[Chem. Pharm. Bull. 33(11)4973—4980(1985)]

Pharmacokinetics in Hepatic Transport of Amaranth in Rats Intoxicated with Carbon Tetrachloride and α-Naphthylisothiocyanate

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(Received January 14, 1985)

The hepatic transport of amaranth (AM) was investigated in normal rats and rats with hepatic intoxication induced by subcutaneous or oral administration of carbon tetrachloride ($CCl_{4s.c.}$ or $CCl_{4p.o.}$) or by oral administration of α-naphthylisothiocyanate (ANIT). The kinetics of the disappearance of AM from plasma as well as the kinetics of its appearance in the bile were studied in rats under pentobarbital anesthesia and the binding activities of plasma and hepatic cytoplasmic proteins to AM were also investigated. A retarded plasma disappearance of AM was observed in CCl_4- or ANIT-intoxicated rats and biliary excretion of AM was decreased in CCl_4-intoxicated rats. Bile flow and biliary excretion of AM were not observed in ANIT-intoxicated rats. The total amount of AM bound to the plasma protein was decreased in ANIT-intoxicated rats, but was not affected in CCl_4-intoxicated groups. The pharmacokinetic parameters were calculated with a three-compartment model, and a decrease in k_{12} (transfer rate constant from plasma to liver) and k_{23} (transfer rate constant from liver to bile) were observed in $CCl_{4p.o.}$-intoxicated rats. In $CCl_{4s.c.}$-intoxicated rats, only k_{12} was decreased. In ANIT-intoxicated rats, significant decreases in k_{12} and increases in k_{24} (rate constant of metabolism) were observed. AM was bound preferentially to X- and Y-fraction rather than to Z-fraction in cytoplasmic proteins. Although the protein concentration in the 110000 *g* supernatant and in the Y-fraction was decreased in $CCl_{4p.o.}$-intoxicated rats, the total protein content of whole liver was not affected. It was considered that changes in the hepatic plasma flow may be a main cause of the retarded disappearance of AM from plasma in the intoxicated rats.

Keywords——amaranth; pharmacokinetic model; intoxicated rats; carbon tetrachloride; α-naphthylisothiocyanate; plasma protein; hepatic cytoplasmic protein

Introduction

Hepatic transport involves various processes, such as influx and efflux across the liver plasma membrane, intracellular transport, metabolism, storage, and secretion from the liver parenchymal cells into canaliculi. A disease state in the liver may affect these processes. Experimental liver necrosis has been studied and discussed from several viewpoint in terms of the biochemical events,[1,2] and phase I[3-12] and phase II[13-19] biotransformation of xenobiotics, but there are few reports on drug disposition or pharmacokinetic analysis in experimental liver necrosis.

Carbon tetrachloride (CCl_4) is a well-known hepatotoxin and produces centrilobular necrosis in many species.[20] Brauer and Pessotti[21] demonstrated that liver slices and isolated perfused liver obtained from CCl_4-intoxicated rats showed no differences in sulfobromophthalein (BSP) uptake as compared to untreated rats. Brauer *et al.*[22] reported that CCl_4 does not affect the ability of the liver to store BSP. However, they demonstrated that the hepatic extraction and biliary excretion of BSP were decreased. Plaa and Hine[23] reported that decreased extraction of BSP from the perfusate rather than decreased biliary excretion seemed

to be one of the factors affected by CCl_4 intoxication. Iga *et al.*[24] suggested that the binding ability of the Y-fraction (cytoplasmic binding protein) to BSP was decreased in chronically CCl_4-intoxicated rats. They[25] also reported that a decrease in the permeability of the sinusoidal plasma membrane of the hepatocyte to indocyanine green might explain the decrease in the uptake of indocyanine green by the livers of chronically CCl_4-intoxicated rats.

Biliary stasis in humans can be caused by tumors, drugs or various pathophysiological alterations. Inhibition of bile flow and biliary excretion may have profound consequences for the pharmacokinetics of drugs. Basseches and DiGregorio[26] demonstrated the altered disposition of procainamide in rats with ligated bile ducts. Drew and Priestly[10] demonstrated that microsomal metabolism of a number of drugs is significantly decreased in rats with ligated bile ducts. α-Naphthylisothiocyanate (ANIT) is a potent cholestatic agent,[27] and Gregus *et al.*[19] reported that the enzyme activities of hepatic phase I biotransformation were affected by ANIT, but those of phase II biotransformation were only minimally affected. Little is known of its effect on drug disposition and pharmacokinetics.

The purpose of this investigation was to study the kinetic behavior of hepatic transport of amaranth (AM), which was chosen as a model xenobiotic, in rats intoxicated with CCl_4 or ANIT and furthermore to study the factors affecting the kinetics. The various effects of these hepatotoxins on drug disposition are discussed.

Experimental

Materials——AM of reagent grade was purchased from a commercial source and was used without further purification. All other reagents were commercial products of analytical grade.

Animals——Adult male Wistar rats (250—300 g) were used in these studies. To induce hepatotoxicity in rats, carbon tetrachloride (CCl_4) was administered by two different routes. One was subcutaneous injection of an olive oil solution of CCl_4.[28] Carbon tetrachloride was dissolved in an equal volume of olive oil, and the mixture was subcutaneously administered at a dose of 4 ml/kg once a day for two successive days. The other was a single oral administration of the same dose of the mixture. ANIT was dissolved in olive oil at a concentration of 12.5% (w/v). The solution was administered orally at a dose of 0.8 ml/kg. No bile flow was observed 24 h after the administration of this dose. The animal study was performed 24 h after the last administration of CCl_4 or ANIT.

Administration of AM and Sampling of Blood and Bile——Rats were anesthetized with sodium pentobarbital (25 mg/kg, *i.p.*). The bile duct was cannulated with polyethylene tubing (PE 10, Clay Adams, U.S.A.). Collection of bile was started 10 min before the intravenous administration of AM. One-fifth milliliters of saline solution containing AM (12.5 μmol) was injected into the left femoral vein of bile-cannulated rats. Bile samples were collected every 5—10 min for 90 min into tared bottles. The volume of bile was considered to be the same as its weight by assuming its density to be unity. The average bile flow (μl/min/kg) before the administration of AM was 107.1 ± 11.2 ($n=5$) in untreated, 94.2 ± 6.6 ($n=6$) in $CCl_{4s.c.}$-intoxicated and 82.4 ± 7.2 ($n=5$) in $CCl_{4p.o.}$-intoxicated rats. The differences among the values were not significant ($p>0.05$). No bile flow was observed in ANIT-intoxicated rats.

One-fifth milliliters of blood was collected from the jugular vein using a heparinized syringe at appropriate time intervals.

Analytical Methods——Plasma was separated by centrifuging the blood sample for 5 min at 10000 rpm. One-tenth milliliters of plasma was appropriately diluted with saline and the concentration of AM was photometrically determined at 520 nm. The concentrations of AM in bile samples were determined in the same way as those in plasma samples after dilution with distilled water.

Plasma Protein Binding of AM——Column chromatography was used to identify the binding protein. A half ml of plasma spiked with 0.3 μmol of AM was loaded on a column (2.3 × 30 cm) packed with Sephadex G-200. Elution was performed at 4 °C with 0.01 M phosphate buffer (pH 7.3) at a flow rate of 12.5 ml/h. Fractions of 2 ml each were collected. The concentrations of protein and protein-bound AM in each fraction were determined photometrically at 280 and 520 nm, respectively.

The Sephadex gel equilibrium method reported by Hirose and Kano[29] was employed to determine the binding parameters. This method has been proved to give comparable results to an equilibrium dialysis method, but a small volume of sample can be used in this method whereas an equilibrium dialysis method needs a relatively large volume of sample. One hundred milligrams of Sephadex G-50 (coarse) was swollen in 0.8 ml of 0.1 M phosphate buffer (pH 7.4). The gel was completely swollen after standing for 4 h at room temperature. Five-hundredth milliliters of the buffer solution of AM (5—10 mM) and 0.35 ml of plasma were added to the gel solution, and the mixture was equilibrated by incubation for 1 h at 37 °C with continuous shaking. One-tenth milliliters of the solution outside the

gel was pipetted off. The concentration of AM in the outside solution was photometrically determined at 520 nm after suitable dilution. The concentration of protein in the solution was determined by the method of Lowry *et al.*[30] The amounts of free and bound AM outside the gel were calculated from the equation derived by Hirose and Kano.[29] The binding parameters were determined by means of Scatchard plots.

Preparation of Supernatant Fractions of Hepatic Homogenates——Rats were anesthetized with pentobarbital, then the liver was perfused with ice-cold saline through the portal vein for 5 min and rapidly removed. Fifty per cent homogenate was prepared in 0.25 M sucrose–0.01 M phosphate buffer (pH 7.4) using a glass homogenizer with a Teflon pestle. The homogenate was ultracentrifuged at 110000 *g* for 120 min at 4 °C. The supernatant fraction was used immediately or after storage at −20 °C.

Binding to Cytosol Fraction——Two mol of AM was dissolved in 2 ml of the 110000 *g* supernatant obtained from a 50% liver homogenate and placed on a Sephadex G-75 column (3.0 × 45 cm). Elution was performed with 0.01 M phosphate buffer (pH 7.4) with a pump-driven downward flow (12 ml/h) at 4 °C. Fractions of 4 ml each were collected. Concentrations of protein and protein-bound AM in each fraction were photometrically determined at 280 nm and at 520 nm, respectively.

Pharmacokinetic Analysis——Pharmacokinetic parameters based on a three-compartment model were calculated by a nonlinear least-squares method using a personal computer. A nonlinear least-squares program (MULTI) reported by Yamaoka *et al.*[31] was used after minor modifications.

Statistical Analysis——Data were represented as the mean with standard error (mean ± S.E.). Statistical evaluation of the data was carried out using Student's *t*-test.

Results and Discussion

Biochemical data in CCl_4- or ANIT-intoxicated rats are shown in Table I. In $CCl_{4s.c.}$-intoxicated rats, there was no significant difference in the biochemical data from those in the untreated rats. On the other hand, in $CCl_{4p.o.}$- or ANIT-intoxicated rats, the activities of transaminases (glutamate oxaloacetate transaminase (GOT), glutamate pyruvate transaminase (GPT)) and lactate dehydrogenase (LDH) in plasma were significantly increased. These results suggest that oral administration of CCl_4 causes more serious hepatotoxicity than subcutaneous administration of an equal dose of CCl_4.

The disappearance of AM from plasma in both untreated and intoxicated rats followed biexponential kinetics (see Fig. 1). A retarded plasma disappearance was observed in the

Table I. Biochemical Parameters in Plasma of CCl_4- or ANIT-Treated Rats

	Untreated	ANIT	$CCl_{4s.c.}$	$CCl_{4p.o.}$
GOT[a]	108.4 ± 9.7	373.3 ± 32.8[c]	137.4 ± 23.0	2222.0 ± 364.7[c]
GPT[a]	26.3 ± 3.5	139.0 ± 26.0[c]	28.4 ± 3.8	671.7 ± 136.1[c]
LDH[b]	2820 ± 697	5112 ± 1910[c]	2814 ± 769	23538 ± 8154[c]

Each value represents the mean ± S.E. of 7—10 rats. *a*) Karemen's unit. *b*) WrÓblewski/ml. *c*) Significantly different from untreated, $p < 0.05$.

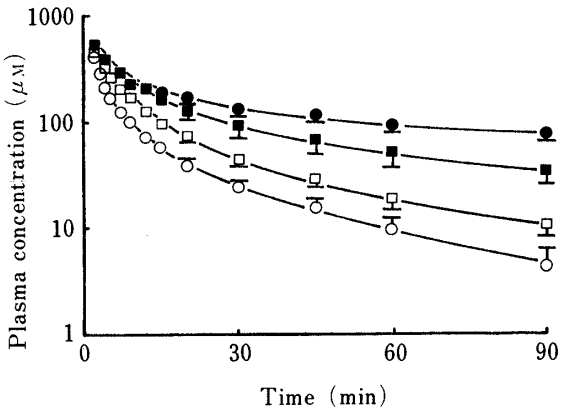

Fig. 1. Disappearance of AM from Plasma after Intravenous Administration to Bile-Cannulated Rats at a Dose of 12.5 μmol/Rat

Each point represents the mean ± S.E. of three rats. Curves were calculated by the non-linear least-squares method on a personal computer.
—○—, untreated; —□—, $CCl_{4s.c.}$; —■—, $CCl_{4p.o.}$; —●—, ANIT.

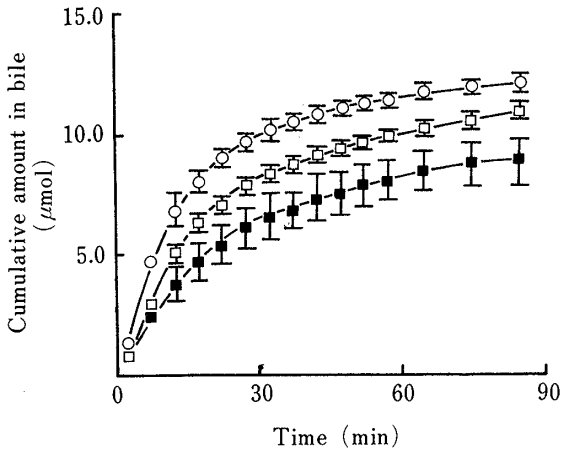

Fig. 2. Biliary Excretion Profiles of AM after Intravenous Administration at a Dose of 12.5 μmol/Rat

Each point represents the mean ± S.E. of three rats. Curves were calculated by the non-linear least-squares method on a personal computer.
—○—, untreated; —□—, $CCl_{4s.c.}$; —■—, $CCl_{4p.o.}$.

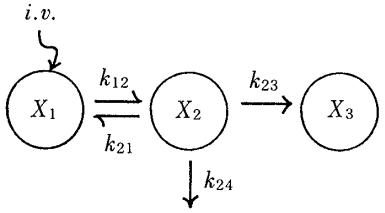

Fig. 3. Schematic Illustration of the Three-Compartment Model

Compartments X_1, X_2, X_3 represent AM in the plasma compartment, in the liver and in the bile, respectively.

TABLE II. Fitted and Derived Pharmacokinetic Parameters of the 3-Compartment Model for *i.v.* Administered AM in Plasma and Bile of Rats

	Untreated	$CCl_{4s.c.}$	$CCl_{4p.o.}$	ANIT
k_{12} (h^{-1})	25.794 ± 4.412	14.720 ± 1.161[a)]	17.043 ± 0.819[a)]	10.291 ± 1.210[a)]
k_{21} (h^{-1})	3.897 ± 0.664	3.849 ± 0.565	4.382 ± 0.575	6.775 ± 1.167
k_{23} (h^{-1})	4.131 ± 0.391	3.607 ± 0.185	2.352 ± 0.379[a)]	—
k_{24} (h^{-1})	0.333 ± 0.089	0.598 ± 0.056	0.646 ± 0.113	1.674 ± 0.240[a)]
V_1 (ml)	15.96 ± 4.06	18.06 ± 1.35	16.36 ± 0.54	22.90 ± 1.71

Each value is the mean ± S.E. of 4—5 rats. *a)* Significantly different from untreated, $p < 0.05$.

intoxicated groups. Biliary excretion profiles of AM are shown in Fig. 2. Decreased biliary excretion of AM was observed in the intoxicated rats. To clarify the hepatic transport processes influenced by intoxication, pharmacokinetic analysis was employed. Plasma disappearance and biliary excretion of AM were expected to be described by a more than three-compartment pharmacokinetic model (Fig. 3), since the plasma concentration time course could be described by a two-compartment model and one more compartment was necessary to connect the plasma and bile data. Although AM is known to be extensively excreted into bile in an unchanged form in rats, Radomski and Mellinger[32)] demonstrated that AM was metabolized to naphthionic acid (1-amino-4-naphthalene sulfonic acid) and excreted in the urine after oral administration of AM. We also identified naphthionic acid as an AM metabolite on a thin-layer chromatogram of the effluent and the liver following the liver perfusion experiment (unpublished data).

Taking account of these results, the pharmacokinetic parameters involving a step of metabolism in the liver were calculated according to the model shown in Fig. 3, with best fitting to observed values. The model contains four rate constants (with the dimension of h^{-1}). X_1 represents the amount of AM in the plasma compartment, X_2 is the amount of AM in the liver, and X_3 represents the amount of AM in the bile. All transfer processes were considered as first-order. Although the rate constant of metabolism, k_{24}, must involve enzymatic kinetics which will be saturated at high dose, the process was regarded as first-order

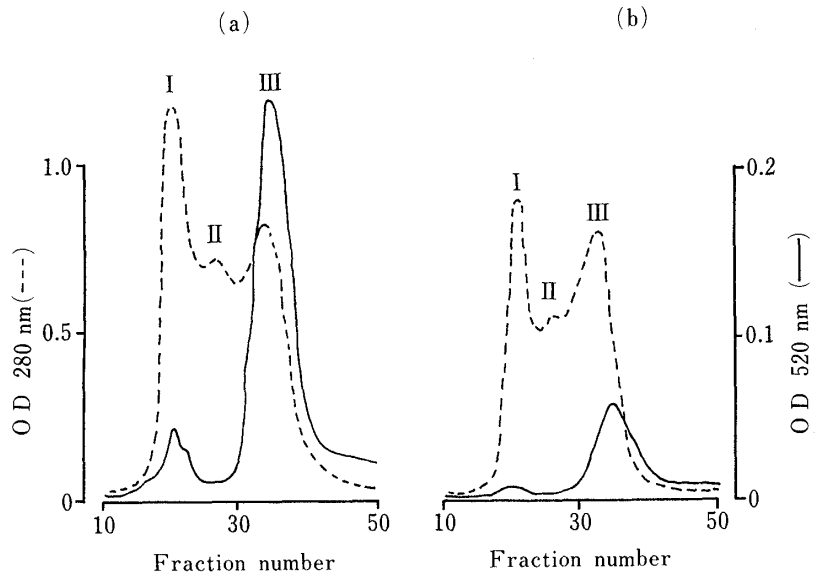

Fig. 4. Elution Patterns (from a Sephadex G-200 Column) of Plasma and AM-Spiked Plasma of an Untreated Rat (a) and of an ANIT-Intoxicated Rat (b)

AM (0.30 μmol) was spiked into 0.5 ml of plasma, which was then applied to a column (2.3 × 30 cm) packed with Sephadex G-200. Elution was performed at 4 °C with 0.01 M phosphate buffer (pH 7.3) at a flow rate of 12.5 ml/h. Fractions of 2 ml each were collected.

because of the low dose of AM employed. In ANIT-intoxicated rats, no bile flow was observed. Assuming k_{23} to be zero, compartment X_3 will be neglected in ANIT-intoxicated rats. The observed values of AM in the plasma and cumulative amount in the bile in each experiment were simultaneously fitted to the proposed model, and pharmacokinetic parameters were calculated by the nonlinear least-squares method (Fig. 3 and Table II). Good fittings were obtained for the amount of AM in plasma and cumulative biliary excretion of AM. Transfer rate constants from plasma to liver (k_{12}) and from liver to bile (k_{23}) were decreased in $CCl_{4p.o.}$-intoxicated rats. However, in $CCl_{4s.c.}$-intoxicated rats, only k_{12} was decreased. In ANIT-intoxicated rats, a significant decrease in the transfer rate constant from plasma to liver (k_{12}) and an increase in k_{24} were observed.

As factors which may cause the changes in the parameters, binding of AM to plasma and to hepatic binding protein, the hepatic blood flow and the permeability of liver sinusoidal membrane may be considered. First, the plasma protein binding of AM in the intoxicated groups was investigated. As shown in Fig. 4, panels (a) and (b), the chromatographic pattern of a plasma sample on Sephadex G-200 showed three peaks, namely I, II and III in order of elution, as described previously.[33,34] Janecki and Krawcynski[33] reported that peak I contained several high-molecular proteins such as β-lipoprotein and α_2-macroglobulin, and that peak II and peak III contained mainly globulin and albumin, respectively. In the present investigation, these three peaks were not clearly separated, but AM was bound only to peak III, namely the albumin fraction. In ANIT-intoxicated rats (panel (b)), although the plasma protein concentration was not much altered, the amount of AM bound to the albumin fraction was decreased. Krstulovic *et al.*[35] suggested that increases of bilirubin, cholesterol and phospholipid occur in the serum of ANIT-intoxicated rats. Bilirubin and cholesterol will bind mainly to albumin in blood, so these substances may interfere with the protein binding of AM, resulting in an increase of plasma clearance of AM. However, plasma clearance of AM was remarkably retarded in ANIT-intoxicated rats. Therefore, the binding of AM to plasma protein may not play a primary role in relation to the decreased clearance of AM in ANIT-intoxicated rats. In the CCl_4-intoxicated rats, on the other hand, the concentration

Table III. Binding Parameters of AM to Plasma Protein

	n	K ($\times 10^3$ M^{-1})	Protein (mg/ml)
Untreated	0.80±0.04	13.23±1.70	67.8±3.8
$CCl_{4s.c.}$	0.72±0.04	8.78±1.20	70.2±1.7
$CCl_{4p.o.}$	0.79±0.05	19.45±3.32	69.3±1.2
ANIT	1.54±0.20[a)]	3.69±0.57[a)]	67.2±3.0

Each value represents the mean±S.E. of 5—8 rats. *a)* Significantly different from untreated, $p<0.05$.

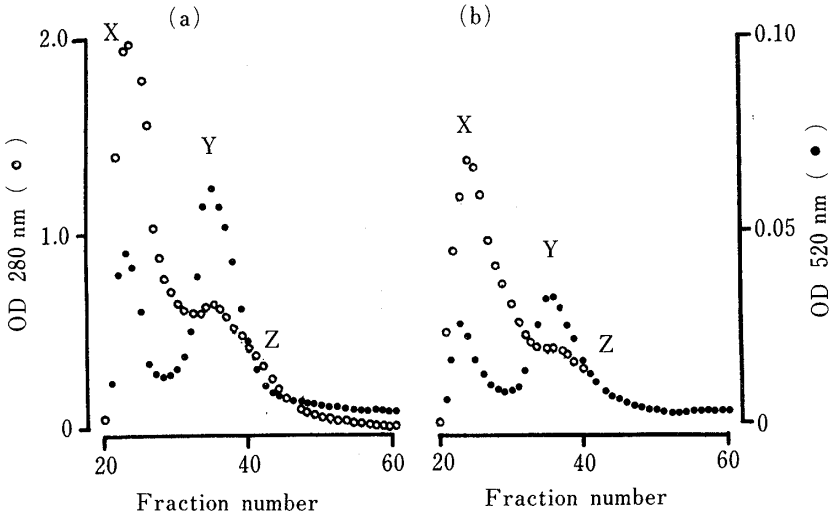

Fig. 5. Elution Patterns (from a Sephadex G-75 Column) of Protein and AM-Spiked Supernatant Fraction of the Liver Homogenate Obtained from an Untreated Rat (a) and a $CCl_{4p.o.}$-Intoxicated Rat (b)

Two μmol of AM was dissolved in 2 ml of the 110000*g* supernatant obtained from a 50% liver homogenate. The solution was placed on a Sephadex G-75 column (3.0×45 cm). Elution was performed at 4 °C with 0.01 M phosphate buffer (pH 7.4) with a pump-driven downward flow (12 ml/h). Fraction of 4 ml each were collected.

of plasma protein and amount of AM bound to plasma protein were not affected.

Binding parameters were calculated from Scatchard plots in which bound and free AM were obtained from Sephadex gel equilibrium data (Table III). The Sephadex gel equilibrium method is reported to be useful for rapid determination of binding parameters simply and without particular apparatus, especially in the case of small sample sizes.[29] In the present investigation, one class of binding site was found in untreated or CCl_4- or ANIT-intoxicated rats. ANIT-intoxicated rats showed a decreased binding constant and increased number of binding sites. The CCl_4-intoxicated groups showed no significant differences in the binding parameters as compared with the untreated rats.

Levi *et al.*[36] suggested that two cytoplasmic organic anion binding proteins, Y-(ligandin) and Z-fraction, are important in the transfer of many organic anions from the plasma into the liver. Iga *et al.*[24] reported a significant decrease in the binding constants of BSP to Y- and Z-fraction as well as a decreased protein concentration of Y-fraction in a chronically CCl_4-intoxicated rats. In order to elucidate whether the changes in the pharmacokinetic parameters observed in the present study are related to the binding of AM to Y- and Z-fraction or not, the binding to cytoplasmic protein was determined.

To study the binding of AM to cytoplasmic binding protein, column chromatography was performed. The elution patterns of a 2 ml supernatant fraction obtained from a 50% liver

TABLE IV. Binding of AM to the Supernatant and Y-Fraction Obtained from 50% Homogenate of Liver in Untreated and CCl_4- or ANIT-Treated Rats

	Untreated	$CCl_{4s.c.}$	$CCl_{4p.o.}$	ANIT
Supernatant (mg protein/ml)	47.98 ±4.55	54.51 ±3.05	26.12 ±4.12[c]	51.66 ±2.25
Y-fraction[a] (mg protein/ml)	0.536±0.019	0.500±0.038	0.411±0.033[c]	0.476±0.035
AM-bound/Y-fraction[b] (nmol/mg protein)	3.392±0.361	2.724±0.152	4.035±0.464	3.858±0.363

Each value represents the mean ± S.E. of 5 rats. *a*) The values are those for the tube giving the highest concentration of protein, *i.e.* tube number 35. *b*) The ratio in tube number 35. *c*) Significantly different from untreated, $p<0.05$.

homogenate and spiked with 2 μmol of AM on a Sephadex G-75 column are shown in Fig. 5, panels (a) and (b). Following the nomenclature of Levi *et al.*[36] the three peaks were designated as X-, Y- and Z-fraction. AM was bound preferentially to X- and Y-fraction rather than to Z-fraction. These results are in agreement with the results of Takada *et al.*[37] The binding of AM to Y-fraction was further analyzed by determining the protein concentration in the supernatant and Y-fraction. These results are summarized in Table IV. The ratio of bound AM to mg protein in the Y-fraction was calculated based on the data obtained from the tube giving the highest concentration of protein, *i.e.* tube number 35. In $CCl_{4p.o.}$-intoxicated rats, significant decreases in the protein concentration of the supernatant and Y-fraction were observed, but the ratio of bound AM to unit concentration of protein in the Y-fraction was not significantly different from that of the untreated rats. These results suggest that there are no structural changes of protein in CCl_4-intoxicated rats. In addition, the ratio of liver wet weight to body weight was increased in $CCl_{4p.o.}$-intoxicated rats. Therefore, the total amount of protein in Y-fraction was not different from that in untreated rats. These results suggest that there is no difference in total protein binding of AM in the whole liver. Thus, the decrease in k_{12} for $CCl_{4p.o.}$-intoxicated rats is probably not due to a change of binding of AM to Y-fraction.

It is well known that the hepatic blood flow is an important factor in hepatic transport of compounds with high clearance. Iga *et al.*[24] suggested that, if $k_{12} \cdot V_1$ is equal to the hepatic plasma flow, the transfer from compartment 1 to compartment 2 corresponds to the hepatic plasma flow and there is a rapid equilibration between the sinusoid and the hepatocyte. In this investigation, $k_{12} \cdot V_1$ was 5.98 ml/min in untreated rats, 4.34 ml/min in CCl_4-intoxicated rats, 3.31 ml/min in $CCl_{4p.o.}$-intoxicated rats, and 3.85 ml/min in ANIT-intoxicated rats. Although the hepatic plasma flow was not measured in this study, the value in untreated rats is comparable to the value reported by Yokota *et al.*,[38] and they reported that the hepatic plasma flow was decreased in chronically CCl_4-intoxicated rats. It is considered that the hepatic plasma flow may also be decreased in acutely CCl_4- or ANIT-intoxicated rats. These results suggest that the retarded disappearance of AM from plasma in these intoxicated rats can be ascribed mainly to the decrease in the hepatic plasma flow. In ANIT-intoxicated rats, k_{24} was significantly increased, which suggests an increase in enzymatic activity in the liver. It is not clear whether the treatment with ANIT causes enzyme induction, and this point is now under study.

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