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# INDUCTION OF METALLOTHIONEIN IN LIVER AND CHANGES OF ESSENTIAL METAL LEVELS IN SELECTED TISSUES BY THREE DEXTRAN DERIVATIVES

TAMIO MAITANI* and KAZUO T. SUZUKI
National Institute for Environmental Studies, Yatabe, Tsukuba, Ibaraki 305, Japan

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**Abstract**—Three dextran derivatives (dextran sulfate, DEAE-dextran and Dextran T500) were injected intraperitoneally into mice to study the induction of zinc–thionein in the liver, and the changes of essential metal levels in the liver, kidneys and spleen. The former was investigated with a high performance liquid chromatograph equipped with an atomic absorption spectrophotometer, and the latter with an inductively coupled plasma–atomic emission spectrometer. Dextran sulfate and DEAE-dextran induced a large amount of hepatic zinc–thionein dose-dependently, while Dextran T500 did not induce metallothionein substantially at the doses studied 20, 40 and 60 mg/kg body wt). The injection of dextran sulfate resulted in a significant increase of spleen weight, with a transitory increase of calcium concentration in the three tissues and a significant decrease of iron level in the spleen. In accord with the induction of zinc–thionein by dextran sulfate and DEAE-dextran, zinc concentration in the liver also increased transitorily after both injections and the time-course of the hepatic zinc level in dextran sulfate showed a close resemblance to that of the calcium one.

For the past ten years, there has been considerable interest in a peculiar metalloprotein, metallothionein (MT) [1]. This low molecular weight protein is characterized by its high cysteine content (about 30%), its complete absence of aromatic amino acids, and can be induced always as a mixture of two isometallothioneins (MT-I and MT-II) in mammals. The protein has seven metal-binding sites for zinc and cadmium, but has no definite metal composition and hence no enzymatic activity (metalloenzyme has, at least at active sites, the definite metal composition). However, MT appears to have important roles in zinc metabolism and protection from toxic metals, though the reasons for its existence have not been definitely established. In recent years in particular, a growing interest has been taken in the former role (zinc metabolism) because MT (zinc–thionein, Zn–Th) is present at a much higher level in pre- and neonatal livers than in adult livers [2–5], and can be induced by stress [6], bacterial infections [7], food restriction [8] and injections of many organic chemicals [9–14].

In the previous paper [13], we reported the induction of MT and the produced changes of essential metal levels in tissues by the administration of carrageenan, a sulfated galactan, in connection with inflammation [15]. We have been prompted to investigate further the correlation between the structure of organic chemicals and the inducibility of MT. We chose three dextran derivatives with the same average molecular weight [dextran sulfate (DS), DEAE-dextran (DE) and Dextran T500 (DT)] as the compounds to be studied because of the following reasons. (1) DS bears a structural resemblance to carrageenan. Both compounds are sulfated polysaccharides and, as viewed at a different standpoint, are polyanions. (2) The oppositely charged polycation is also commercially available as DE. This is advantageous to study the relationship between MT-induction and electric charge. (3) The biological properties of the compounds can be well investigated both *in vitro* and *in vivo* [16–22].

MT level was determined by a high performance liquid chromatograph (equipped with a gel permeation column) with an atomic absorption spectrophotometer as a detector (HPLC–AAS) [23]. The produced changes of several essential metal levels in tissues were measured by an inductively coupled plasma–atomic emission spectrometer (ICP–AES) [24].

## MATERIALS AND METHODS

*Injection to animals.* DS, DE and DT (mol. wt = 500,000 for all derivatives) were purchased from Pharmacia Fine Chemicals (Uppsala, Sweden) and used without further purification. Since the commercially obtained DS contains 0.5–2.0% phosphate (0.24% phosphorus by ICP–AES), all compounds were dissolved in phosphate-buffered saline (PBS), pH 7.4.

Experiment 1: 54 male ICR mice (JCL, Clea Japan, Tokyo; 6–7 weeks old; body wt ± S.D., 36.2 ± 1.3 g) were injected intraperitoneally (i.p.) with three dextran derivatives at three doses (20, 40, 60 mg/kg body wt, six mice/group) and killed 24 hr after the injection by cardiac puncture under ether anaesthesia. Control mice were killed 24 hr after the injection of PBS.

Experiment 2: 42 male ICR mice (JCL, Clea Japan, Tokyo; 5 weeks old; body wt ± S.D., 30.8 ± 1.3 g) were injected i.p. with DS at a dose of 40 mg/kg body wt and sacrificed after 8 and 16 hr, 1, 2, 3, 4 and 7 days after the injection. Control mice

* To whom correspondence should be addressed.

were given PBS and killed one day after the injection.

The liver, kidneys and spleen were excised and washed in chilled isotonic sucrose solution.

*Determination of essential metal concentration.* The liver (about 0.4 g portion), both kidneys and whole spleen from each mouse were digested with 1 or 0.5 ml of mixed acid ($HNO_3$:$HClO_4$ = 5:1, v/v) and the solutions were diluted to 10, 10 and 5 ml, respectively, with twice distilled water. The concentrations of several essential metal levels were determined simultaneously by ICP–AES (Jarrell–Ash Model 975 Plasma Atomcomp) with an autosampler. Experimental conditions for ICP–AES were: incident power, 1.1 kW; reflected power, ≈5 W; sample intake rate, 1.1 ml/min; viewing height above coil, 19 mm, coolant gas flow rate, 20 l/min; sample gas flow rate, 0.6 l/min; preburn, 1 sec; exposure, 10 sec.

*Preparation of supernatant for HPLC–AAS.* The livers (about 0.4 g portion) from mice were combined in each group and homogenized in 3 vol. of 0.1 M Tris–HCl buffer solution (pH 7.4, 0.25 M glucose) with a Polytron homogenizer under iced-water cooling in an atmosphere of nitrogen. The homogenates were centrifuged at 170,000 *g* for 60 min at 2°. To detect the induced Zn–Th as Cd–Th [13], cadmium acetate solution (1000 ppm, 10 μl) was added to a 300 μl portion of each liver supernatant and the excess cadmium was removed as Cd-containing denatured high molecular weight proteins by heat treatment (80°, 5 min) and centrifugation (10,000 *g*, 1 min).

*Determination of induced MT by HPLC–AAS.* A 100 μl portion of the Cd-replaced liver supernatant fraction was applied to HPLC [Toyo Soda HLC 803A equipped with a gel permeation column (TSK GEL SW 3000 column, Toyo Soda, 7.5 × 600 mm with a precolumn, 7.5 × 75 mm)], and the column was eluted with 50 mM Tris–HCl buffer solution (pH 8.0 at 25°) at a flow rate of 1 ml/min, and the cadmium level of the eluate was continuously monitored by a flame atomic absorption spectrophotometer (Hitachi 170-50A). The quantity of induced MT was calculated from the relative peak area on the chromatogram to a standard Cd–Th solution.

## RESULTS

Three dextran derivatives were injected i.p. and the amount of induced hepatic Zn–Th was compared by using HPLC–AAS. The typical gel permeation–cadmium atomic absorption chromatogram is represented in Fig. 1 in the case of DS administration (40 mg/kg, 1 day). As shown in the figure, the amount of Zn–Th could be easily determined in the Cd-replaced and then heat-treated supernatant by the HPLC–AAS method without any errors produced by the presence of other SH-containing compounds and zinc-containing proteins. Since MT shows two cadmium peaks corresponding to MT-I and MT-II at the pH, it can be determined at isometallothionein levels if necessary. The peak for MT-I was larger in every supernatant than that for MT-II. The recovery of cadmium (applied as Cd–Th) into the eluate was almost 100% (unpublished observation).

Figure 2 shows the dose-dependencies of the amount of MT induced in the liver by the three dextran derivatives. The absolute values of cadmium content in Cd–Th (namely, Zn content in the induced Zn–Th) were determined by the relative peak area to a standard Cd–Th solution with a known cadmium concentration. As expected from the structural similarity to carrageenan, DS induced a large amount

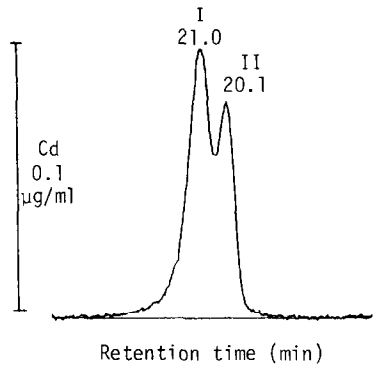


Fig. 1. Gel permeation–cadmium atomic absorption chromatogram of liver supernatant after injection of DS. Male mice were injected i.p. with DS dissolved in PBS (40 mg/kg body wt) and were killed 24 hr after the injection. A 0.4 g portion of liver from each mouse was combined and homogenized in 3 vol. of 0.1 M Tris–HCl buffer solution (pH 7.4, 0.25 M glucose) and the homogenate was centrifuged at 170,000 *g* for 60 min. Cadmium acetate solution was added to the supernatant and the excess cadmium was removed by heat-treatment and centrifugation. A 100 μl portion of the Cd-replaced supernatant was applied to HPLC–AAS, and the column equipped to HPLC was eluted with 50 mM Tris–HCl buffer solution (pH 8.0 at 25°), and atomic absorbance of cadmium was monitored continuously. The detector level of AAS was set as indicated by the vertical bar. I and II indicate MT-I and MT-II, respectively.

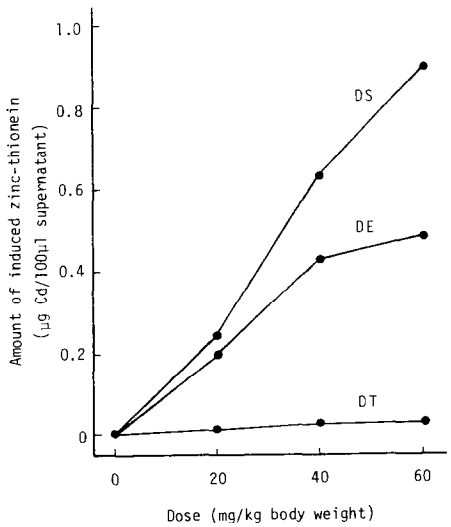


Fig. 2. Dose-dependencies of the amount of induced MT. Mice were injected with respective dose of three dextran derivatives and were killed 24 hr later. Control mice were injected with PBS. Cadmium contents in Cd-replaced supernatants were determined from the relative peak area to a standard Cd–Th solution on gel permeation–cadmium atomic absorption chromatograms (see legend to Fig. 1).

Table 1. Organ weights (tissue wt/body wt $\times 10^3$)*

| | Liver | Kidney | Spleen |
|---|---|---|---|
| PBS | 62.9 ± 2.8 | 17.4 ± 2.1 | 3.43 ± 0.44 |
| DS | 67.1 ± 4.0 | 18.5 ± 1.8 | 5.77 ± 1.64† |
| DE | 60.4 ± 3.2 | 16.9 ± 1.6 | 3.31 ± 0.48 |
| DT | 63.3 ± 5.0 | 17.8 ± 1.2 | 3.54 ± 0.56 |

* Mice were injected i.p. with dextran derivatives at a dose of 40 mg/kg body wt and killed 24 hr after the injection. Values are expressed as mean ± S.D. for six mice.
† Significantly different from PBS treatment ($P < 0.05$).

of MT dose-dependently. DE, which has the oppositely charged substituent to DS, also induced a large quantity of Zn–Th. On the other hand, DT, which has no substituent, induced only a negligible amount of MT at every dose.

The organ weights after the administrations are given in Table 1 as the ratio to body wt. A significant change was observed in the spleen weight after the injection of DS. The increase of spleen weight became progressively greater up to 7 days during the experimental period, though there was no significance between the values at 1 and 7 days (data not shown).

The alterations of several essential metal levels after injection of the dextran derivatives were followed so as to find a clue to the cause of MT-induction. The changes of metal concentrations in the liver, kidneys and spleen produced by the dose of 40 mg/kg body wt are summarized in Table 2. Since the injection of DS produced a significant increase of spleen weight, the table also includes the changes of total metal contents in spleen. In agreement with the results in Fig. 2, zinc level showed a significant increase in DS and DE. In DS treatment, calcium level increased significantly in every tissue examined along with the decrease of iron concentration in spleen. However, when expressed as total content in spleen, the iron level was not significantly altered due to the increase of the weight, while zinc (and magnesium, though not significant) showed a higher level than control.

The time-courses of the metal concentrations in liver and spleen, which were influenced significantly by the injection of DS, are represented in Figs. 3 and 4, respectively. The time-course of calcium level in the liver showed a close resemblance to that of zinc (namely, Zn–Th level). The similarity seems to suggest the correlation between them. As shown in Fig. 4, the calcium and iron concentrations in the spleen showed a maximum and a minimum, respectively, at 16 hr after the injection, and thereafter, both levels showed the tendency to recover to control levels. However, the iron concentration still remained at a lower level than control even 7 days after the injection.

## DISCUSSION

Dextran derivatives dissolved in PBS were injected

Table 2. Metal concentrations and contents in tissues*

| Liver | Mg | Ca | Fe | Zn |
|---|---|---|---|---|
| | ($\mu$g/g wet wt) | | | |
| PBS | 238 ± 11 | 33.9 ± 2.5 | 134 ± 32 | 26.6 ± 1.4 |
| DS | 249 ± 12 | 45.5 ± 4.3§ | 138 ± 21 | 35.8 ± 2.2§ |
| DE | 233 ± 5 | 34.5 ± 2.5 | 139 ± 19 | 32.2 ± 2.9‡ |
| DT | 233 ± 8 | 32.2 ± 2.1 | 153 ± 8 | 27.2 ± 1.9 |
| **Kidney** | **Mg** | **Ca** | **Fe** | **Zn** |
| | ($\mu$g/g wet wt) | | | |
| PBS | 204 ± 11 | 49.0 ± 2.0 | 84 ± 5 | 19.7 ± 1.0 |
| DS | 201 ± 9 | 52.8 ± 2.7† | 76 ± 9 | 19.6 ± 0.5 |
| DE | 207 ± 7 | 51.7 ± 3.8 | 84 ± 7 | 20.7 ± 0.8 |
| DT | 200 ± 4 | 49.2 ± 2.1 | 82 ± 6 | 19.3 ± 0.4 |
| **Spleen** | **Mg** | **Ca** | **Fe** | **Zn** |
| | ($\mu$g/g wet wt) | | | |
| PBS | 247 ± 6 | 36.0 ± 3.2 | 212 ± 41 | 19.9 ± 1.5 |
| DS | 252 ± 11 | 51.5 ± 11.3† | 133 ± 20‡ | 19.2 ± 0.9 |
| DE | 247 ± 17 | 40.8 ± 5.0 | 232 ± 49 | 20.4 ± 1.4 |
| DT | 254 ± 13 | 33.7 ± 1.3 | 246 ± 52 | 19.8 ± 0.7 |
| **Spleen** | **Mg** | **Ca** | **Fe** | **Zn** |
| | ($\mu$g/total organ) | | | |
| PBS | 29.8 ± 4.2 | 4.18 ± 0.34 | 25.8 ± 3.6 | 2.39 ± 0.34 |
| DS | 44.5 ± 15.0 | 9.22 ± 1.75§ | 22.8 ± 9.0 | 3.57 ± 1.08† |
| DE | 24.9 ± 3.8 | 4.21 ± 0.44 | 24.4 ± 7.5 | 2.14 ± 0.30 |
| DT | 31.6 ± 4.6 | 4.32 ± 0.51 | 30.1 ± 7.8 | 2.60 ± 0.36 |

* Values are expressed as mean ± S.D. for six mice. Significant differences from PBS treatment are represented as follows: †$P < 0.05$, ‡$P < 0.01$, §$P < 0.001$.

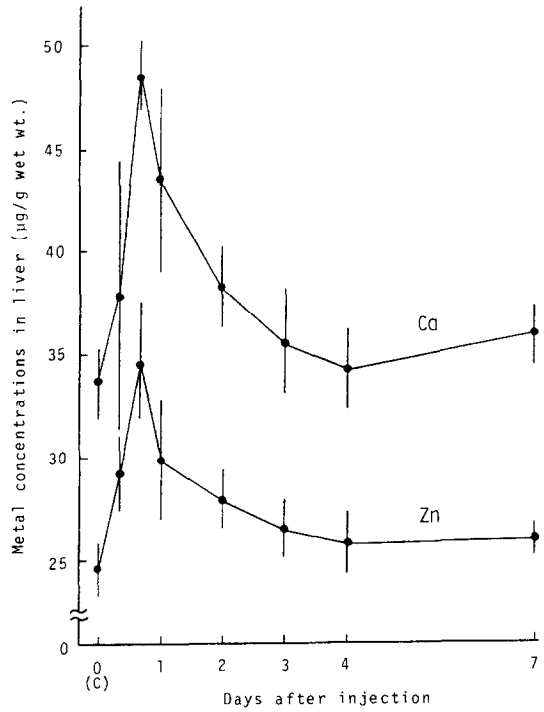


Fig. 3. Changes of calcium and zinc concentrations in liver after injection of DS. Mice were injected i.p. with DS at a dose of 40 mg/kg body wt. The animals were killed after 8 and 16 hr, 1, 2, 3, 4 and 7 days. Control mice were injected with PBS and were killed 1 day after the injection. The value is mean ± S.D. of six samples.

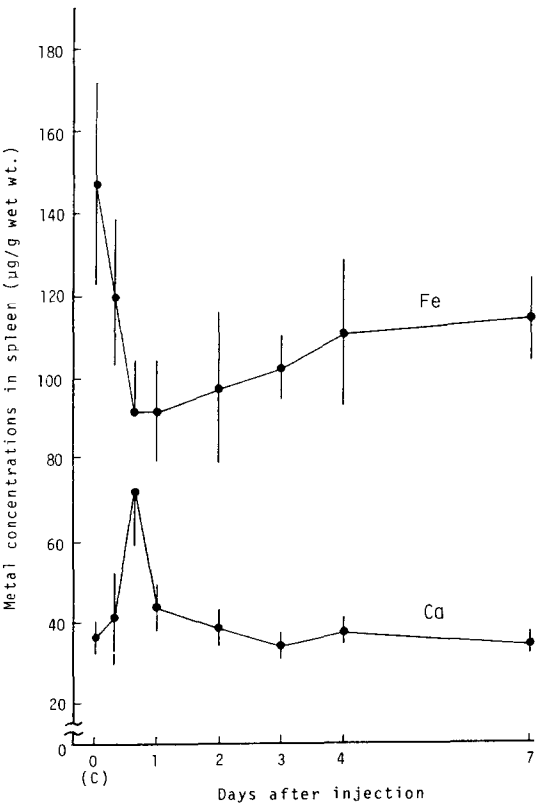


Fig. 4. Changes of iron and calcium concentrations in spleen after injection of DS (see legend to Fig. 3).

i.p. into mice to investigate the relationship between the structure and the ability to induce hepatic Zn–Th. The injection of a high dose of polysaccharides with the same average molecular weight might be anticipated to induce Zn–Th in all three cases to the same extent due to stress [6]. However, the induction of Zn–Th was observed in quite different manners among them, namely DS and DE induced a large amount of MT with and without the disturbances of metal (Ca and Fe) levels, respectively, and DT did not induce MT substantially.

DS is a sulfated polysaccharide. We have recently reported that carrageenan with a similar structure (sulfated galactan) to DS, also induced Zn–Th with the increase of spleen weight and the changes of calcium and iron levels [13]. It is not clear whether or not all the sulfated polysaccharides with high molecular weight can induce Zn–Th in the same manner. The clarification seems to be interesting because there exist many natural compounds which belong to sulfated polysaccharides such as heparin and chondroitin sulfate. Moreover, acid polysaccharides or, more generally, polyanions may be attractive substances for the examination of the correlation between the structure and the MT-inducing ability. Polyanions, including DS, have a number of immunity-related activities [16, 17]. In this respect, the increase of spleen weight observed only after injection of DS appears to be very suggestive. Although both sulfated polysaccharides (DS and carrageenan) can induce Zn–Th with the changes of calcium and iron levels, the MT-induction with this pattern is not restricted to the saccharides but rather it seems to be common for a wide variety of substances because even the injection of suspended cadmium salt also produced this pattern [25].

Unexpectedly, DE also induced a large quantity of Zn–Th. No changes of essential metal levels were detected except the increase of hepatic zinc level due to the induction of Zn–Th. This fact suggests that the increase of hepatic calcium level is not always the requisite for the induction of MT. However, conversely, in the cases that the increase of calcium level in liver (and spleen) was detected on our studies, a large amount of MT was always detected in the liver [13, 25].

DE is a polycation. The difference in electric charge between DE (polycation) and DS (polyanion) must be reflected in their behaviors after their administrations, since the positive charges of DE can be available for complex formation with the negatively charged (due to, for example, sialic acid residues) cell surface [20]. Moreoever, the negative charges of DS may influence the transglomerular passage of DS because the glomerular capillary wall is negatively charged [18]. However, dextran sulfates injected intravenously into rats are metabolized sooner or later depending on the molecular weight [22]. Consequently, it cannot be elucidated yet to what extent the difference in charge explains the difference in MT-inducing ability and influences on essential metal levels.

DT, with the mother structure for DS and DE, could neither induce Zn–Th in the liver nor influence the essential metal levels in the tissues examined. This result seems quite reasonable, because DT

(dextran) is clinically utilized as a substitute for plasma.

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