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HEPATIC TRANSPORT OF INDOCYANINE GREEN IN RATS CHRONICALLY INTOXICATED WITH CARBON TETRACHLORIDE*

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Abstract—Hepatic transport of indocyanine green (ICG) and probable factors altering the disposition of ICG were examined in rats chronically intoxicated with carbon tetrachloride. Delays were shown in both plasma disappearance and biliary excretion of ICG in intoxicated rats. No significant difference was shown in the total amount of ICG bound to the plasma proteins. In the intoxicated rats, significant decreases were observed in the pharmacokinetic parameters, k_{12}, k_{34} and V_2, calculated by a three-compartment model, while a significant increase was observed in k_{21}; V_1 was not altered. In both the control and intoxicated rats, the values of $k_{12} \cdot V_1$ were significantly smaller than the hepatic plasma flow, and it was suggested that the plasma flow does not play a primary role in the hepatic uptake of ICG. No significant difference was observed in the elution profiles of the 100,000 *g* supernatant fraction on a Sephadex G-75 column, and ICG bound mainly to the X-fraction in both the control and intoxicated rats. About 90 per cent of the ICG administered intravenously was distributed to the 9000 *g* precipitate fraction by 5 min in both groups of rats. It was concluded that a decrease in the permeability of the sinusoidal plasma membrane of the hepatocyte may explain the decrease of ICG uptake rate by the livers of the intoxicated rats.

Indocyanine green (ICG) is widely used to assess hepatic function as measured by the plasma retention of this dye after intravenous injection. Therefore, to elucidate the hepatic transport characteristics of ICG is not only of basic interest but also of practical importance. After intravenous administration, ICG is distributed in the plasma volume without extravascular distribution and is removed exclusively by the liver into the bile without biotransformation [1, 2]. With a low dose (0.5 mg/kg), ICG has a high hepatic extraction ratio in man (60–80 per cent) [2] and hepatic blood flow is the rate-determining factor in the hepatic uptake of this dye [3, 4], but in rats, a remarkably low hepatic extraction ratio was reported with a dose above 1 mg/kg [5]. The disposition in man of ICG in the presence of liver disease has been studied by many investigators [6, 7], but little has been reported with respect to basic information in experimental animals.

The purpose of this study is to elucidate the hepatic transport of ICG in rats chronically intoxicated with CCl_4, and to determine the probable factors altering its disposition.

MATERIALS AND METHODS

Animals. Adult male Donryu (SD strain) rats (Nihon Rat Co. Ltd., Tokyo) weighing 120–140 g were used. CCl_4-intoxication was produced by repeated injection of CCl_4 in olive oil (3 : 4, v/v), 0.1 ml/100 g body wt, under the skin of the back twice a week for 8–9 weeks. Control rats were given repeated injections of olive oil. The patho-physiological consequences of chronic CCl_4 intoxication were reported in a previous paper [8].

Blood and bile sampling. Femoral artery cannulation (PE50) and bile fistula production (PE10) were used to study excretion of the dye into bile and its 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 through the femoral artery cannula at a dose of 0.1 ml/100 g body wt (100 units). Ether anesthesia was used for the operation and during the sampling period. The body temperature was 32 ± 1°.

Materials. ICG was purchased from the Daiichi Pure Chemicals Co. Ltd., Tokyo. All other reagents were commercially available and of analytical grade.

Analytical methods. For the blood samples, 0.1 ml samples of plasma were diluted with 3 ml of deionized water and immediately measured at 800 nm in a Hitachi 124 spectrophotometer. There was no significant decrease in the absorbance of ICG within 30 min after dilution without the addition of 0.1% crystalline bovine serum albumin as a stabilizer. For the bile samples, after 0.1 ml was diluted with 4 ml of deionized water, the samples were centrifuged at 4° for 10 min, 12,000 r.p.m. (about 12,000*g*), in a Kubota KRP-65 centrifuge (Kubota Manufactory Co. Ltd., Tokyo). The supernatant fractions were

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diluted ten times with deionized water, and the optical densities were measured at 795 nm in a Hitachi 124 spectrophotometer.

Binding to plasma protein. The column chromatography method was used in the same manner as reported previously [8]. To an aliquot of 0.5 ml plasma, ICG (0.24 μmole) was added. After 30 min at room temperatue, the mixture was applied to a descending column system (1.75 × 60 cm) packed with Sephadex G-200 (Pharmacia Fine Chemical, Uppsala). Elution was performed with 0.01 M phosphate buffer, pH 7.3, at a flow rate of 12.5 ml/hr at 4°; (3-ml) fractions were collected. The concentrations of protein and ICG in the effluent were measured in a Hitachi 124 spectrophotometer at 280 and 800 nm respectively.

Binding to cytoplasmic fraction. The supernatant fractions were prepared in the manner described previously [8]. One milliliter of the 100,000 *g* supernatant fraction from a 50 per cent liver homogenate was mixed with 0.2 μmole of ICG and placed on a Sephadex G-75 column (2.0 × 75 cm). Elution was performed with 0.05 M Tris–HCl buffer (pH 7.4) using a pump-driven downward flow (11 ml/hr) at 4°. Protein concentration was estimated by absorbance at 280 nm, and protein bound ICG by absorbance at 790 nm in a Hitachi 124 spectrophotometer.

Distribution in liver fraction. The procedure for determining ICG in the liver, reported by Paumgartner *et al.* [9], was used. After 3 μmoles of ICG were injected through the femoral vein under light ether anesthesia, rats were killed by bleeding from a carotid artery; liver perfusion with ice-cold 0.9 per cent saline through the portal vein was started within 1 min. After a 10-min perfusion, the liver was removed and a 50 per cent homogenate was prepared by the same procedure described previously [8]. The homogenate was centrifuged at 9000 *g* for 20 min in a Kubota KRP-65 centrifuge at 4°. An aliquot of the 9000 *g* supernatant fraction was centrifuged at 100,000 *g* for 120 min in a Hitachi model 65P ultracentrifuge (Hitachi Koki Co. Ltd., Tokyo) at 4° and the 100,000 *g* supernatant fraction was prepared. Four milliliters of acetone were added to 1 ml of each of these two supernatant fractions (9000 *g* and 100,000 *g*) and, after vigorous shaking, the mixtures were centrifuged at 3500 r.p.m. for 15 min. The optical density of the acetone layer was measured at 790 nm in a Hitachi 124 spectrophotometer. Ten milliliters of acetone were added to 1 ml of a 50 per cent homogenate and the same procedure as described for the supernatant fraction was carried out. ICG was quantitated by a standard curve prepared with known amounts of the dye. The recovery of ICG added to a homogenate, the 9000 *g* supernatant fraction, and the 100,000 *g* supernatant fraction, respectively, ranged from 97 to 99 per cent.

Pharmacokinetic analysis. Parameters in plasma and bile kinetics were calculated with a non-linear iterative least squares method [10] by the use of a Hitachi 8700/8800 digital computer.

Statistical analysis. All means are presented with their standard errors (mean ± S.E.). Student's *t*-test was utilized to determine a significant difference between the control and the intoxicated groups, with $P = 0.05$ as the minimal level of significance.

RESULTS

Pharmacokinetic aspects. Plasma disappearance curves for 120 min after intravenous administration of 3 μmoles ICG are shown in Fig. 1. A typical delay of plasma disappearance was observed in the intoxicated rats. Biliary excretion profiles of ICG are shown in Fig. 2. The amount excreted in 4 hr in the control rats was 58 ± 3.8 per cent of the dose (N = 3), while that of the intoxicated rats was 44 ± 3.5 per cent (N = 5). The mean bile flow rate for 4 hr was not significantly altered by CCl_4 intoxication. The time course of 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 necessary to connect the plasma and bile data according to the precursor successor rule proposed by Beck and Rescignio [11]. The pharmacokinetic parameters were computed by a non-linear iterative least squares method and are listed in Table 1. In the intoxicated rats, significant decreases were observed in k_{12}, k_{34} and V_2, while a significant increase was observed in k_{21}; V_1 was not altered.

Plasma protein binding. As shown in Fig. 4, panels a and b, the chromatographic pattern of plasma on Sephadex G-200 showed three peaks, namely peak I, a high molecular protein fraction such as β-lipoprotein and α_2-macroglobulin, peak II, mainly a globulin fraction, and peak III, mainly an albumin fraction [12]. The three peaks did not show a clear-cut separation, while at this dose ICG was bound to both peaks I and III, namely a high molecular protein fraction and an albumin fraction respectively. The amount of ICG bound to peak III was about five times larger than that bound to peak I. On the other hand, in the intoxicated rats, peak I increased relative to peaks II and III, and the amount of ICG bound to peak I increased remarkably (Fig. 4b).

Binding to cytoplasmic binding fractions. The elution pattern of a 1-ml supernatant fraction from a

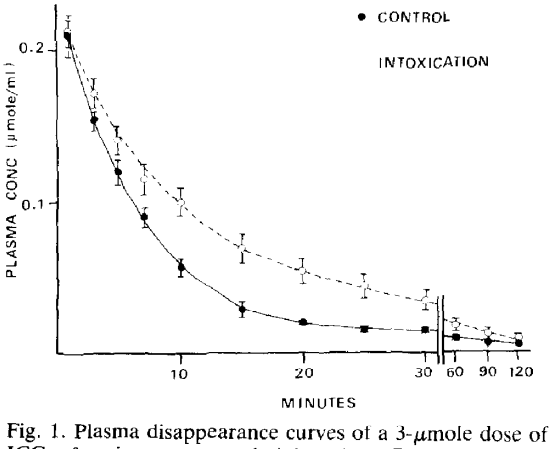

Fig. 1. Plasma disappearance curves of a 3-μmole dose of ICG after intravenous administration. Each point represents the mean ± S.E. of three to five rats. Curves were calculated by an iterative least squares method [10] using a digital computer. Key: (——) control; and (– – – – –) intoxication.

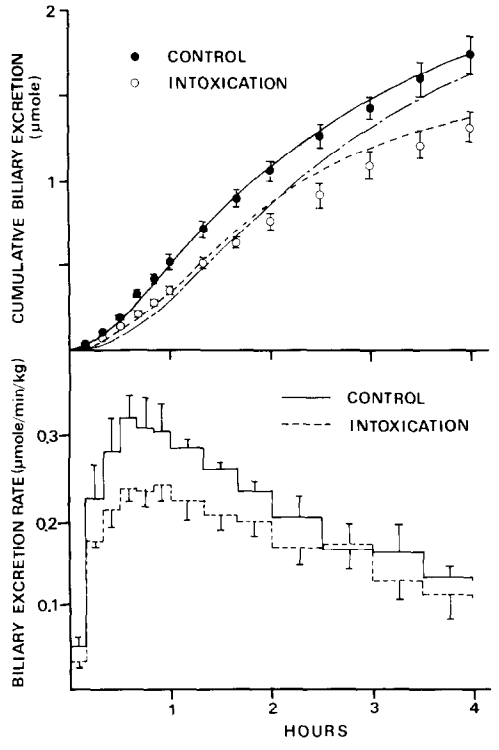

Fig. 2. Biliary excretion profile of ICG after a 3-μmole dose administered intravenously. Top panel: cumulative biliary excretion curves. Each point represents the mean ± S.E. of three to five rats. Curves were calculated by an iterative least squares method [10], using a digital computer. Key: (——) control [the ratio (R) of the total recovery of ICG from the bile to the administered dose was fixed at 0.70]; (– – – – –) intoxication, R = 0.50; and (— – —) intoxication, R = 0.70 (see text). Bottom panel: biliary excretion rate. Each data point represents the mean ± S.E. of three to five rats. Key: (——) control; (– – – – –) intoxication. No significant difference was shown between the control and the intoxicated rats.

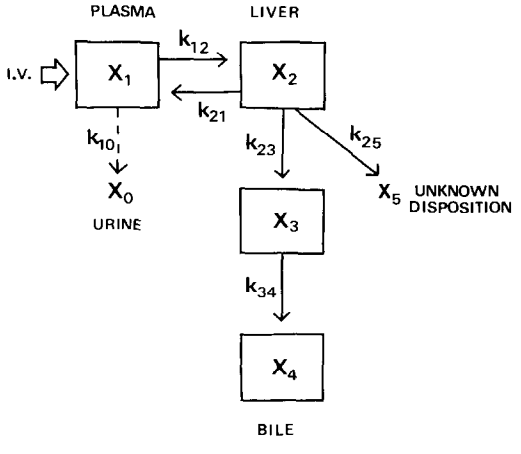

Fig. 3. Schematic illustration of a four-compartment open model. The data of ICG kinetics were fitted in the plasma compartment (X_1) and in the bile compartment (X_4).

Table 1. Pharmacokinetic parameters calculated with a four-compartment model*

Pharmacokinetic parameters	Control rats (N = 3)	Intoxicated rats (N = 5)
k_{12} (min^{-1})	0.1642 ± 0.0051	0.0987 ± 0.0096†
k_{21} (min^{-1})	0.0083 ± 0.0011	0.0194 ± 0.0058†
k_{23} (min^{-1})	0.0062 ± 0.0016	0.0115 ± 0.0028
k_{25} (min^{-1})	0.0027 ± 0.0007	0.0115 ± 0.0028
k_{34} (min^{-1})	0.0472 ± 0.0027	0.0170 ± 0.0010†
V_1 (ml)	11.9 ± 0.3	13.2 ± 0.5
V_2 (ml)	235.0 ± 31.0	67.2 ± 21.4†

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

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

50 per cent liver homogenate containing 0.6 μmole ICG after Sephadex G-75 column chromatography is shown in Fig. 5, panels a and b. Following the nomenclature of Levi *et al.* [13], three peaks have been labeled X-, Y- and Z-fractions, respectively. In this eluted condition, most of the ICG was bound to the X-fraction; the ICG, bound to the Y-fraction, was one-tenth of that bound to the X-fraction. No significant difference was shown in the elution patterns between the control and the intoxicated rats.

Distribution in liver homogenate fraction. The distribution of ICG in a 50 per cent liver homogenate 5 min after the intravenous administration of 3 μmoles ICG is shown in Fig. 6. In both the intoxicated and the control rats, only 10 per cent of the dose was distributed in the 100,000 *g* supernatant fraction, while about 75 per cent of the dose was distributed in the 9000 *g* precipitate fraction. These findings are contrary to a previous report by Levi *et al.* [13]. The time course of ICG distribution in the liver homogenate after the intravenous administration of 3 μmoles ICG revealed a maximum at 15 min, while the amount distributed in the 100,000 *g* supernatant fraction remained constant after 5 min until 60 min (Fig. 7).

DISCUSSION

In the present investigation, we have examined the hepatic transport of ICG in rats with a chronic liver lesion produced by multiple doses of CCl_4, as a model for liver cirrhosis [14, 15], and compared the pharmacokinetic results with biochemical and physiological data in control rats.

The reduction in ICG clearance from the plasma is a useful index for diagnostic evaluation of liver function. In this study, a delay in the plasma clearance was observed in the intoxicated rats (Fig. 1), but the retention was not as conspicuous as that of sulfobromophthalein (BSP) [8]. In blood, ICG is bound to both the high molecular protein fraction (peak I) and the albumin fraction (peak III) (see Fig. 4, panels a and b). Although the plasma albumin concentration in the intoxicated rats was significantly decreased, as reported previously [8], and this resulted in the decrease of the amount of ICG bound to the albumin fraction (peak III), this decrease was

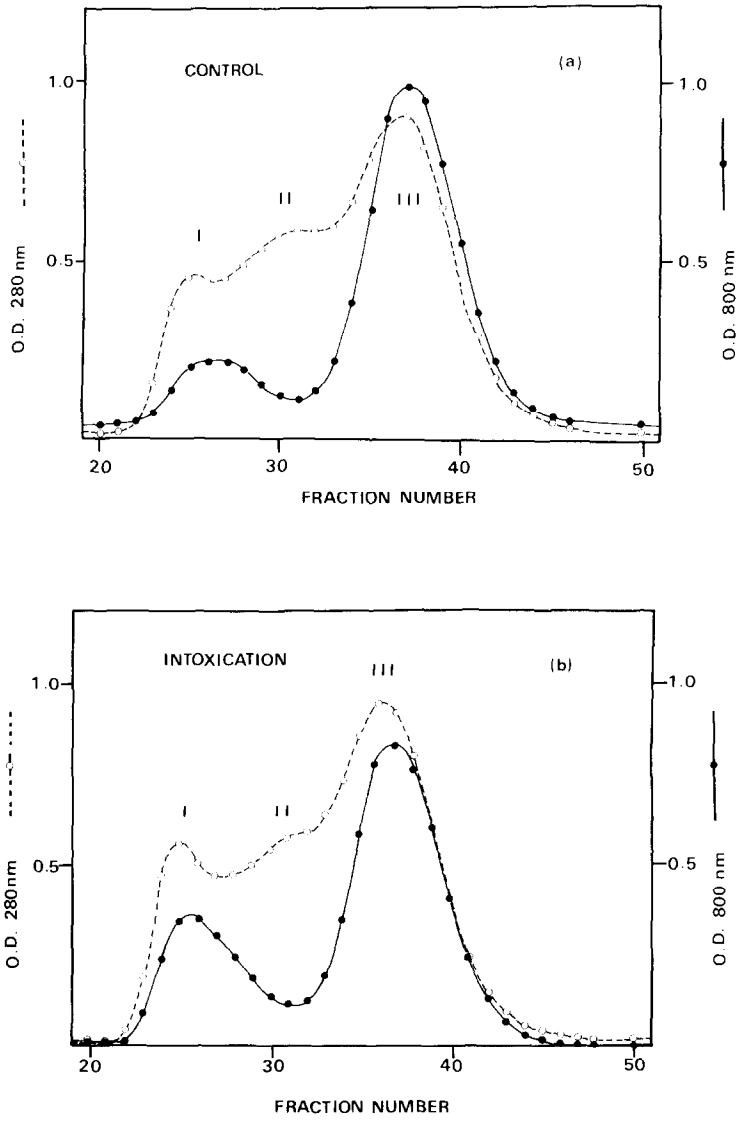

Fig. 4. Binding patterns of ICG with plasma protein from control rats (a) and intoxicated rats (b). To an aliquot of 0.5 ml plasma, 0.24 μmole ICG was added. After 30 min standing at room temperature, the mixture was applied to a descending column system (1.75 × 60 cm) packed with Sephadex G-200. Elution was performed with 0.01 M phosphate buffer, pH 7.3, at a flow rate of 12.5 ml/hr at 4°. Each fraction was 3 ml and fifty fractions were collected. Key: (——) optical density at 800 nm; and (– – – –) optical density at 280 nm.

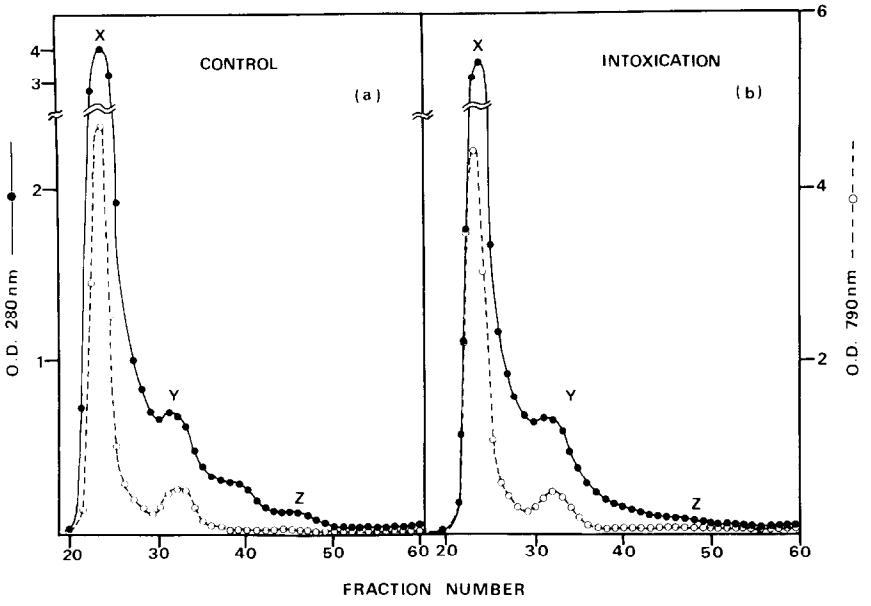

Fig. 5. Elution patterns (from a Sephadex G-75 column) of ICG added *in vitro* to the supernatant fraction of the liver of a control rat (a) and an intoxicated rat (b). One milliliter of the 100,000 *g* supernatant fraction from a 50 per cent liver homogenate was mixed with 0.2 μmole ICG and placed on a Sephadex G-75 column (2.0 × 75 cm). Elution was performed with 0.05 M Tris–HCl buffer (pH 7.4) by use of a pump-driven downward flow system (11 ml/hr) at 4°.

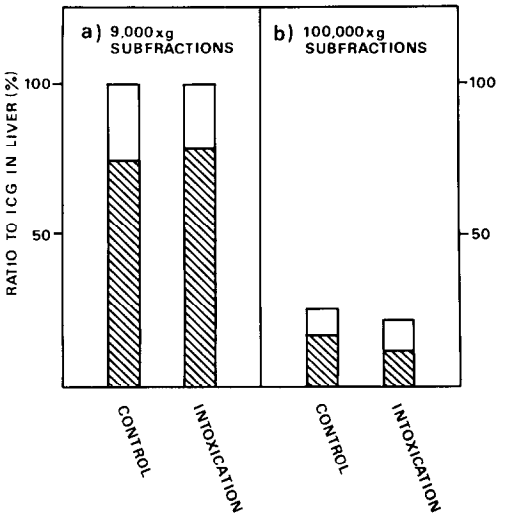

Fig. 6. Hepatic intracellular distribution of ICG. After a 3 μmole dose of ICG administered intravenously, rats were killed at 5 min by bleeding from a carotid artery. The liver was perfused with ice-cold 0.9 per cent saline through the portal vein for 10 min. A 50 per cent homogenate was prepared as described previously [8]. Panel a: 9000 *g* subfractions were obtained after centrifugation at 9000 *g* for 20 min at 4°. Panel b: an aliquot of 9000 *g* supernatant fraction was centrifuged at 100,000 *g* for 120 min at 4° and the 100,000 *g* subfractions were obtained. Open squares represent supernatant fractions after the centrifugations at 9000 *g* and 100,000 *g*, respectively, while hatched squares represent precipitate fractions after the centrifugations at 9000 *g* and 100,000 *g*, respectively. The procedure for determining ICG in the liver reported by Paumgartner *et al.* [9] was used to calculate the amount of ICG in each subfraction. The recovery of ICG added to a homogenate and each subfraction ranged from 97 to 99 per cent.

offset by the increase of the ICG bound to the high molecular protein fraction (peak I). Consequently, no significant difference was observed in the total amount of ICG bound to the plasma protein. It was difficult to determine the binding parameter of the plasma protein using an equilibrium dialysis method due to the impermeability of the dialysis membrane to ICG. On the other hand, the volume of compartment 1 (V_1) is equal to the plasma volume and did not show a significant difference between the control and the intoxicated rats (see Table 1). The binding of ICG to plasma protein, thus, may not play a primary role in decreasing the clearance of ICG.

A dose-dependent plasma disappearance of ICG has been reported by Klaassen and Plaa [16] and Paumgartner *et al.* [9] showed that the hepatic uptake of ICG followed Michaelis–Menten kinetics in rats.

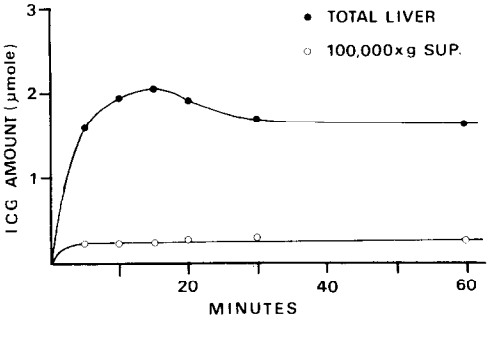

Fig. 7. Time course of the ICG distribution to the 100,000 *g* supernatant fraction after a 3 μmole dose of ICG in the control rats. Each point represents the mean of three rats. Key: (●) total amount of ICG in the liver; and (○) amount of ICG in the 100,000 *g* supernatant fraction.

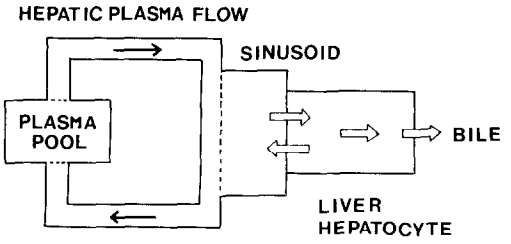

Fig. 8. A general model for the hepatic transport of ICG (see text).

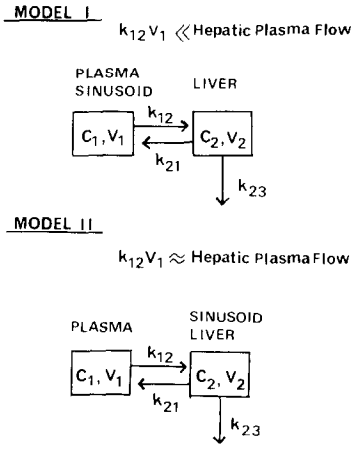

Fig. 9. Two conceivable compartment models for the hepatic transport of ICG. In model I, the sinusoid is included in the plasma compartment (compartment 1) where the transfer from compartment 1 to compartment 2 represents the translocation of the sinusoidal plasma membrane of the hepatocytes. In model II, the sinusoid belongs to the liver compartment (compartment 2) where the transfer from compartment 1 to compartment 2 corresponds to the hepatic plasma flow and there is an exceedingly rapid equilibration between the sinusoid and the hepatocyte.

However, these previous studies with IGG in laboratory animals were performed with relatively high doses. Recently, Iga and Klaassen [5] reported a linear relation between the AUC (area under the plasma concentration time curve) and the dose (ranging from 1 to 10 mg/kg) with the intravenous administration of ICG. The dose of ICG employed in the present study (3 μmoles/rat) corresponds to 8.4 mg/kg; this dose is in the linear range.

In pharmacokinetic analysis, the hepatic overall transport of ICG can be represented schematically using a compartment model, as shown in Fig. 8, involving four distinct processes: (1) transport from plasma pool to sinusoid, (2) influx and efflux across the sinusoidal plasma membrane of the hepatocyte, (3) intracellular transport, and (4) translocation across the bile canalicular membrane into the bile, since after intravenous administration ICG is distributed in the plasma volume without extravascular distribution and is removed exclusively by the liver into the bile without biotransformation [2, 3]. Based on this model, the plasma disappearance curves were best represented by 2-exponential terms in both the control and intoxicated rats, as shown in Fig. 1. Accordingly, two different compartment models (model I and model II), as shown in Fig. 9, were conceivable for the plasma disappearance of ICG. In model I, the sinusoid is included in the plasma compartment (compartment 1) where the transfer from compartment 1 to compartment 2 represents the translocation of the sinusoidal plasma membrane of the hepatocyte. On the other hand, in model II, the sinusoid belongs to the liver compartment (compartment 2) where the transfer from compartment 1 to compartment 2 corresponds to the hepatic plasma flow and there is an exceedingly rapid equilibration between the sinusoid and hepatocytes. Comparing the value of $k_{12} \cdot V_1$ with the hepatic plasma flow, we can determine the best model for the hepatic transport of ICG, since the product of $k_{12} \cdot V_1$ is equal to the hepatic blood (plasma) flow in model II [3]. In a previous paper [17] we reported a significant decrease of the hepatic blood (plasma) flow in CCl_4 chronically intoxicated rats, as shown in Table 2. A comparison of the values of $k_{12} \cdot V_1$ in the present study with the hepatic plasma flow from the previous paper [17] is also summarized in Table 2. In both the control and the intoxicated rats, the values of $k_{12} \cdot V_1$ are significantly smaller than the hepatic plasma flow. Thus, it appears that model I is the best representation of the hepatic transport of ICG, in which the permeability of the sinusoidal plasma membrane of the hepatocyte is the rate-determining step. Consequently, $k_{12} \cdot V_1$ will represent the translocation across the sinusoidal plasma membrane of the hepatocyte, and the decrease of $k_{12} \cdot V_1$ in the intoxicated rats is due mainly to the decrease of k_{12}, since V_1 did not show a significant difference between the control and the intoxicated rats (see Table 1). These findings suggest that the

Table 2. Comparison of pharmacokinetic parameters* with hepatic plasma flow†

	Control rats	Intoxicated rats
$k_{12} \cdot V_1$ (ml/min)	1.95 ± 0.002‡	1.30 ± 0.005§‖
Hepatic plasma flow (ml/min)	7.25 ± 0.22‡	5.06 ± 0.63‡‖

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

† Data from a previous paper [17].

‡ N = 3.

§ N = 5.

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

decrease of k_{12} is due mainly to the decrease in the permeability of the sinusoidal plasma membrane of the hepatocyte. The increase of k_{21} might also play a role in the reduction of ICG clearance from the plasma, but it is difficult to interpret its physiological meanings from the limited present results.

Levi *et al.* [13] suggested that two cytoplasmic organic anion binding proteins, Y-protein (Ligandin) and Z-protein, are important determinants in the transfer of many organic anions, such as BSP, ICG and bilirubin, from the plasma into the liver. In the present study, only 10 per cent of the ICG in the liver was distributed in the 100,000 *g* supernatant fraction at all times up to 60 min after the intravenous administration of ICG (see Figs. 6 and 7). In the elution patterns of the 100,000 *g* supernatant fraction on Sephadex G-75 (see Fig. 5, panels a and b), ICG bound mainly to the X-fraction, and no significant difference was observed in the elution profiles between the control and the intoxicated rats. Also, we obtained a remarkably large association constant for ICG (about $1 \times 10^7\ M^{-1}$) to the 9000 *g* precipitate fraction, using a centrifugal method, and no significant difference was shown between the control and the intoxicated rats (unpublished data). These findings indicate that ICG has a large binding affinity to the 9000 *g* precipitate fraction. This would explain why no significant difference was shown in k_{23} (see Table 1).

The transfer constant from liver to bile, k_{34} was also decreased in the intoxicated rats (see Table 1). In the intoxicated rats (see Fig. 2, top panel) 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 ICG from the bile to the administration dose (R) was fixed at 0.50. Using this ratio, we calculated the pharmacokinetic parameters, k_{23} and k_{25}, for biliary transport and unknown disposition, respectively [18]. This ratio was not identical with the value obtained for the total recovery in the bile in our study (approximately 0.70). These discrepancies were also reported for BSP [8, 18]. This suggests that a more elaborate model might be necessary to explain the biliary excretion data for ICG and BSP in pathological conditions.

In conclusion, the decrease in permeability of the sinusoidal plasma membrane of the hepatocyte might explain the decrease in ICG uptake rate into the liver which was observed in the intoxicated rats.

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