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Studies on Poisonous Metals. XI.[1] Effect of Chelating Agents on Biliary Excretion of Cadmium in Rats

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The mechanism of the stimulatory effect of chelating agents, such as DL-penicillamine and citric acid, on the biliary excretion of cadmium in rats was studied. The results of Sephadex G-75 gel filtration of liver soluble fraction of rats administered cadmium with or without these chelating agents indicated that the chelating agents significantly depressed the binding of cadmium to a high molecular weight protein of liver tissue. The cumulative biliary excretion of DL-penicillamine and citric acid in a 9 h period after administration were about 1% and about 0.04% of the dose, respectively. It is suggested that the stimulatory effect of DL-penicillamine on the biliary excretion of cadmium was due to the formation of DL-penicillamine–cadmium complex, and that citric acid depressed the binding of cadmium to high molecular protein in the liver cytosol, resulting in enhanced biliary excretion of cadmium.

Keywords——cadmium; biliary excretion; DL-penicillamine; citric acid; rat

Cadmium administered to animals is predominantly accumulated in the liver, kidney, and intestine[2-4] and most of the excreted cadmium is found in the feces.[4-7] It has been suggested that biliary excretion of cadmium may be important after a single high-dose exposure.[8-12] Recently, we reported that a large portion of cadmium administered intraperitoneally to rats was excreted in the gastrointestinal lumen through the bile and gastrointestinal mucosa, and that an intravenous administration of chelating agents, such as citric acid, DL-penicillamine, and D-cysteine, stimulated the biliary excretion of cadmium.[13] Further, Cherian reported that when various chelating agents were administered intraperitoneally 30 min after cadmium injection, the thiol-containing agents, such as 2,3-dimercapto-1-propanol, DL-penicillamine, *N*-acetylpenicillamine, and dithioerythritol, stimulated the biliary excretion of cadmium.[14]

The present study was undertaken to clarify the mechanism of the stimulatory effects of DL-penicillamine and citric acid on the biliary excretion of cadmium in rats.

Experimental

Materials and Equipment——Cadmium chloride and citric acid were obtained from Wako Pure Chemical Ind., Osaka. DL-Penicillamine was obtained from Tokyo Kasei Kogyo Co., Ltd., Tokyo. Penicillaminedisulfide was prepared from DL-penicillamine according to the method of Butenandt *et al.*[15] [1,5-^{14}C]Citric acid was obtained from The Radiochemical Centre Ltd., Amersham, Buckinghamshire, England. PCS™ liquid scintilation cocktail was purchased from Amersham Corporation, Arlington Heights, Ill., U.S.A. and Sephadex G-75 was obtained from Pharmacia Fine Chemicals, Uppsala, Sweden. Cellulose plates, 0.1 mm thick, were obtained from E. Merck AG, Darmstadt, Germany. All other chemicals were of reagent grade.

A Shimadzu AA-610S atomic absorption spectrophotometer, a Shimadzu QV-50 spectrophotometer, a Hitachi-Horiba F-7 pH meter, a Hitachi KLA-3B amino acid analyzer, and an Aloka LSC-502 liquid scintillation counter were utilized.

Test Animal——Male Wister rats weighing 180—240 g were used for the experiments. They were fasted for about 20 h with drinking water *ad libitum* prior to the experiments.

Experimental Procedures——Rats were anesthetized with urethane (1 g/kg, *i.p.*) and the bile duct was cannulated with polyethylene tubing (PE 10) as described previously.[13] Cadmium chloride and chelating agents administered to rats were dissolved in 0.9% NaCl solution. Cadmium chloride (3 mg Cd/kg) was administered intraperitoneally to rats. Chelating agents (60—120 mg/kg) were administered intravenously to rats from the femoral vein immediately after the administration of cadmium. Bile samples were collected for an experimental period of 9 h.

Analytical Procedures——1) DL-Penicillamine: DL-Penicillamine in the bile was oxidized to penicillaminedisulfide by a modification of the method of Butenandt *et al.*[15]; *trans*-1,2-cyclohexanediamine-*N*,*N*,*N'*,*N'*-tetraacetic acid (14 mg) was added to the bile containing DL-penicillamine and the pH was adjusted to 10 with 1 N NaOH. DL-Penicillamine was oxidized to penicillaminedisulfide by bubbling O_2 gas through the mixture for about 9 h. The oxidized products contained D-, L- and DL-forms of penicillaminedisulfide. The qualitative and quantitative analyses of penicillaminedisulfide were performed on an amino acid analyzer using a 0.9×50 cm column of globular resin #3105 and sodium citrate buffer (0.2 N Na^+, pH 3.34) according to the amino acid analysis method of Moore and Stein.[16]

2) Citric Acid: The bile from rats injected with citric acid containing [1,5-^{14}C]citric acid (2 μCi) was acidified with concentrated HCl and centrifuged for 15 min at $900 \times g$. The supernatant was chromatographed on a Dowex 50 (H^+) column (1.2×3 cm). The resulting eluate was concentrated to a small volume and subjected to cellulose thin-layer chromatography. Cellulose was scraped off the plate in 1 cm sections and extracted with PCS™ scintillation fluid. Then the ^{14}C radioactivity of each portion was determined by using a liquid scintillation counter, and a histogram was plotted to locate the ^{14}C peaks on the plate.

3) Cadmium: The determination of cadmium was carried out by the procedure reported previously.[17]

Gel Filtration of Liver Soluble Fraction——Liver was homogenized in 3 volumes of chilled Tris-HCl buffer (0.01 M Tris-HCl, 0.05 M NaCl, pH 8.0), using a glass-Teflon homogenizer. The homogenate was centrifuged at $10000 \times g$ for 30 min at 4 °C to remove nuclei and mitochondria. The postmitochondrial supernatant was further centrifuged at $102000 \times g$ for 1 h. An aliquot (0.5 ml) of the supernatant was applied to a Sephadex G-75 column (1.5×41 cm). The column was eluted with Tris-HCl buffer at a flow rate of 3.5—4.0 ml/h at 4 °C and the effluent was collected in 2 ml fractions. The absorbance at 280 nm and the concentration of cadmium were determined.

Results and Discussion

Mechanism of Stimulatory Effect of DL-Penicillamine on Biliary Excretion of Cadmium

In order to elucidate the behavior of cadmium in the liver in relation to the stimulated biliary excretion of cadmium, the effect of DL-penicillamine on the accumulation and binding of cadmium in the liver tissues was investigated by fractionating liver soluble supernatant of rats given cadmium with and without DL-penicillamine on a Sephadex G-75 column. Representative elution profiles of liver supernatant of rats 4 h after injection of cadmium alone or both cadmium and DL-penicillamine are shown in Fig. 1. Cadmium content in the liver soluble fraction and the amounts of cadmium in fractions I and II are listed in Table I. DL-Penicillamine tended to decrease the cadmium content in the liver supernatant. The

Table I. Distribution of Cadmium in Soluble Fraction Components of Rat Liver 4 h after Administration of Cadmium Chloride with and without Chelating Agents

	Cadmium content of soluble fraction (μg/0.5 ml)	Cadmium distribution (μg)[a] Fraction	
		I	II
Cadmium alone	5.47 ± 0.41	1.83 ± 0.25	3.48 ± 0.26
Cadmium + DL-penicillamine	4.80 ± 0.40	0.85 ± 0.22[b]	3.71 ± 0.57
Cadmium + citric acid	4.87 ± 0.44	1.23 ± 0.17[c]	3.48 ± 0.36

Doses of cadmium and chelating agents were cadmium chloride 3 mg Cd/kg, DL-penicillamine 120 mg/kg, and citric acid 60 mg/kg.
The values are the means for 3 to 5 animals.
a) One-half ml was subjected to gel filtration.
Significantly different from cadmium alone, *b*) $p < 0.01$; *c*) $p < 0.05$.

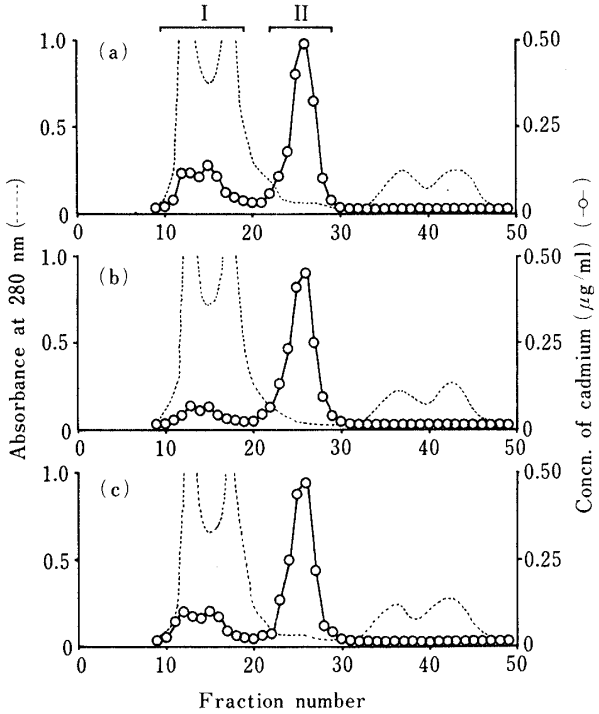

Fig. 1. Sephadex G-75 Chromatographic Profile of Rat Liver Soluble Fraction 4 h after Administration of Cadmium with and without Chelating Agents

Rats were given (a) cadmium (3 mg/kg), (b) cadmium (3 mg/kg) and DL-penicillamine (120 mg/kg), (c) cadmium (3 mg/kg) and citric acid (60 mg/kg).

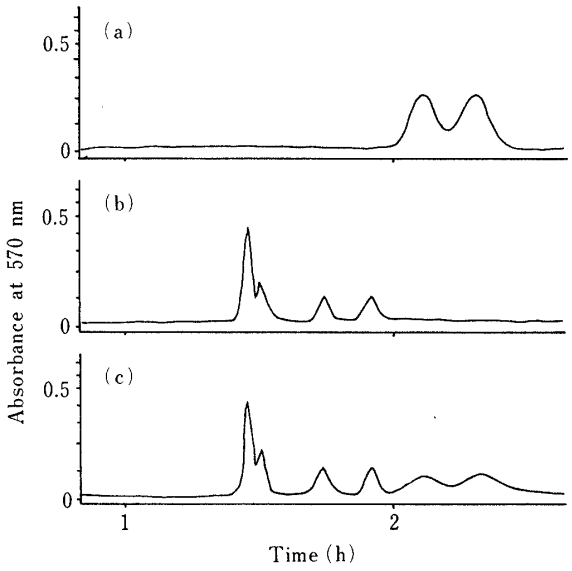

Fig. 2. Analysis of DL-Penicillamine in Rat Bile, Using an Amino Acid Analyzer

(a) authentic penicillaminedisulfide, (b) normal rat bile (treated with O_2 gas), (c) bile of rat given DL-penicillamine (120 mg/kg) with cadmium (3 mg/kg) (treated with O_2 gas).

amount of cadmium bound to a low molecular weight protein (fraction II) corresponding to metallothionein,[18] which has a high binding affinity for cadmium, was little influenced by the simultaneous administration of DL-penicillamine. However, this chelating agent significantly depressed the binding of cadmium to a high molecular weight protein (fraction I), which probably has a lower binding affinity for cadmium.

Our previous study[13] suggested that cadmium in the bile of rats given cadmium in combination with chelating agents, such as DL-penicillamine and citric acid, is largely bound to substance with low molecular weight. A qualitative experiment was performed to examine whether DL-penicillamine administered intravenously to rats is excreted into the bile. As described in the experimental section, the bile of a rat given an intravenous injection of DL-penicillamine was treated with O_2 gas and applied to an amino acid analyzer. As shown in Fig. 2, two peaks of penicillaminedisulfides consisting of D-, L- and DL-forms, were detected on the chromatogram. This result shows that a portion of administered DL-penicillamine is excreted into the bile.

Furthermore, the biliary excretion of cadmium and DL-penicillamine was investigated after simultaneous administration of these compounds (Table II). The biliary excretion of cadmium was remarkably enhanced by the use of DL-penicillamine, as reported previously.[19] The cumulative biliary excretion of DL-penicillamine in a 9 h period after administration was 1% of the dose, and was extremly large as compared with that after the administration of DL-penicillamine alone. In addition, the molar ratio of DL-penicillamine to cadmium excreted into the bile was 2.76 and thus, such an amount of DL-penicillamine was sufficient for the formation of a stable DL-penicillamine–cadmium complex, which is known to have a ligand-to-metal molar ratio of 1 : 1.[19,20] These results suggest that cadmium in the bile of rats given

TABLE II. Biliary Excretion of Cadmium or Chelating Agents in Rats Given Cadmium, Chelating Agents or Both Cadmium and Chelating Agents

	Biliary excretion (% of dose)/9 h[a)]		(Molar ratio Chelating agent/ cadmium)
	Cadmium	Chelating agent	
Cadmium alone	0.95±0.25	—	—
DL-Penicillamine alone	—	0.17±0.09	—
Cadmium+DL-penicillamine	10.94±2.84	1.00±0.23	2.76±0.83
Citric acid alone	—	0.035±0.018	—
Cadmium+citric acid	4.57±0.78	0.042±0.012	0.11±0.03

Doses of cadmium and chelating agents were cadmium chloride 3 mg/kg, DL-penicillamine 120 mg/kg, and citric acid 60 mg/kg.
a) The values are the means±standard deviation for 3 to 6 animals.

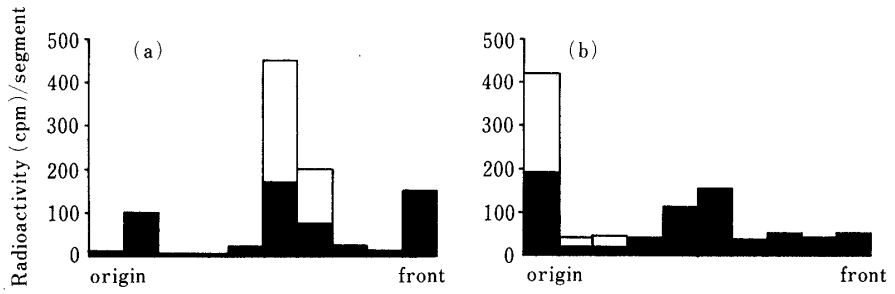

Fig. 3. Thin-layer Chromatography of Rat Bile after Administration of ^{14}C-Citric Acid with Cadmium

Plate: cellulose.
Solvent system:
(a) AcOH–HCOOH–H_2O (5:1:1); (b) EtOH–28% NH_4OH–H_2O (80:4:16).
■, bile of rat given ^{14}C-citric acid with cadmium; □, authentic ^{14}C-citric acid.

cadmium together with DL-penicillamine is largely bound to DL-penicillamine.

These results suggest that DL-penicillamine depressed the binding of cadmium to a high molecular weight protein of liver tissue, and that cadmium is excreted into the bile in the form of a DL-penicillamine–cadmium complex, resulting in the enhanced biliary excretion of the metal.

Mechanism of Stimulatory Effect of Citric Acid on Biliary Excretion of Cadmium

As in the case of DL-penicillamine described above, the liver soluble fraction of rats injected with cadmium together with citric acid was chromatographed on a Sephadex G-75 column (Fig. 1). As shown in Table I, the amount of cadmium bound to a low molecular weight fraction (fraction II) was little influenced by the administration of citric acid. However, citric acid significantly depressed the binding of cadmium to a high molecular weight protein (fraction I).

Furthermore, the bile of rats simultaneously given cadmium and citric acid containing ^{14}C-citric acid was examined by using a Dowex 50 (H^+) column as described in the experimental section, and was also subjected to cellulose thin-layer chromatography. As shown in Fig. 3, the distribution pattern of ^{14}C-radioactivity detected on the chromatogram suggests the presence of citric acid in the bile. However, the cumulative biliary excretion of citric acid in a 9 h period after administration was only about 0.04% of the dose and was much less than that of cadmium; the citric acid-to-cadmium molar ratio was 0.11 : 1 (Table II). Thus

it is suggested that only a portion of cadmium can be excreted in the bile in the form of cadmium–citrate complex. In addition, Cherian and Vostal[11] recently reported that a main cadmium-binding compound in the bile of rat administered cadmium was glutathione.

From these results, the stimulatory effect of citric acid on the biliary excretion of cadmium seems to be due to the inhibitory action of citric acid on the binding of cadmium to a high molecular weight protein in the liver cytosol. Cadmium may then be mainly excreted in the bile as cadmium–citrate complex and cadmium–glutathione complex.

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