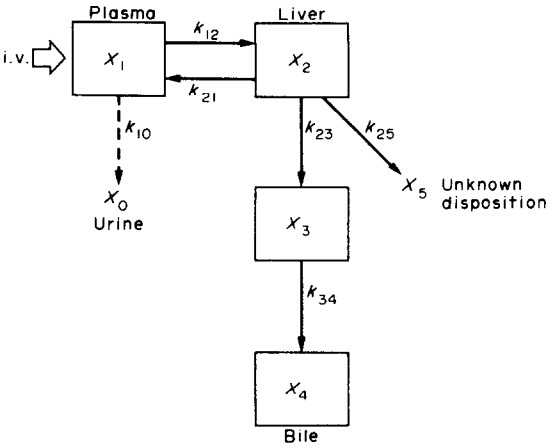


Fig. 3. Schematic illustration of a four-compartment open model. The data of BSP kinetics were fitted in the plasma compartment (X_1) and in the bile compartment (X_4).

peak I contained several high molecular proteins such as β-lipoprotein and α_2-macroglobulin, peak II contained mainly golbulin, and peak III contained low molecular proteins, mainly albumin. In the present investigation, these three peaks did not show a clear-cut separation, while BSP bound only to peak III, namely the albumin fraction. On the other hand, in the intoxicated rats (Fig. 4b), peak I increased relative to peak II and III, while BSP bound only to peak III as in the control animals.

Scatchard plots of BSP binding to plasma protein obtained from equilibrium dialysis are shown in Fig. 5. Two classes of affinity binding sites, namely one with high and the other with low affinity, are evident. The binding parameters calculated from these plots are listed in Table 3. Although the binding constant of the low affinity binding site (K_2) showed a decrease in the intoxicated rats, the binding constant of the

Table 2. Pharmacokinetic parameters calculated with a four-compartment model*

Pharmacokinetic parameters	Control rats (n = 4)	Intoxicated rats (n = 4)
k_{12} (min^{-1})	0.4612 ± 0.0219	0.1455 ± 0.0372†
k_{21} (min^{-1})	0.0635 + 0.0216	0.0862 ± 0.0220
k_{23}‡ (min^{-1})	0.3270 ± 0.0405	0.0257 ± 0.0041† (0.0206 ± 0.0020)†
k_{25}‡ (min^{-1})	0.0363 ± 0.0045	0.0086 ± 0.0021† (0.0137 ± 0.0006)†
k_{34}‡ (min^{-1})	0.0560 ± 0.0032	0.0104 ± 0.0008§ (0.0243 ± 0.0026)§
V_1 (ml)	11.35 ± 0.22	13.30 ± 1.50
V_2 (ml)	82.40 ± 28.30	22.45 ± 8.50

* Results are given as the mean ± S. E. Parameters were calculated by a non-linear iterative least squares method, using a digital computer.

† Significantly different ($P < 0.01$) from the control.

‡ Calculations were carried out under conditions such that the ratios (R) of the total recovery of BSP in the bile to the administered dose were fixed at 0.90 in the control and 0.75 in the intoxicated rats respectively. The values in parentheses were calculated from $R = 0.60$ (see text).

§ Significantly different ($P < 0.05$) from the control.

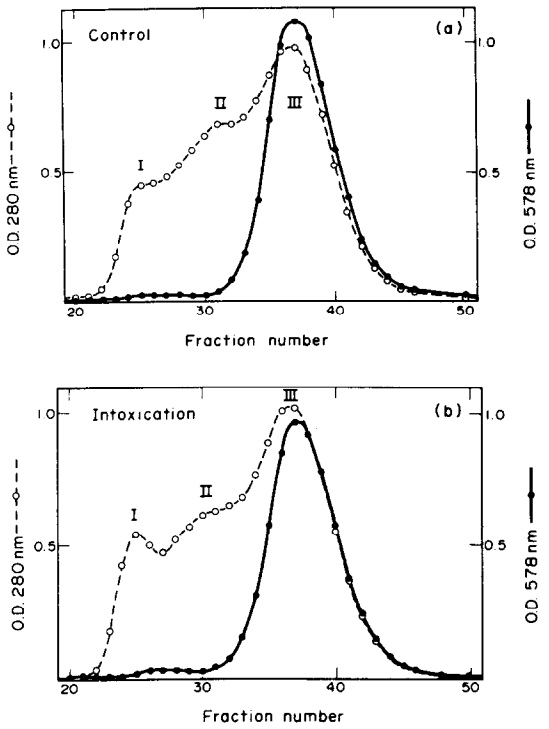


Fig. 4. Binding patterns of BSP with plasma protein from control rats (a) and intoxicated rats (b). To an aliquot of 0.5 ml plasma, 0.3 μmole BSP was added. After 30 min standing at room temperature, the mixture was applied to a descending column system (1.75 × 60 cm) packed with Sephadex G-200. Elution was performed with 0.01 M phosphate buffer, pH 7.3, at the flow rate of 12.5 ml/hr at 4°. Each fraction was 3 ml and fifty fractions were collected.

high affinity binding sites (K_1) and the number of high and low affinity binding sites (n_1 and n_2) were not decreased in the intoxicated rats. The downward

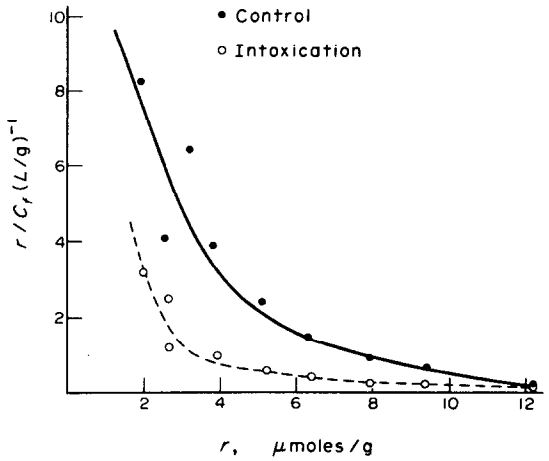


Fig. 5. Scatchard plot of BSP binding to plasma protein obtained by an equilibrium dialysis method. Plasma samples were previously dialyzed in 0.05 M Tris–HCl buffer, pH 7.4, for 24 hr at 4°. The concentration range of BSP in the buffer solution was from 30 to 200 μM. Equilibrium dialysis was performed for 96 hr at 4° (see text). Curves were calculated by a non-linear iterative least squares method, using a digital computer. Key: (——) control; and (-----) intoxication.

Table 3. Binding parameters of BSP to plasma protein by an equilibrium dialysis method*

Binding parameters	Control rats	Intoxicated rats
K_1 (M^{-1})	3.6×10^7	3.8×10^7
K_2 (M^{-1})	8.2×10^5	2.0×10^5
n_1 (μmoles/g)	3.6	2.6
n_2 (μmoles/g)	10.9	15.0

* Parameters were calculated by a non-linear iterative least squares method, using a digital computer.

parallel transition of the calculated curve in the intoxicated rats might be due to the decrease in the concentration of albumin which has the highest affinity for BSP.

Binding to cytoplasmic binding fractions. The elution pattern of a 1-ml supernatant from a 25% liver homogenate containing 1 μmole BSP after Sephadex G-75 column chromatography is shown in Fig. 6, panels

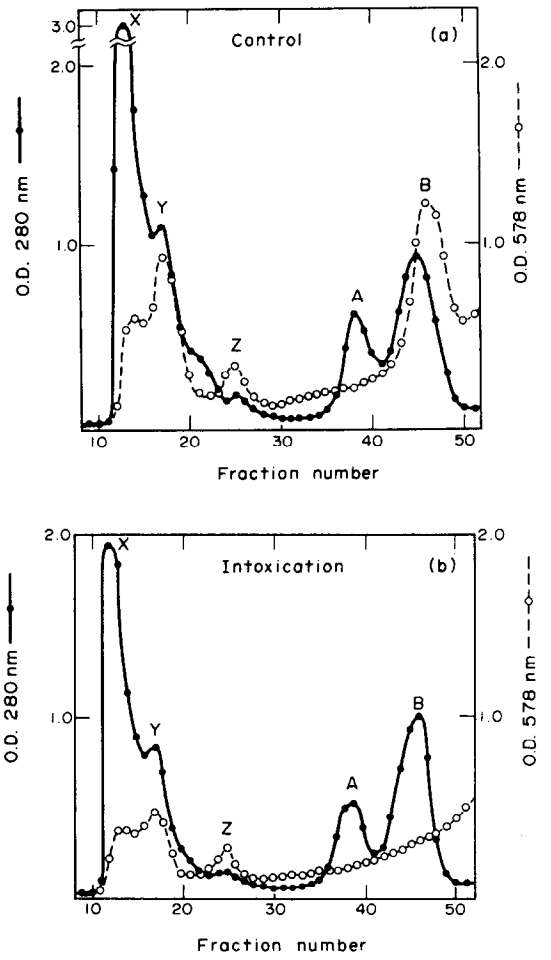


Fig. 6. Elution patterns (from a Sephadex G-75 column) of BSP added *in vitro* to the supernatant fraction of the liver of a control rat (a) and an intoxicated rat (b). One ml of the 100,000 *g* supernatant from a 25% liver homogenate was mixed with 1 μmole BSP and placed on a Sephadex G-75 column (2.0 × 65 cm). Elution was performed with 0.05 M Tris–HCl buffer (pH 7.4) by use of a pump-driven downward flow system (12 ml/hr) at 4°.

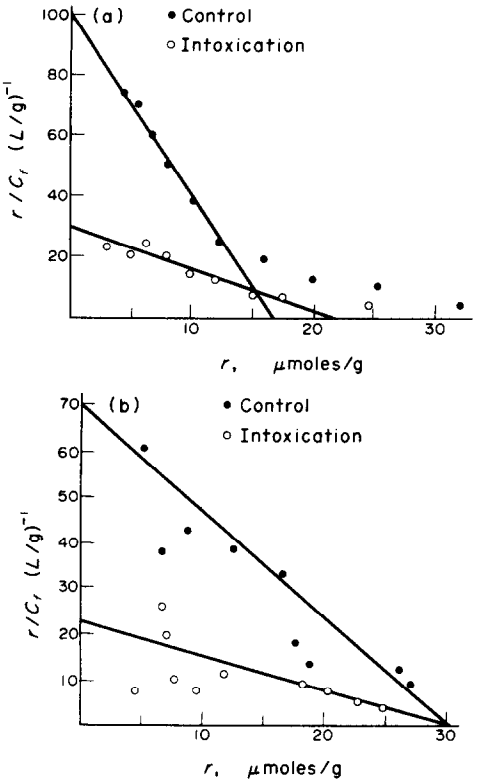


Fig. 7. Scatchard plots of BSP binding to a Y-fraction (a) and a Z-fraction (b) with an equilibrium dialysis method. The concentration range of BSP in 0.05 M Tris–HCl buffer (pH 7.4) solution was from 0.05 to 20 μM. Equilibrium dialysis was performed for 96 hr at 4°. Lines were drawn visually. Key: (——) control; and (-----) intoxication.

a and b. In both, the protein absorbance at 280 nm showed five peaks. Following the nomenclature of Levi *et al.* [1], the first, the second and third peaks have been labeled X-, Y- and Z fractions respectively. The fourth and the fifth peaks are smaller molecules such as nucleic acid residue, but have not been identified yet. In the control animals, most of the BSP was bound to the Y-fraction and less was bound to the X- and Z-fractions. A characteristic peak was seen at tube 46 named B-fraction, which appears identical with the fraction reported as BSP–GSH by Klaassen [25]. The free BSP peak which was eluted at tube 60 is not shown in the figures. In the intoxicated animals, the protein absorbance of the X-, Y- and Z-fractions at 280 nm tended to decrease, and a remarkable decrease of BSP binding to the Y-fraction was observed. Furthermore, the peak at tube 46 (B-fraction) disappeared. To confirm the decrease in binding of BSP to the Y-fraction, the binding activity was calculated by dividing the maximal absorbance at $O.D._{578\,nm}$ by that at $O.D._{280\,nm}$. The ratio was 0.83 ± 0.01 (n = 4) in the control and 0.53 ± 0.035 (n = 3) in the intoxicated rats.

The binding of BSP to Y- and Z-fractions was further studied by equilibrium dialysis. The results are shown in Fig. 7, panels a and b, as Scatchard plots. The binding parameters calculated from these plots are listed in Table 4. The binding constant of the high affinity binding site (K_1) showed a decrease in both Y- and Z-fractions, while the number of high affinity binding sites (n_1) showed little difference.

Glutathione S-transferase activity of the Y-fraction. It is well known that BSP is excreted into bile mainly as the glutathione conjugate [26–29]. It has been reported that glutathione conjugation is the rate-limiting step in the overall transfer of BSP from blood into bile [20, 30], and that the soluble fraction of the liver cytosol contains the enzyme which catalyzes BSP–glutathione conjugation, i.e. glutathione *S*-aryltransferase (EC 2.5.1.13) [31, 32]. Recently, Kaplowitz *et al.* [33, 34] reported that the Y-fraction was a heterogenous enzyme family catalyzing the conjugation of glutathione, namely glutathione *S*-aryl [35], *S*-epoxide [36], *S*-aralkyl [37] and *S*-alkyl [38]. Habig *et al.* [39–41] also reported that the Y-fraction contained five glutathione conjugation enzymes, namely glutathione *S*-transferase A, B, C, D and E, following their nomenclature in the chromatographic and electrophoretic studies, and furthermore that Ligandin (Y-protein) was identical to glutathione *S*-transferase B (EC 2.5.1.18).

No significant decrease was observed in the GSH concentration in the liver as shown in Table 5. This result is consistent with earlier reports, although in those studies acute CCl_4-intoxicated rats were examined [42, 43]. In order to elucidate whether a change of BSP–glutathione conjugation activity of the Y-fraction caused the conspicuous delay in the plasma and bile kinetics, the specific enzyme activity of the Y-fraction was determined. As shown in Table 5, no significant difference was observed.

DISCUSSION

As an experimental model of liver injury, CCl_4 hepatotoxicity has been widely used. Although many

Table 4. Binding parameters of BSP to Y- and Z-fractions by an equilibrium dialysis method*

	Y-fraction		Z-fraction	
Binding parameters	Controls rats (n = 4)	Intoxicated rats (n = 3)	Control rats (n = 4)	Intoxicated rats (n = 3)
K_1 (× 10^6 M^{-1})	7.8 ± 0.5	4.3 ± 1.8†	2.5 ± 0.8	0.4 ± 0.1†
n_1 (μmoles/g)	14.9 ± 0.3	14.8 ± 1.2	27.2 ± 1.2	25.0 ± 3.5

* Parameters were calculated by visual approximation. Results are given as the mean ± S.E.

† Significantly different ($P < 0.01$) from the control.

Table 5. BSP–glutathione conjugation enzyme activity of the Y-fraction and hepatic reduced glutathione (GSH) content

	Control rats	CCl_4-intoxicated rats
Specific enzyme activity *in vitro* of Y-fraction for BSP conjugation (μmoles/mg protein/min)*	0.098 ± 0.004	0.085 ± 0.009
Hepatic glutathione (GSH) content† (mg/g liver)	1.75 ± 0.26	1.79 ± 0.05

* Values are given as the mean ± S. E. of four rats. The procedure for the spectrophotometric assay for BSP–glutathione conjugation enzyme activity proposed by Goldstein and Combes [16] was used (see text).

† Values are given as the mean ± S. E. of three rats. The procedure for the fluorometric assay for glutathione proposed by Cohn and Lyle [17] was used.

investigators have reported on the effect of CCl_4 on the hepatic transport of BSP [10, 44–47], these have been restricted to acute hepatotoxicity, i.e. effect of a single dose of CCl_4. As Cameron and Karunaratne [7] reported, the chronic liver lesion produced by multiple doses of CCl_4 is distinctly different from the acute liver lesion produced by a single dose of CCl_4. Recently, Paquet and Kamphausen [48] reported that CCl_4, with a dosage regimen similar to that in the present study, produced an increase in the content of hydroxyproline, a criteria for fibrosis, and a marked increase in triglycerides. Tsurufuji and Ouchi [12] have also reported similar findings. In the present investigation, we examined the hepatic transport of BSP in rats with the chronic liver lesion produced by multiple doses of CCl_4 as a model for the liver cirrhosis and compared the pharmacokinetic results with biochemical and physiological data in control rats.

It is well known that the reduction of BSP clearance from the plasma is an important index for the diagnostic evaluation of liver function. In the present investigation, a conspicuous retention of BSP clearance from the plasma was observed in the intoxicated rats (see Fig. 1). In the blood, BSP is bound mainly to albumin [14]. Although in the intoxicated rats the concentration of plasma albumin was decreased (see Table 1), no significant difference was observed in the elution profiles or the binding parameters [see Figs. 4 (panels a and b) and 5 and Table 3]. In spite of the decrease in the plasma albumin concentration in the intoxicated rats, BSP was still bound mainly to plasma albumin. The decrease in albumin concentration probably did not play a primary role in decreasing the clearance of BSP, since V_1 did not show a significant difference between the control and intoxicated rats.

In the pharmacokinetic analysis, the plasma disappearance curves of BSP can be described by two exponential terms, namely a two-compartment model, in both the control and the intoxicated rats (see Fig. 1), and these observations are in agreement with reports in rats [10], rabbits [49], dogs [13] and men [50, 51]. The liver can be considered to belong to a distinctly different compartment from that of the plasma because the volume of compartment 1(V_1) equals the plasma volume (see Table 2), and BSP does not appear in the hepatocyte immediately after intravenous administration [52]. From these observations, it might be reasonable to represent the physiological meaning of the two compartments as shown in Fig. 3. Our proposed model is in agreement with that of Giorgi and Segre [10], which was proposed to describe the plasma disappearance of BSP in acute CCl_4-treated rats. It should be noted that they took into account the losses of BSP from the circulation not due to liver uptake as the outflow from compartment 1 (k_{10}), which is largely due to urinary excretion, while in our model (see Fig. 3) we taken into account the losses not due to biliary excretion and refer to it as the unknown disposition from compartment 2, which is represented as the transfer constant k_{25}, since the urinary excretion of BSP was negligible in both the intoxicated and normal rats [53], as well as in men with cirrhotic livers [51]. Giorgi and Segre [10] also reported that, in acute CCl_4-treated rats, the two transfer constants, k_{12} and k_{23}, showed a significant decrease with respect to those of the control rats. In the present investigation in chronically intoxicated rats, a similar tendency was also observed (see Table 2), although a significant decrease in k_{34} was observed. Giorgi and Segre [10] defined k_{12} as the rate constant of hepatic uptake process from the plasma into the liver cytoplasm and k_{21} as the efflux rate constant from the liver cytoplasm into the plasma respectively.

Levi *et al.* [1] suggested that two cytoplasmic organic anion binding proteins, Y-protein (Ligandin) and Z-protein, are important determinants in the transfer of many organic anions, especially BSP, from the plasma into the liver. In the present investigation, a significant decrease in the binding constants [see Fig. 7 (panels a and b) and Table 4] for Y- and Z-fractions for BSP as well as the protein concentration of the Y-fraction [see Fig. 6 (panels a and b)] was observed. A significant decrease in k_{12} was also observed. These findings support the conclusion that CCl_4 decreases the clearance of BSP by decreasing its uptake into the liver due to the lower amount and affinity of Y-fraction, including Ligandin, for BSP. The glutathione-conjugating activity (μmoles BSP conjugated/mg of Y-fraction) was not decreased nor was the concentration of GSH in the liver altered by chronic CCl_4 treatment (see Table 5). However, in the elution patterns on Sephadex G-75 [see Fig. 6 (panels a and b)], a remarkable decrease in the B peak, which corresponds to the BSP–GSH synthesized during elution, was observed in the intoxicated rats. This decrease in the BSP–GSH fraction might suggest that the ability of the Y-fraction to conjugate

BSP was decreased per rat. In order to elucidate whether this decrease in the conjugation ability is due to the quantitative decrease in the Y-fraction per rat or due to an increase in Michaelis constants (K_m), further detailed enzymatic kinetics for the Y-fraction would be necessary. It is difficult to determine whether the decrease in k_{23} is caused by the decrease of BSP-conjugating activity, or by the impairment of the intrahepatic transport process.

It is still uncertain whether one can assume that the transport rate of BSP from the blood pool into the liver sinusoid is too fast to be measured by compartmental analysis. If the transport rate could be measured, $k_{12}V_1$ would be equal to the hepatic plasma flow [54]. In the present investigation, $k_{12}V_1$ was 5.23 ml/min/rat in the control group, and 1.94 ml/min/rat in the intoxicated group. Although these values are somewhat lower than those reported in the previous papers [11], they are comparable. Thus, the hepatic transport process due to the plasma flow might not be too fast to be negligible for the plasma disappearance of BSP. Accordingly, on the basis of this point of view, k_{23} will correspond to the hepatic uptake process, and, furthermore, the decrease of k_{23} in the intoxicated rats will be coincident with the decreased binding activity of the Y-fraction [see Figs. 6 and 7 (panels a and b) Table 4], and k_{34} will correspond to the rate-limiting process in the intrahepatic transport or biliary excretion.

The defect of this model, however, is that the efflux from the liver cytoplasm into the plasma which was revealed by Scharschmidt *et al.* [52] is disregarded. Such a defect can also be seen in the Giorgi model, since it disregards the transport process from the blood pool to the liver. But when either the efflux or the transport is considered, the plasma concentration time course should be described by three exponential terms. In general, it might be impossible to define the pharmacokinetic parameters by the restricted experimental data points as obtained in the present investigation, and this might explain why the hepatic plasma flow ($k_{12}V_1$) calculated by an insufficient two-compartment model did not completely agree with the observed values in a previous paper [11].

From these findings, it might be impossible to elucidate what process is represented by the initial plasma disappearance rate (indicated usually K), which is widely used for the index of the clinical diagnostic test. Although the decrease of K has been interpreted as a decrease in the hepatic uptake rate, it might be necessary to take into account the decrease of hepatic blood flow as a primary factor.

The transfer constant k_{34}, which includes all processes after the disappearance of BSP from the plasma such as intrahepatic transport, metabolism and biliary excretion, was also decreased in the intoxicated rats. In the intoxicated rats (see Fig. 2a), the calculated curves for the biliary excretion data showed a discrepancy from the observed values. The best-fitting curve was obtained when the ratio of the total recovery of BSP from the bile to the administration dose (R) was fixed at 0.60. This ratio was not identical with the value obtained for the total recovery in the bile in our study (approximately 0.75). Giorgi and Segre [10] also obtained decreased ratios with simulated curves in the intoxicated rats (approximately 0.50). These findings suggest that the pharmacokinetic analysis of the biliary excretion behavior in the CCl_4-intoxicated rats with a compartment model based on the linear first-order kinetics might not be feasible. A more elaborate model might be necessary to explain the biliary excretion data in pathological conditions.

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