Biochemical Pharmacology, Vol. 31, No. 11, pp. 2039–2046, 1982.
Printed in Great Britain.

0006-2952/82/112039-08 $03.00/0

INHIBITION OF HEPATIC MICROSOMAL DRUG-METABOLIZING ENZYMES BY HABU SNAKE (*TRIMERESURUS FLAVOVIRIDIS*) VENOM FRACTIONS

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(*Received* 27 *April* 1981; *accepted* 9 *October* 1981)

Abstract—An inhibitor of hepatic microsomal drug-metabolizing enzyme activity was isolated from the venom of the Habu snake (*Trimeresurus flavoviridis*) by gel filtration through Sephadex G-100 and Amberlite CG-50 column chromatography. The inhibitor, designated R-CG-50-2, gave one band on sodium dodecylsulfate (SDS) polyacrylamide gel electrophoresis and caused high hemorrhagic activity when administered i.c. to rabbits. R-CG-50-2 inhibited the drug-metabolizing enzyme system even after being heated at 70° for 5 min, in spite of a complete loss of hemorrhagic activity. Cytochrome P-450 content and NADPH–cytochrome *c* reductase activity of rat hepatic microsomes were decreased by administration *in vivo* either R-CG-50-2 or heated R-CG-50-2. The extent of the decrease was greater with unheated R-CG-50-2 than with heated R-CG-50-2. In both cases, cytochrome P-420, the inactive form of cytochrome P-450, was not detected. Lipid peroxidation in hepatic microsomes was also decreased by administration of unheated R-CG-50-2 but the decrease was not significant. In an *in vitro* experiment, both heated and unheated R-CG-50-2 decreased the cytochrome P-450 content and the NADPH–cytochrome *c* reductase activity of microsomes, but, unlike the results *in vivo*, cytochrome P-450 was converted to cytochrome P-420. Microsomal lipid peroxidation was greatly inhibited by either heated or unheated R-CG-50-2 *in vitro*. It was concluded that the inhibition of the hepatic microsomal drug-metabolizing enzyme system by either heated or unheated R-CG-50-2 may have been due to the decrease in the cytochrome P-450 content and the NADPH–cytochrome *c* reductase activity, and that lipid peroxidation may not have had an effect on the inhibition.

It is well established that the metabolism of a large number of drugs and foreign compounds is catalyzed by the hepatic microsomal drug-metabolizing enzyme system that consists of the flavoprotein NADPH–cytochrome *c* reductase and cytochrome P-450. The activities of drug-metabolizing enzymes vary with the age and strain, hormonal and nutritional status, and drug pretreatment of the animal [1–5]. More recently there have been some studies which show that hepatic microsomal drug-metabolizing enzyme activities are also altered by injection of bacteria [6, 7]. Thus, the metabolism of drugs and, consequently, their activities and toxicities are influenced by many conditions. We demonstrated previously that the administration of Habu venom to rats prolonged the hexobarbital-produced sleeping time and inhibited the activities of hepatic microsomal drug-metabolizing enzymes [8]. Habu venom contains various enzymes and toxic proteins [9–11]. When Habu venom is injected parentally into animals, it causes severe local effects such as hemorrhage, edema, and necrosis. Studies have been made to determine what components of the venom contribute to producing such local reactions. Takahashi and Ohsaka [12] isolated the hemorrhagic principles from Habu venom. An edema-forming factor and a necrotizing factor have also been reported [11]. We are interested in whether inhibition of hepatic microsomal drug-metabolizing enzymes is caused by the same component that causes hemorrhage, edema, and necrosis or by a separate, specific component. This work was undertaken to isolate from Habu venom the inhibitor of the hepatic microsomal drug-metabolizing enzyme system and to determine the enzymatic and biologic properties of the isolated inhibitor. Furthermore, the mechanism of inhibition of the drug-metabolizing enzyme system by the isolated inhibitor was examined and discussed.

MATERIALS AND METHODS

Snake venom. The venom used in this study was a pool of lyophilized venom taken from specimens of Habu (*Trimeresurus flavoviridis*) collected on Okinawa Island in 1967.

Chemicals. Nicotinamide adenine dinucleotide phosphate, glucose-6-phosphate and cytochrome *c* (type III) were purchased from the Sigma Chemical Co., St. Louis, MO. Reduced nicotinamide adenine dinucleotide phosphate and glucose-6-phosphate dehydrogenase were from Orientalkobo Ltd., Tokyo. Sephadex G-100 (particle size 40–120 nm) was provided by Pharmacia Fine Chemicals, Uppsala, Sweden. Amberlite CG-50 (type II) was pur-

* This paper was presented by Y. Aniya in partial fulfilment of the requirement of the Ph.D. degree in the Department of Pharmacology, Faculty of Medicine, Kagoshima University, 1980.

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chased from the Rohm & Hass Co., Philadelphia, PA. *p*-Tosyl-L-arginine methyl ester (TAME) hydrochloride was obtained from Nakarai Chemicals Ltd., Kyoto.

Animals and preparation of microsomes. Male Wistar rats, weighing 100–150 g, were obtained from Nihonrat Ltd., Saitama. Animals were fed with a commercial diet (Orientalkobo Ltd.) and water *ad lib.* Lyophilized venom was freshly dissolved in a physiological saline solution and injected into rats intraperitoneally at a dose of 5 mg/kg animal weight. Drug-metabolizing enzyme activity in hepatic microsomes was determined 18 hr after the venom injection. In the case of heated venom, the same dose (5 mg/kg) was heated at 70° for 5 min, and then the centrifuged supernatant fraction was injected. Control animals received an equivalent volume of saline solution only. Liver microsomes were prepared as follows. The animal was decapitated and the liver was removed after perfusion with 1.15% KCl solution *in situ*. The liver was homogenized in a Teflon–glass homogenizer with 3 vol. of ice-cold 1.15% KCl solution. The homogenate was centrifuged at 9,000 *g* for 30 min, and some of the supernatant fraction was used to assay the inhibition of drug-metabolizing enzyme activity by venom fractions obtained from column chromatography. The rest of 9,000 *g* supernatant fraction was further centrifuged at 105,000 *g* for 60 min. The resulting microsomal pellet was then resuspended in 1.15% KCl solution, and the suspension was used to study the mechanism of the inhibition of drug-metabolizing enzymes.

Analytical methods and assay of microsomal components. The standard reaction mixture for the assay of aminopyrine *N*-demethylation contained 1 mM aminopyrine, microsomes (0.7 to 1.0 mg protein), 80 mM Tris–HCl buffer (pH 7.4) and an NADPH-generating system, in a final volume of 1.0 ml. The NADPH-generating system consisted of 0.33 mM NADP, 8 mM glucose-6-phospate, 6 mM $MgCl_2$, and 0.2 units of glucose-6-phosphate dehydrogenase. When necessary, the venom was added to the standard mixture. For the purpose of determining the inhibitory effect on aminopyrine *N*-demethylation by each venom fraction during the purification, the 9,000 *g* supernatant fraction was used as microsomes. The mixture was incubated in a water bath shaker for 20 min at 37° under air. The *N*-demethylation of aminopyrine was determined by measuring the formation of formaldehyde using the Nash method [13]. Lipid peroxidation was determined by using the same standard mixture and conditions as were used for aminopyrine *N*-demethylation. Peroxide formation was determined by the thiobarbituric acid method as described by Wills [14] and modified by Kamataki and Kitagawa [15]. Cytochrome P-450 and NADPH–cytochrome *c* reductase were measured by the method of Omura and Sato [16], and the method of Mazel [17] respectively. Microsomal protein was determined by the method of Lowry *et al.* [18], using bovine serum albumin as reference protein.

Isolation of the inhibitor of drug-metabolizing enzymes. Gel filtration of crude Habu venom through a Sephadex G-100 column was carried out at 5° using 0.005 M Tris–HCl buffer (pH 8.5) which contained 0.15 M NaCl. The fraction that had an inhibitory effect on drug-metabolizing enzymes was dialyzed against distilled water at 5° for 15 hr and then lyophilized. The lyophilized fraction was applied to a column of Amberlite CG-50 and eluted with a linear gradient of 0.2 to 0.4 M NaCl in 0.005 M borate–HCl buffer (pH 8.3). The fraction that inhibited the drug-metabolizing enzymes was rechromatographed on the Amberlite CG-50 column using the same conditions except that the gradient was of 0.2 to 0.3 M NaCl. The protein content of the eluent was measured by u.v. absorption at 280 nm, based on the assumption that the absorbancy of a 100 μg/ml solution of dried crude venom was 0.15.

Polyacrylamide gel electrophoresis. Sodium dodecylsulfate polyacrylamide gel electrophoresis was carried out as described by Takagi and Yanagi [19]. After the R-CG-50-2 solution was dialyzed against distilled water for 6 hr and lyophilized, 10 μg of the unheated R-CG-50-2 and 12 μg of heated R-CG-50-2 were subjected to gel electrophoresis. Protein in the gel was stained with Coomassie Brilliant Blue G-250.

Determination of enzymatic and biologic activities of Habu venom. Hemorrhagic activity was determined by the method of Kondo *et al.* [20] and was represented as a hemorrhagic spot (cm^2) per 10 μg of the venom. Proteolytic activity was estimated by the method of Takahashi and Ohsaka [10] and was expressed as the O.D. at 280 nm per mg of the venom. Phospholipase A activity was determined according to the method of Habermann and Neumann [21] with some modification: each sample (0.2 ml, 4 μg protein) of venom was incubated with 1 ml of 50% egg yolk solution (0.04 M Veronal–HCl buffer, pH 7.0) at 37° for 60 min, this was followed by measuring the coagulation time of the reaction mixture in a boiling water bath. Arginine ester hydrolase activity was determined with *p*-tosyl-L-arginine methyl ester as substrate according to the method of Roberts [22] and was expressed as the amount of hydrolyzed substrate (μmoles) per mg of each fraction.

RESULTS

Isolation from Habu venom of an inhibitor of drug-metabolizing enzymes. Figure 1 shows the gel filtration of crude Habu venom (300 mg) on Sephadex G-100. Protein was recovered in three fractions which were designated G-100-1, G-100-2, and G-100-3 in order of elution. The effects of the three fractions on *in vivo* aminopyrine *N*-demethylation in hepatic microsomes of rats are shown in Fig. 1 as bars. After the injection of G-100-1, aminopyridine *N*-demethylation appeared to have decreased to about 70% of the control, and after the injection of G-100-2 to 50%, but only the decrease produced by G-100-2 was statistically significant. The recovery of protein in G-100-2 was about 50% of that in the crude venom. The lyophilized G-100-2 fraction (200 mg) was then chromatographed using Amberlite CG-50. The chromatographic profile is indicated in Fig. 2. Four fractions were obtained and designated CG-50-1, CG-50-2, CG-50-3, and CG-50-4 in order of elution. A statistically significant decrease

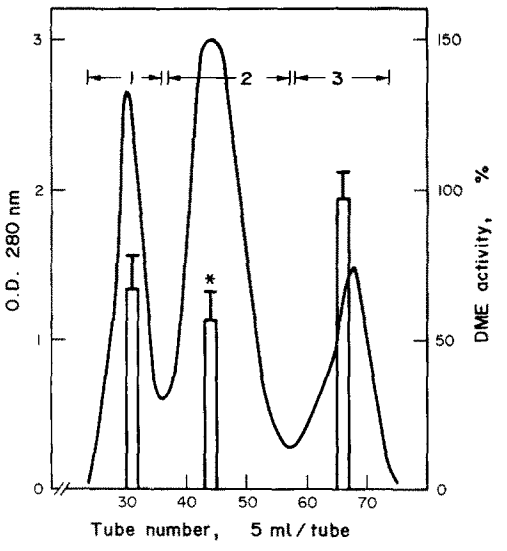

Fig. 1. Gel filtration of crude Habu venom on Sephadex G-100. The column (2.6 × 65 cm) of Sephadex G-100 was equilibrated with 0.005 M Tris–HCl buffer (pH 8.5) containing 0.15 M NaCl. Habu venom (300 mg), dissolved in 4 ml of the same buffer, was applied to the column and eluted at 5°. Each Sephadex G-100 fraction was lyophilized and injected i.p. (5 mg/kg) into rats; microsomal drug-metabolizing enzyme (DME) activities were determined 18 hr later as described in Materials and Methods. The control value of formaldehyde formation from aminopyrine *N*-demethylation was 32.40 ± 3.60 nmoles · (mg protein)$^{-1}$ · (20 min)$^{-1}$. DME activities are presented as bars showing the percentages of the control value. Vertical bars indicate ± S.E. (N = 5). Key: (*)P < 0.05.

in microsomal aminopyrine *N*-demethylation was found after CG-50-3 injection into rats (Fig. 2). Phospholipase A activity *in vitro* was detected in both CG-50-1 and CG-50-4 but not in CG-50-3 or CG-50-2; proteolytic activity was mainly in CG-50-2. Thus, CG-50-3, the fraction that inhibited aminopyrine *N*-demethylation, was almost completely separate from the fractions possessing phospholipase A and proteinase activities. Therefore, we regarded CG-50-3 as the fraction that contained an inhibitor of drug-metabolizing enzymes and performed further purification. Figure 3 shows the chromatographic profile and the enzymatic activities after rechromatography of CG-50-3 on Amberlite CG-50. Two peaks were obtained by rechromatography. Inhibition *in vivo* of aminopyrine *N*-demethylation was caused by injection of the fraction constituting the second peak, which had been designated R-CG-50-2. R-CG-50-2 was free of phospholipase A and proteolytic activities. Upon electrophoresis on SDS polyacrylamide gel, both R-CG-50-2 and heated R-CG-50-2 gave a single band, and both relative molecular weights ranged from 26,000 to 28,000, as shown in Fig. 4. The purification procedure and yield at each step are summarized in Table 1. The protein recovered from R-CG-50-2 was about 1% of that in the crude Habu venom.

Properties of R-CG-50-2. All fractions obtained from column chromatography of Habu venom were lyophilized, and their enzymatic and biologic activities were determined (Table 2). R-CG-50-2 contained high hemorrhagic activity and trace amounts of proteolytic activity, but phospholipase A and arginine ester hydrolase, which are known to be

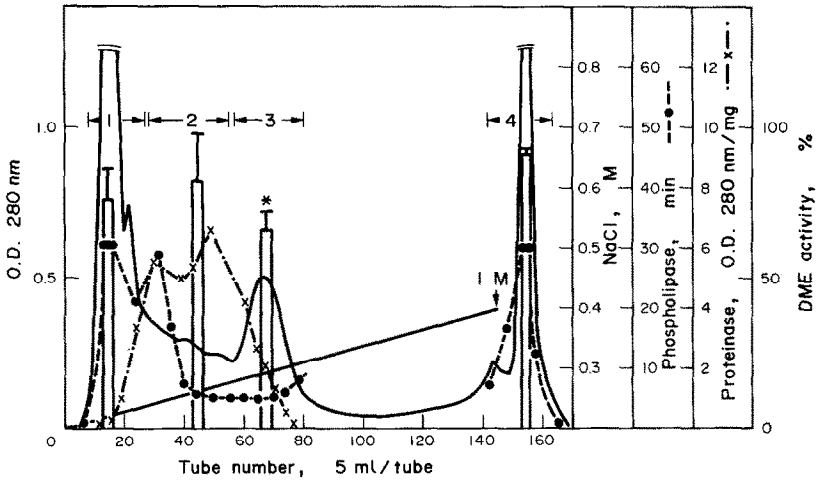

Fig. 2. Chromatography on Amberlite CG-50 of venom fraction II (G-100-2) from Sephadex G-100. The column (1.6 × 35 cm) was equilibrated with 0.005 M borate–HCl buffer (pH 8.3) containing 0.2 M NaCl. G-100-2 (200 mg) was dissolved in 5 ml of the same buffer and applied to the column. Linear gradient elution was carried out at 5° with 250 ml of the buffer in the mixing vessel and buffer of the same volume containing 0.4 M NaCl in the reservoir. DME activity *in vivo* of hepatic microsomes of rats was measured using the same conditions as described in Fig. 1. The control value of formaldehyde from aminopyrine *N*-demethylation in microsomes obtained from five rats was 43.32 ± 1.64 nmoles · (mg protein)$^{-1}$ · (20 min)$^{-1}$. DME activities are presented as bars showing them as percentages of the control aminopyrine *N*-demethylation. Vertical bars indicate ± S.E. Key: (*) P < 0.05. The NaCl gradient, phospholipase activity (as coagulation), and proteinase activity (as absorbance) are also shown.

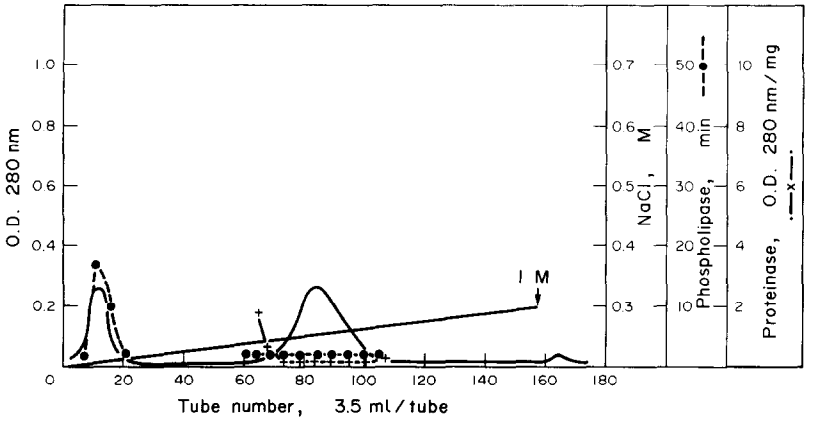

Fig. 3. Rechromatography of venom fraction III (CG-50-3) on Amberlite CG-50. CG-50-3 (20 mg) was dissolved in 4 ml of 0.005 M borate–HCl buffer (pH 8.3) containing 0.2 M NaCl and was eluted under the same conditions as Fig. 2, the only difference being the linear gradient elution from 0.2 to 0.3 M NaCl.

heat-stable enzymes, were not detected. Hemorrhagic and proteolytic activities in R-CG-50-2 were lost completely by heating at 70° for 5 min. However, R-CG-50-2 inhibition of aminopyrine *N*-demethylation was observed even after heating, as shown in Fig. 5; the decrease was statistically significant. To clarify the contribution of the heat-stable component to the inhibition of a drug-metabolizing enzyme *in vivo*, experiments *in vitro* were conducted (Fig. 6). After heating at each temperature for 5 min, R-CG-50-2 was incubated with microsomes from normal rats. Aminopyrine *N*-demethylation was decreased at 25° to 57% of the control, to 77% at 55°, and to 80% at 85° (Fig. 6). Hemorrhagic activity was completely lost above 60°. Thus, a heat-stable component, other than phospholipase A or arginine

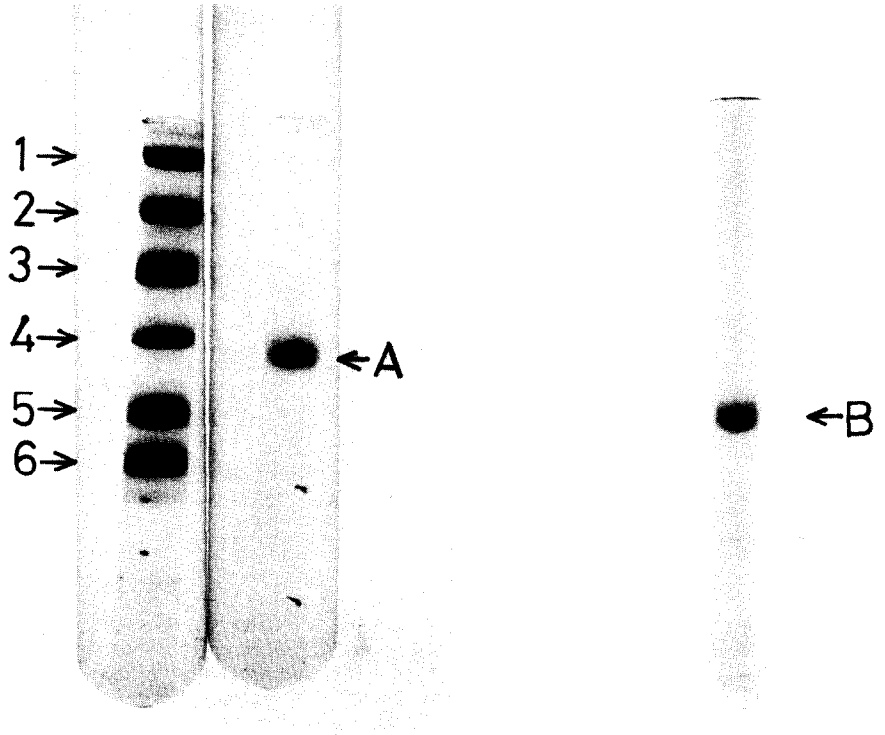

Fig. 4. SDS polyacrylamide gel electrophoresis of R-CG-50-2 and heated R-CG-50-2. Current, 6 mA/tube; time, 2.5 hr; sample, (A) R-CG-50-2 (10 μg) and (B) heated R-CG-50-2 (12 μg). Left gel (calibration kits for low molecular weight, Pharmacia Fine Chemicals): (1) phosphorylase b, 94,000; (2) albumin, 67,000; (3) ovalbumin, 43,000; (4) carbonic anhydrase, 30,000; (5) trypsin inhibitor, 20,100; and (6) α-lactalbumin, 14,400.

Table 1. Recovery of protein and inhibitory activity by serial column chromatography

Steps	Total protein (mg)	Recovery of protein (%)	Inhibitory activity	
			*In vivo** (%)	*In vitro*† (%/μg)
Crude venom	1600.0	100	48	1.38 (1.0)
Sephadex G-100	832.0	52.0	42	1.45 (1.05)
Amberlite CG-50 (First)	63.0	3.9	42	3.31 (2.39)
Amberlite CG-50 (Second)	12.9	0.8	47	3.90 (2.81)

* Inhibitory activities *in vivo* are represented as percentages of aminopyrine *N*-demethylation by microsomes from rats that had been injected with the respective lyophilized preparations (5 mg/kg).

† Inhibitory activity *in vitro* is represented as the percentage decrease in aminopyrine *N*-demethylation, per μg protein, produced by the eluate from each column without lyophilization.

Table 2. Some enzymatic and biologic activities of each preparation

Preparation	Phospholipase A (min/4 μg)	Proteinase (O.D. at 280 nm/mg)	Arginine ester hydrolase (TAME, μmoles/mg)	Hemorrhage (cm^2/10 μg)
Crude	>30	4.62	50.0	3.4
G-100-2	>30	2.87	15.4	2.4
CG-50-3	7	0.37	0	2.5
R-CG-50-2	<1.5	0.14	0	3.7
Heated R-CG-50-2	<1.5	0	0	0

ester hydrolase, was present in R-CG-50-2 and contributed to the inhibition of the drug-metabolizing enzymes.

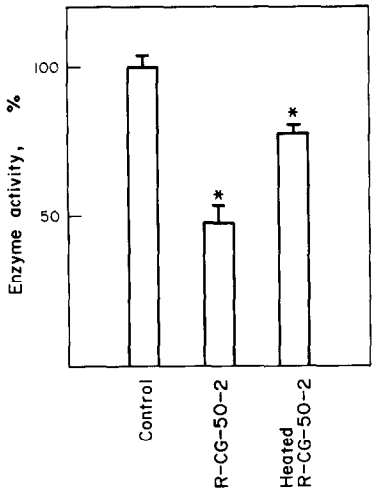

Fig. 5. Activity of aminopyrine *N*-demethylation after the injection of R-CG-50-2 and heated R-CG-50-2. Lyophilized unheated R-CG-50-2 [5 mg · $(3 ml)^{-1}$ · kg^{-1}] was injected into rats intraperitoneally. Heated R-CG-50-2 (5 mg/3 ml of 0.9% NaCl) was kept at 70° for 5 min, and the centrifuged supernatant fraction of 3 ml/kg was injected. DME activity was determined as described in the legend of Fig. 1 except that the 9,000 *g* liver fraction in the incubation mixture was substituted by the 105,000 *g* pellet. The control value obtained from four rats was formaldehyde, 53.82 ± 7.80 nmoles · (mg protein)$^{-1}$ · $(20 min)^{-1}$ as aminopyrine *N*-demethylation. DME activity is represented as a histogram of the percentage of control aminopyrine *N*-demethylation. Vertical bars indicate ± S.E. Key: (*) $P < 0.05$.

Mechanism of inhibition of Habu venom of drug-metabolizing enzymes. The mechanism of inhibition of hepatic microsomal drug-metabolizing enzymes was examined by using R-CG-50-2 and heated R-CG-50-2. Table 3 shows the effect of administration of R-CG-50-2 and heated R-CG-50-2 on rat hepatic microsomes. Aminopyrine *N*-demethylation, cytochrome P-450, and NADPH–cytochrome *c* reductase were decreased significantly by R-CG-50-2 to 48, 60 and 75% of the control respectively. Only a small decrease in lipid peroxidation was caused by R-CG-50-2. After administration of heated R-CG-50-2, a significant decrease in

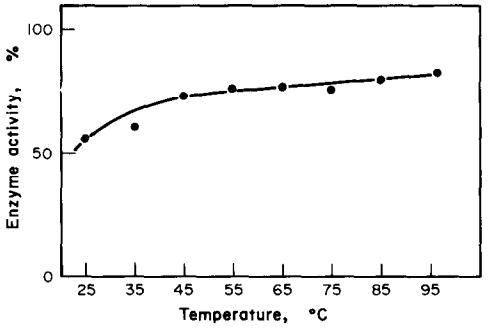

Fig. 6. Effect of heating on R-CG-50-2 inhibition of aminopyrine *N*-demethylation. R-CG-50-2 (100 μg) was dissolved in 1.0 ml of 0.1 M phosphate buffer (pH 7.4) and heated at each temperature for 5 min. Then 0.1 ml of the centrifuged supernatant fraction was added to the incubation mixture as described in the legend of Fig. 5 and aminopyrine *N*-demethylation activity was determined. Enzyme activity is represented as the percentage of control aminopyrine *N*-demethylation (control value of formaldehyde, 35.8 nmoles · (mg protein)$^{-1}$ · $(20 min)^{-1}$).

Table 3. Effects of R-CG-50-2 and heated R-CG-50-2 on aminopyrine *N*-demethylation, cytochrome P-450, NADPH–cytochrome *c* reductase, and lipid peroxidation *in vivo**

	Aminopyrine *N*-demethylation (formaldehyde, (nmoles/mg protein/20 min)	Cytochrome P-450 (nmoles/mg protein)	NADPH–cytochrome *c* reductase (nmoles/min/mg protein)	Lipid peroxidation (TBA value)
Control	53.82 ± 7.80	0.669 ± 0.01	414.15 ± 15.90	0.283 ± 0.09
R-CG-50-2	25.80 ± 2.37†	0.402 ± 0.05†	312.08 ± 11.42†	0.253 ± 0.03
Control	55.58 ± 2.34	0.569 ± 0.12	441.20 ± 72.99	0.208 ± 0.02
Heated R-CG-50-2	44.12 ± 2.34†	0.523 ± 0.11	399.50 ± 12.80	0.206 ± 0.01

* R-CG-50-2 and heated R-CG-50-2 were injected into rats, and aminopyrine *N*-demethylation of hepatic microsomes was determined by the same conditions as described in the legend of Fig. 5. Microsomal cytochrome P-450, NADPH–cytochrome *c* reductase, and lipid peroxidation were measured as described in Materials and Methods. TBA value indicates O.D. (at 532 nm) per mg protein, and each value is the mean ± S.E. of three to four rats.
† $P < 0.05$.

aminopyrine *N*-demethylation was measured, but the decrease of cytochrome P-450 and NADPH–cytochrome *c* reductase was small. Lipid peroxidation was not changed by heated R-CG-50-2. No cytochrome P-420 was observed in either the R-CG-50-2 or the heated R-CG-50-2.

Liver microsomes of normal rats were treated with R-CG-50-2 and heated R-CG-50-2 *in vitro*. As shown in Table 4, aminopyrine *N*-demethylation was decreased to 11% of the control by both R-CG-50-2 and heated R-CG-50-2, but only about 55% of cytochrome P-450 was converted to cytochrome P-420 by R-CG-50-2 treatment and only about 40% was converted by heated R-CG-50-2. Figure 7 shows the conversion of cytochrome P-450 to cytochrome P-420 with R-CG-50-2 and heated R-CG-50-2, spectrophotometrically. Table 5 indicates the relationships among the aminopyrine *N*-demethylation, NADPH–cytochrome *c* reductase, and lipid peroxidation *in vitro* tests. R-CG-50-2 decreased aminopyrine *N*-demethylation to 59% of the control, decreased NADPH–cytochrome *c* reductase to 45%, and decreased lipid peroxidation to 33%. The decreases in aminopyrine *N*-demethylation, NADPH–cytochrome *c* reductase, and lipid peroxidation were smaller with heated R-CG-50-2 than with unheated R-CG-50-2.

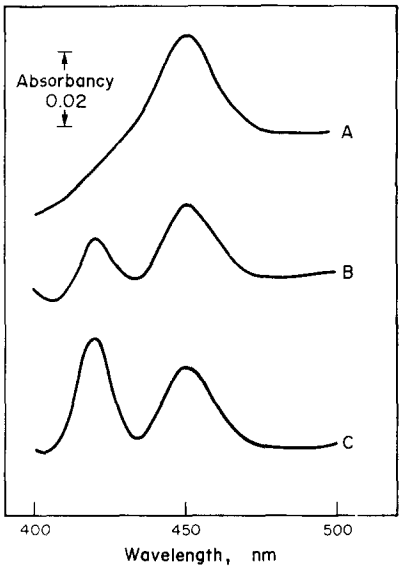

Fig. 7. Carbon monoxide difference spectra of hepatic microsomes treated with R-CG-50-2 or heated R-CG-50-2. Curve A: control. Curve B: microsomes treated with heated R-CG-50-2. Curve C: microsomes treated with R-CG-50-2.

DISCUSSION

The inhibitor of the hepatic microsomal drug-metabolizing enzymes was isolated from Habu venom by gel filtration and ion exchange column chromatography. This inhibitor of drug-metabolizing enzymes, designated R-CG-50-2, had high hemorrhagic activity but almost no proteolytic activity and no phospholipase activity at all. Though it has been reported that phospholipase and trypsin inactivate hepatic microsomal cytochrome P-450 [16, 23], we were able to separate R-CG-50-2 from the proteinase

Table 4. Effects of R-CG-50-2 and heated R-CG-50-2 on aminopyrine *N*-demethylation and cytochrome P-450 *in vitro**

	Aminopyrine *N*-demethylation (formaldehyde, nmoles/mg protein/20 min)	Cytochrome P-450 (nmoles/mg protein)	Cytochrome P-420 (nmoles/mg protein)
Control	40.38 (100)	0.743 (100)	0
R-CG-50-2	2.5 (11.8)	0.346 (46.7)	0.407
Heated R-CG-50-2	2.5 (11.8)	0.458 (61.7)	0.196

* Hepatic microsomes (2.6 mg in 1.0 ml of 0.1 M phosphate buffer, pH 7.4) prepared from normal rats were treated with R-CG-50-2 (900 μg) or heated R-CG-50-2 in a nitrogen atmosphere at 4° for 18 hr. Values in parentheses are percentages of control values.

Table 5. Effects of R-CG-50-2 and heated R-CG-50-2 on aminopyrine *N*-demethylation, NADPH–cytochrome *c* reductase, and lipid peroxidation *in vitro**

	Aminopyrine *N*-demethylation (formaldehyde, nmoles/mg protein/20 min)	NADPH–cytochrome *c* reductase (nmoles/min/mg protein)	Lipid peroxidation (TBA value)
Control	59.45 ± 0.86	499.86 ± 56.49	0.166 ± 0.06
R-CG-50-2	35.07 ± 2.47†	174.69 ± 36.95†	0.056 ± 0.02†
Heated R-CG-50-2	43.52 ± 1.22†	211.38 ± 7.76†	0.066 ± 0.02†

* R-CG-50-2 (12 μg in 0.1 ml of 0.1 M phosphate buffer, pH 7.4) or heated R-CG-50-2 (70°, 5 min) was added to the incubation mixture described in the text and incubated at 37° for 20 min. TBA value indicates O.D. (at 532 nm) per mg protein, and each value is the mean ± S.E. of three samples.
† $P < 0.05$.

and the phospholipase included in Habu venom. The hemorrhagic principle (HR) 1 and HR 2 have been purified from Habu venom, and their activities are destroyed by heating to above 60° [10, 12, 24]. In view of the process of purification of the inhibitor of the drug-metabolizing enzyme, it seemed reasonable to assume that R-CG-50-2 was the same as HR 2 and that it was the hemorrhagic activity which caused the inhibition of drug-metabolizing enzymes. However, it was found that, R-CG-50-2 when heated (70°, 5 min) and administered to rats, retained the ability to inhibit drug-metabolizing enzymes in spite of a complete loss of hemorrhagic activity (Fig. 5). Therefore, it was assumed that a heat-stable component was contained in R-CG-50-2. This was also supported by the *in vitro* tests which showed that the inhibition of drug-metabolizing enzymes by R-CG-50-2 decreased with an increase in temperature, and that a constant inhibition of drug-metabolizing enzymes was observed above 60°, the temperature at which the hemorrhagic activity was lost. R-CG-50-2 and heated R-CG-50-2 showed a single band on SDS polyacrylamide gel electrophoresis and had almost the same molecular weights (26,000 to 28,000). Furthermore, heated R-CG-50-2 showed a maximum absorption at 280 nm, as did the unheated R-CG-50-2. Thus, it was concluded that R-CG-50-2 contained not only the heat-sensitive hemorrhagic principle (HR 2) but also the heat-stable component and that both components were proteins which resembled each other very closely in molecular weight and charge. It was reported recently that HR 2 consists of several components with hemorrhagic activity [12, 25] but there was no study of its relation to the heat-stable component. Since neither phospholipase nor arginine ester hydrolase, which are known to be heat-stable, was detected in R-CG-50-2, the heat-stable component included in R-CG-50-2 is presumed to be a specific protein that has an inhibitory effect on drug-metabolizing enzymes.

Administration of 5 mg/kg of fractions obtained from column chromatography caused almost the same degree of inhibition of drug-metabolizing enzymes. Two reasons can be advanced to explain this lack of an increase in inhibitory potency. First, dialysis and the lyophilization of eluates from each column caused a loss of drug-metabolizing enzyme inhibitory activity. It was necessary to concentrate eluates until an adequate volume for administration to the rat was obtained, but a suitable method to concentrate the activity, other than lyophilization, was not found. However, during the *in vitro* tests an increase in drug-metabolizing enzyme inhibitory action was observed when using eluates without lyophilization. Second, it was assumed that the proteolytic, hemorrhagic, and phospholytic activities of the crude venom contributed to the inhibition of the drug-metabolizing enzymes, because the fractions possessing those activities (G-100-1 and CG-50-1) weakly decreased the drug-metabolizing enzyme activity. Such a contribution to the inhibition of drug-metabolizing enzymes was also suggested from the fact that the administration of phospholipase A from *Crotalus terr. terr.* or trypsin caused a lesser degree of inhibition of the drug-metabolizing enzymes than did Habu venom (unpublished observation).

A significant decrease in cytochrome P-450 content and in NADPH–cytochrome *c* reductase activity of hepatic microsomes was observed after administration of R-CG-50-2 but the decrease of lipid peroxidation was minor. As described in the beginning of this paper, the hepatic microsomal drug-metabolizing enzyme system consists of NADPH–cytochrome *c* reductase and cytochrome P-450. The latter is the terminal oxidase in the system. Therefore, it is reasonable to assume that the inhibition of drug-metabolizing enzymes by R-CG-50-2 may have been due to the decrease in the cytochrome P-450 content of hepatic microsomes. Though it was reported that administration of carbon tetrachloride [26] and adjuvant [27] decreased cytochrome P-450 content by causing the conversion of cytochrome P-450 to cytochrome P-420, no cytochrome P-420 was detected when R-CG-50-2 was administered. Thus, it appeared that a mechanism different from the conversion of cytochrome P-450 to cytochrome P-420 was involved in the decrease in cytochrome P-450 content caused by R-CG-50-2. Miyagi *et al.* [28] showed that δ-aminolevulinic acid synthetase, the rate-limiting enzyme among all heme synthesis processes, was depressed in mice by the administration of Habu venom. We also observed a depression of δ-aminolevulinic acid synthetase in rats (unpublished observations). Since cytochrome P-450 is a hemoprotein, it is reasonable to assume that the decrease of cytochrome P-450 content by R-CG-50-2 may have been due to the depression of heme synthesis which was followed by the reduction of cytochrome P-450 synthesis.

NADPH–cytochrome *c* reductase is believed to be a rate-limiting enzyme of the hepatic microsomal

drug-metabolizing enzyme system [29] and is also believed to catalyze microsomal lipid peroxidation [30]. It has been reported that enhancement of microsomal lipid peroxidation causes inhibition of microsomal drug-metabolizing enzymes by means of degradation of cytochrome P-450 heme [31], of competition for electrons from NADPH–cytochrome *c* reductase [29, 30], and of destruction of lipid essential for drug-metabolizing enzyme activity [32]. After R-CG-50-2 was administered, NADPH–cytochrome *c* reductase and lipid peroxidation were always diminished. Therefore, it was concluded that the inhibition of drug-metabolizing enzymes by R-CG-50-2 was a result of the decrease in cytochrome P-450 content and the decrease of NADPH–cytochrome *c* reductase activity but not of the increase in lipid peroxidation.

Since the administration of heated R-CG-50-2 decreased cytochrome P-450 content and NADPH–cytochrome *c* reductase activity to a lesser degree than did the administration of R-CG-50-2, it is reasonable to conclude that heated R-CG-50-2 inhibited hepatic microsomal drug-metabolizing enzymes in the same manner as did R-CG-50-2.

Furthermore, we examined the mechanism of inhibition by R-CG-50-2 microsomal drug-metabolizing enzymes *in vitro*. Treatment of microsomes with R-CG-50-2 or heated R-CG-50-2 resulted in a marked decrease in aminopyrine *N*-demethylation and in the conversion of cytochrome P-450 to cytochrome P-420. NADPH–cytochrome *c* reductase activity and lipid peroxidation were also decreased markedly with R-CG-50-2 or heated R-CG-50-2 *in vitro*. Cytochrome P-450 content and NADPH–cytochrome *c* reductase activity were also decreased by R-CG-50-2 and heated R-CG-50-2 *in vitro*, but there was a difference in the decrease of cytochrome P-450 content *in vivo* and *in vitro*. The conversion of cytochrome P-450 to cytochrome P-420 was not observed *in vivo* but the conversion seemed to play a major part in decreasing cytochrome P-450 content *in vitro*.

According to Imai and Sato [33], the conversion of cytochrome P-450 to cytochrome P-420 results from a disturbance in the hydrophobic environment around the heme of cytochrome P-450. The hemorrhagic principle destroys the basement membranes in vessel walls [34]. It is therefore suggested that the hemorrhagic principle included in R-CG-50-2 impaired microsomal membranes and changed the hydrophobic interaction around cytochrome P-450. Even though the heat-stable component involved in R-CG-50-2 converted cytochrome P-450 to cytochrome P-420, no satisfactory explanation for the conversion is at present available.

Acknowledgement—We are indebted to Dr. Zenji Hokama for supplying the snake venom.

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