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Protective Effect of Molybdenum on the Acute Toxicity of Mercuric Chloride. II.

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The protective mechanism of Na_2MoO_4 against the acute toxicity of $HgCl_2$ was studied by investigating the subcellular distributions of mercury and molybdenum in the liver and kidney of rats treated with either Na_2MoO_4 or $HgCl_2$ or both, and by performing Sephadex G-75 gel chromatography of the liver and kidney cytosol obtained from these groups.

The rats given $HgCl_2$ (0.03 mmol/kg, once, *s.c.*) after pretreatment with Na_2MoO_4 (1.24 mmol/kg, once a day for 3 d, *i.p.*) has decreased the mercury contents in the liver mitochondrial and microsomal fractions and in the renal nuclear fraction, in comparison with those of the rats given $HgCl_2$ alone. However, the mercury content in the renal cytosol of this group was increased in comparison with that of the rats given $HgCl_2$ alone. A gel filtration study showed that the increase in mercury content of the renal cytosol of the rats pretreated with Na_2MoO_4 was associated with an increase in the metal content in the metallothionein-like fraction.

These results suggest that molybdenum alleviated the acute $HgCl_2$ toxicity not only by reducing the mercury contents in the mitochondrial and microsomal fractions of liver and in the nuclear fraction of kidney, but also by enhancing the retention of mercury in the renal metallothionein-like fraction.

Keywords——acute toxicity; mercuric chloride; protective effect; sodium molybdate; metallothionein

Molybdenum is an important factor affecting copper metabolism in animals.[1] Several mechanisms for this interaction have been reported,[2] but the detailed mechanism is not known. Recent investigations concerning molybdenum–copper interaction suggest that the formation of tetrathiomolybdate anion, decreasing the concentration of available copper in the plasma, liver and kidney and affecting the subcellular distribution of the metal in the kidney, is a causative factor in the subsequent impediment of copper metabolism.[3,4]

On the other hand, we recently found that the acute toxicity of $HgCl_2$ in rats was alleviated by pretreatment of the rats with Na_2MoO_4 and that such a suppressive effect appeared as a result of the reduction in mercury contents in the liver, kidney and spleen.[5] This decrease in the mercury contents of these tissues was not due to inhibition of absorption of mercury but was due to enhancement of urinary excretion of the metal. However, the mechanism of Na_2MoO_4 protection against the acute toxicity of $HgCl_2$ is unclear at present.

In the present study, therefore, we investigated the subcellular distributions of mercury and molybdenum in the liver and kidney after exposure to $HgCl_2$, and conducted Sephadex G-75 gel chromatography of the cytosol obtained from these tissues, in order to check the hypothesis that molybdenum may affect the metabolism of mercury at the cellular level, as in the case of copper.

Materials and Methods

All reagents were of high analytical grade. Calibration proteins for gel chromatography were purchased from

Boehringer Mannheim Yamanouchi. Sephadex G-75 was obtained from Pharmacia Japan. Male Wister strain rats (Matumoto Labo-Animals Laboratory) were fed a commercial solid diet (CE-2, Clea Japan) and were given water *ad libitum*. The average body weight of the rats used in this study was 170 g.

Subcellular Distributions of Mercury and Molybdenum in the Liver and Kidney——A total of 160 rats was randomly divided into four treatment groups of 40 rats each. Group 1 was not treated. Group 2 received *i.p.* injection of Na_2MoO_4 dissolved in saline at a dose of 1.24 mmol/kg once a day for 3 d. Group 3 was given one *s.c.* injection of $HgCl_2$ dissolved in saline at a dose of 0.03 mmol/kg. Group 4 received the same dose of $HgCl_2$ as group 3, 24 h after the final *i.p.* injection of Na_2MoO_4 as in group 2. Twenty rats from each group were sacrificed by cervical dislocation 24 of 40—48 h after exposure to $HgCl_2$ and the liver (only at the latter time point) and kidney were perfused with 1.15% KCl. Then the tissues were rapidly removed, rinsed with the same solution and blotted. The tissues were weighed and homogenized immediately with 5 vol of iced 1.15% KCl in a Waring blender. The homogenates were fractionated into four subcellular fractions by centrifugation according to the method of Schneider and Hogeboom[6] After centrifugation, mercury and molybdenum present in the respective subcellular fractions were independently determined by using the preparations from liver and kidney of ten rats.

Sephadex G-75 Gel Chromatography of Liver and Kidney Cytosol——Three groups of 3 rats were treated with either Na_2MoO_4 or $HgCl_2$ or both as in the above experiment, and were sacrificed by cervical dislocation 40—48 h after exposure to $HgCl_2$. After perfusion of the liver and kidney, a soluble fraction was prepared as in the above experiment. A 5 ml portion of the soluble fraction from liver or kidney was applied to a Sephadex G-75 column (2 × 70 cm) equilibrated with 0.02 M Tris-HCl buffer (pH 8.6) at 4 °C. The cytosol was eluted with the same buffer at a flow rate of 0.23 ml/min and 4 ml of column effluent per tube was collected. Absorbance at 280 nm was monitored for determining relative protein concentration in column effluents. The following molecular weight standards were used to calibrate the column: ferritin, 450000; aldolase, 158000; albumin, 68000; cytochrome c, 12500.

Determination of Mercury and Molybdenum——Mercury found in the subcellular fractions and the column effluents was determined by flameless atomic absorption spectrophotometry[7] after digesting the samples in sulfuric–nitric acid.[8]

Molybdenum found in the subcellular fractions was determined by a Hitachi 170-50A atomic absorption spectrophotometer equipped with a graphite atomizer (GA-2) according to the method of Cardenas and Mortenson.[9] Molybdenum found in the column effluents was determined according to the method of Gary and Gaston.[10]

Results

Table I shows the subcellular distribution of mercury in the liver 48 h after exposure to $HgCl_2$. At this time point, there was no difference in distribution pattern of mercury between group 3, the Hg-dosed group, and group 4, the Mo–Hg-dosed group. However, the mercury

TABLE I. Subcellular Distribution of Mercury in the Liver of Rats Given Mercuric Chloride with or without Sodium Molybdate Pretreatment

	Treatment[a] and dose (mmol/kg)	Mercury content (μg/g wet liver) in				
		Homogenate	Nuclei and debris	Mitochondria	Microsomes	Supernatant
Group 1	(No treatment)	N.D[c]	N.D	N.D	N.D	N.D
Group 2	Na_2MoO_4 (1.24)	N.D	N.D	N.D	N.D	N.D
Group 3	$HgCl_2$ (0.03)	8.85 ± 0.24	2.33 ± 0.32	1.69 ± 0.15	1.18 ± 0.11	3.08 ± 0.20
Group 4	Na_2MoO_4 (1.24) + $HgCl_2$ (0.03)	6.18 ± 0.25[b]	1.98 ± 0.25	1.10 ± 0.09[b]	0.74 ± 0.07[b]	2.22 ± 0.37

a) Group 1 was not treated. Group 2 received *i.p.* injection of sodium molybdate dissolved in saline at a dose of 1.24 mmol/kg once a day for 3 d. Group 3 was given one *s.c.* injection of mercuric chloride dissolved in saline at a dose of 0.03 mmol/kg. Group 4 received the same dose of mercuric chloride as group 3, 24 h after the final *i.p.* injection of sodium molybdate as in group 2. The rats were killed by cervical dislocation 40—48 h after exposure to $HgCl_2$. Each value represents the mean ± S.E. from ten rats.
b) $p < 0.01$. Significant differences from group 3.
c) N.D. Not detected (determination limit; 0.01 μg/g wet liver).

TABLE II. Subcellular Distribution of Mercury in the Kidney of Rats Given Mercuric Chloride with or without Sodium Molybdate Pretreatment

	Treatment[a] and dose (mmol/kg)	Time (h)	Mercury content (μg/g wet kidney) in				
			Homogenate	Nuclei and debris	Mitochondria	Microsomes	Supernatant
Group 1	(No treatment)	24	N.D[d]	N.D	N.D	N.D	N.D
		48	N.D	N.D	N.D	N.D	N.D
Group 2	Na_2MoO_4 (1.24)	24	N.D	N.D	N.D	N.D	N.D
		48	N.D	N.D	N.D	N.D	N.D
Group 3	$HgCl_2$ (0.03)	24	79.72±0.98	51.90±2.33	12.26±0.82	4.76±0.15	13.69±0.35
		48	53.14±0.95	24.78±0.86	6.57±0.95	5.68±0.45	16.77±1.15
Group 4	$Na_2MoO_4+HgCl_2$ (1.24) (0.03)	24	71.22±0.61[b]	31.67±0.68[b]	11.97±1.02	5.58±0.50	20.31±1.39[b]
		48	49.13±0.43[c]	15.33±0.60[b]	5.28±0.38	5.20±0.26	25.44±1.21[b]

a) The rats were treated with molybdenum and mercury as in Table I and were killed by cervical dislocation 24 and 40—48 h after exposure to mercuric chloride.
Each value represents the mean±S.E. from ten rats.
b) $p<0.01$. *c*) $p<0.05$. Significant differences from group 3.
d) N.D. Not detected (determination limit; 0.01 μg/g wet kidney).

TABLE III. Subcellular Distribution of Molybdenum in the Kidney of Rats after Administration of Mercuric Chloride

	Treatment[a] and dose (mmol/kg)	Time (h)	Molybdenum content (μg/g wet kidney) in				
			Homogenate	Nuclei and debris	Mitochondria	Microsomes	Supernatant
Group 1	(No treatment)	24	N.D[c]	N.D	N.D	N.D	N.D
		48	N.D	N.D	N.D	N.D	N.D
Group 2	Na_2MoO_4 (1.24)	24	2.37±0.10	1.30±0.04	N.D	N.D	0.82±0.03
		48	1.91±0.07	0.60±0.06	N.D	N.D	1.00±0.13
Group 3	$HgCl_2$ (0.03)	24	N.D	N.D	N.D	N.D	N.D
		48	N.D	N.D	N.D	N.D	N.D
Group 4	$Na_2MoO_4+HgCl_2$ (1.24) (0.03)	24	3.90±0.09[b]	1.80±0.38	N.D	N.D	1.84±0.16[b]
		48	3.36±0.05[b]	0.83±0.14	N.D	N.D	2.35±0.27[b]

a) The rats were treated with molybdenum and mercury as in Table I and were killed by cervical dislocation 24 and 40—48 h after exposure to $HgCl_2$. Each value represents the mean±S.E. from ten rats.
b) $p<0.01$. Significant differences from group 2.
c) N.D. Not detected (determination limit; 0.50 μg/g wet kidney).

content in the mitochondrial and microsomal fractions was significantly decreased in the Mo–Hg-dosed group as compared with the Hg-dosed group. On the other hand, the mercury content in subcellular fractions of liver from the rats in group 1, the no-treatment group, and the rats in group 2, the Mo-dosed group, were not estimated because they were too low for determination by the analytical method used in this study. In these groups, the mercury contents in the subcellular fractions from the kidney were also not estimated at any time points for the same reason.

Table II shows the subcellular distribution of mercury in the kidney 24 and 48 h after administration of $HgCl_2$. When mercury contents in corresponding subcellular fractions were

compared between the Hg-dosed and Mo–Hg-dosed groups at each time point, the metal content in the nuclear fraction was decreased in the Mo-Hg-dosed group as compared with the Hg-dosed group at both time points, but the metal content in the soluble fraction was rather increased at both time points. These results suggest that pretreatment with Na_2MoO_4 affected the subcellular distribution of mercury in the kidney.

The subcellular distribution of molybdenum in the liver and kidney after exposure to $HgCl_2$ was investigated. In the no-treatment and Hg-dosed group, the molybdenum contents were not exactly estimated in any fractions from the tissues, because they were too low for determination by the analytical method used in this study. Appreciable amounts of molybdenum were found only in the nuclear and soluble fractions of kidney obtained from Mo-dosed and Mo–Hg-dosed rats, 24 and 48 h after exposure to $HgCl_2$. The results are shown in Table III. The data indicate that molybdenum content in the soluble fraction was consistently increased in the Mo–Hg-dosed group as compare with the Mo-dosed group at both time points. Molybdenum content in the nuclear fraction was also increased to some extent in the Mo–Hg-dosed group, although there was no statistical difference in the metal content of this fraction between these groups.

Figures 1 and 2, respectively, show Sephadex G-75 gel filtration profiles of mercury from the liver and kidney cytosols obtained from Hg-dosed and Mo-Hg-dosed rats at 48 h after exposure to $HgCl_2$. Two mercury-binding fractions were found in both the liver and kidney cytosols of these groups. The first mercury-binding fraction in the liver cytosol was eluted in a high molecular weight fraction near a ferritin with a molecular weight of 450000 (tube number

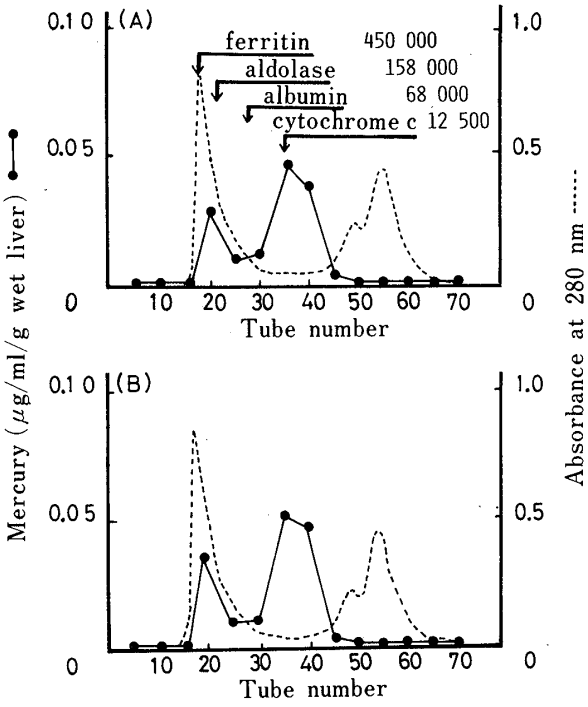

Fig. 1. Sephadex G-75 Gel Filtration Profiles of Mercury from the Liver Supernatant of Hg-Dosed (A) and Mo–Hg-Dosed (B) Rats

The 2 × 70 cm Sephadex G-75 column was eluted at 4 °C with 0.02 M Tris-HCl buffer (pH 8.6) at a flow rate of 0.23 ml/min and 4 ml of column effluent per tube was collected. Absorbance at 280 nm was monitored for determining relative protein concentration in each column effluent, then five consecutive collection tubes were combined for the determination of mercury found in the column effluent.

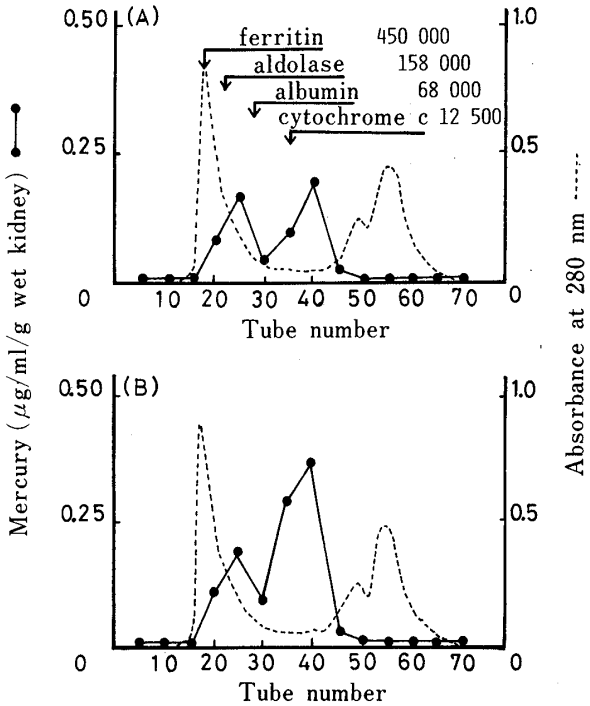

Fig. 2. Sephadex G-75 Gel Filtration Profiles of Mercury from the Kidney Supernatant of Hg-Dosed (A) and Mo–Hg-Dosed (B) Rats

The column size and conditions were the same as in Fig. 1. Absorbance at 280 nm was monitored for determining relative protein concentration in each column effluent, then five consecutive collection tube were combined for the determination of mercury found in the column effluent.

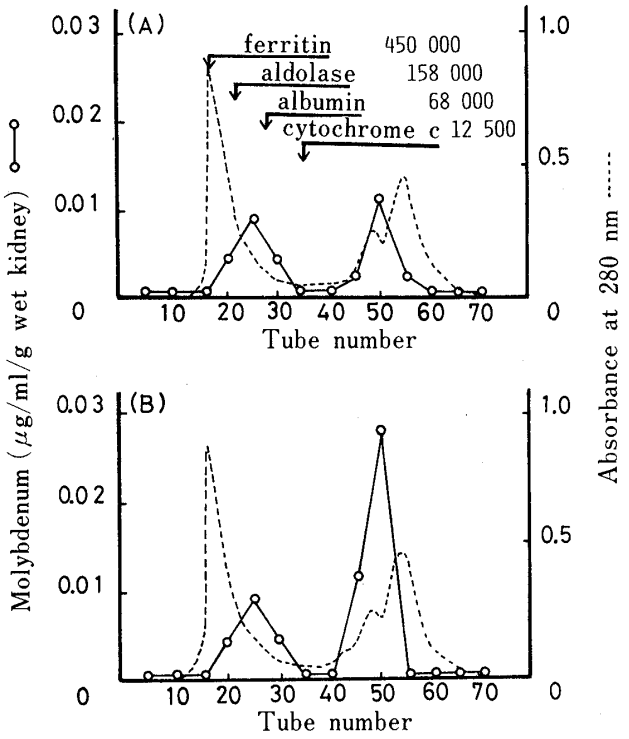

Fig. 3. Sephadex G-75 Gel Filtration Profiles of Molybdenum from the Kidney Supernatant of Mo-Dosed (A) and Mo–Hg-Dosed (B) Rats

The column size and conditions were the same as in Fig. 1. Absorbance at 280 nm was monitored for determining relative protein concentration in each column effluent, then five consecutive collection tubes were combined for the determination of molybdenum found in the column effluent.

18) and that in the kidney cytosol was eluted in a high molecular weight fraction near an aldolase with a molecular weight of 158000 (tube number 22). The second mercury-binding fraction of both organs had gel chromatographically almost identical elution positions, namely they were both eluted in a low molecular weight fraction near a cytochrome c with a molecular weight of 12500 (tube number 36). The low molecular mercury binding fraction was considered to be metallothionein-like fraction in view of the lack of absorption at 280 nm. There was no significant difference as regards mercury content between the two mercury-binding fractions of liver cytosol in both the Hg-dosed and Mo–Hg-dosed groups. In the case of kidney cytosol, however, a remarkable difference with respect to mercury content in the metallothionein-like fraction was observed between the groups, namely the mercury content of this fraction was considerably increased in the Mo–Hg-dosed group as compared with the Hg-dosed group.

Figure 3 shows Sephadex G-75 gel filtration profiles of molybdenum from the renal cytosol of Mo-dosed and Mo–Hg-dosed rats, 48 h after exposure to $HgCl_2$. Two molybdenum-binding fractions were found in the renal cytosol from these groups. The first molybdenum-binding fraction was eluted in a high molecular weight fraction near-an-aldolase. The second molybdenum-binding fraction was eluted in a lower molecular weight fraction than metallothionein-like protein, but the exact molecular weight of the fraction was not determined. When the molybdenum contents in corresponding fractions were compared between the Mo-dosed and Mo–Hg-dosed groups, the metal content in the low molecular weight fraction was consistently higher in the Mo–Hg-dosed group than in the Mo-dosed group.

Discussion

The present study showed that the pretreatment of rats with Na_2MoO_4 reduced the mercury content in their mitochondrial and microsomal fractions of liver, and such pretreatment with Na_2MoO_4 also decreased the mercury content in their renal nuclear fraction but rather enhanced the retention of the metal in the cytosol of the tissue.

The results on the subcellular distribution of molybdenum showed that molybdenum content in the renal cytosol was also increased in Mo–Hg-dosed rats as compared with Mo-dosed rats.

The gel filtration study demonstrated that mercury and molybdenum were independently increased in the renal cytosol of Mo–Hg-dosed rats, namely mercury was increased in the metallothionein-like fraction and molybdenum was increased in a lower molecular weight fraction than the metallothionein-like fraction.

These results suggest that molybdenum did not alter the subcellular distribution of mercury in the liver and kidney by directly interacting with mercury. Certainly, a change in subcellular distribution of mercury connected with the pretreatment with Na_2MoO_4 was observed in the kidney of Mo–Hg-dosed rats, but this was a metallothionein-associated change. It is known that metallothionein affects the subcellular distribution of mercury in the kidney.[11] Therefore, the possibility that molybdenum alleviated the acute $HgCl_2$ toxicity by modifying the subcellular distribution of mercury in the liver and kidney through direct interaction with mercury may be ruled out.

Thus, the increase in mercury content of the renal metallothionein-like fraction of the rats pretreated with Na_2MoO_4 suggests that metallothionein may be involved in the mechanism of protective action of Na_2MoO_4 against the acute $HgCl_2$ toxicity. The low molecular weight fraction has not yet been identified, but possessed several properties in common with metallothionein. Namely, the low molecular weight fraction had almost the same molecular weight as cytochrome c with a molecular weight of 12500 and did not show absorption at 280 nm (results of Sephadex G-75 gel chromatography). The apparent molecular weight of metallothionein on gel chromatography is approximately 10000, and this protein does not show absorption at 280 nm.[12] It is also known that metallothionein is induced by various heavy metals, such as mercury,[13] cadmium[14] and zinc.[15]

We have no explanation for the increase in mercury content of the renal metallothionein-like fraction of Mo–Hg-dosed rats, but this increase suggests that molybdenum affects the rate of biosynthesis or degradation of metallothionein-like protein, or the binding ratio of mercury and metallothionein-like protein. These problems remain to be investigated. However, it is generally accepted that metallothionein is involved in the detoxification process of toxic heavy metals, such as mercury and cadmium.[16]

In conclusion, it is considered that molybdenum alleviated the acute $HgCl_2$ toxicity not only by reducing the mercury content in the mitochondrial and microsomal fractions of liver and in the nuclear fractions of kidney, but also by enhancing the retention of mercury in the renal metallothionein-like fraction.

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