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Age- and Sex-Dependent Variations of Essential Metal Levels in Tissues and Responses to Dextran Sulfate Treatment Which Induces Zinc-Thionein

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Age and sex dependencies of iron, calcium and zinc levels in liver, kidney and spleen were investigated in male and female mice (3—12 weeks old). To clarify the age and sex dependencies of responses to dextran sulfate treatment, 4- and 12-week-old mice were injected intraperitoneally with dextran sulfate and killed 24 h later. The most striking change was observed in splenic iron level. The level increased with age and a five (male)- or eight (female)-fold increase was observed within the experimental period. Dextran sulfate treatment produced a marked decrease of the splenic iron level and the response was larger in 12-week-old mice. The alterations of metal levels after dextran sulfate treatment in the supernatant and precipitated fractions were also studied.

Keywords——age dependence; sex dependence; dextran sulfate; iron; calcium; spleen; liver; zinc-thionein; zinc; metallothionein

With the recent development of analytical techniques for metals, much interest has been shown in the changes of essential metal levels during diseases,[1,2] or reversely, to the various diseases due to the abnormal metabolism of essential metals (for example, acrodermatitis enteropathica in zinc[3-6]). The metabolism of zinc and copper has recently become of particular interest in relation to a unique low molecular weight protein, metallothionein (MT),[7] which was isolated first as a cadmium- and zinc-binding protein.[8] The unusually high level of hepatic zinc in the mammalian fetus and neonate has been suggested to be related to the accumulation of zinc-thionein (Zn-Th).[9-12] The participation of MT was also studied in Wilson's disease[13] and Menkes disease,[14-19] which are due to the abnormal metabolism of copper. Thus, the metabolism of zinc and copper is closely related to that of MT.

We have been very interested in the changes of other essential metal levels in tissues produced with the induction of Zn-Th and have reported the results of injection of carrageenan,[20] dextran sulfate,[21] suspended cadmium salt[22] and soluble heavy metals.[23] Although a group of common responses (increases of zinc and calcium in liver, and increase of calcium and decrease of iron in spleen along with the increase of spleen weight) of metal levels were observed on the injection of those substances, the magnitudes of some responses were quite different, and might have depended on the age and sex of the animals used.

In this paper, we report the developmental changes of iron, calcium and zinc levels in tissues and the dependencies of the above-mentioned responses induced by dextran sulfate treatment on age in the developmental period. Metal concentrations were determined by the use of an inductively coupled plasma-atomic emission spectrometer (ICP-AES).[24] To elucidate sex-dependent differences, both male and female animals were studied.

Materials and Methods

Animals——Three-week-old male and female ICR mice were purchased from Clea Japan and given a standard laboratory chow (MF, Oriental Yeast Co.) and water *ad libitum* throughout the experimental period. The animals were killed at the ages of 3 (at two days after introduction into the laboratory), 4, 6, 8, 10 and 12 weeks under ether anaesthesia without any injection. To compare the responses produced by the injection of dextran sulfate, other 4- and 12-week-old mice were killed 24 h after the injection of dextran sulfate dissolved in phosphate-buffered saline (40 mg/kg body weight).[21] The liver (about 0.4 g portion) and a half of the spleen from each mouse were separately combined in each group of age and sex, and homogenized in 3 and 6 volumes of 0.1 M Tris-HCl buffer solution (pH 7.4, 0.25 M glucose), respectively. In the cases of 4- and 12-week-old mice, all liver samples were homogenized under ice-water cooling in an atmosphere of nitrogen. The homogenates were centrifuged at 170000 × *g* for 60 min at 2 °C.

Metal Determinations of Tissues, Homogenates and Supernatants——The liver (about 0.3 g portion), one kidney and a half of the spleen from each animal were digested with 1, 0.5 and 0.3 ml of mixed acid (HNO_3 : $HClO_4$, 5 : 1, v/v) and then the solutions were diluted to 10, 5 and 3 ml, respectively, with doubly distilled water. Homogenates and supernatants from liver (1 ml) and spleen (0.3 ml) were digested with the same volume of the mixed acid and the solutions were diluted to 5 and 3 ml, respectively, with doubly distilled water. Essential metal concentrations were determined simultaneously with an ICP-AES machine (Jarrell-Ash Model 975 Plasma Atomcomp) with an autosampler.

Determination of Zn-Th——To detect induced Zn-Th as Cd-Th, cadmium acetate solution (1000 ppm Cd, 10 μl) was added to 300 μl of liver supernatant obtained from 4- and 12-week-old mice and the excess cadmium bound to high molecular weight proteins was removed by heat treatment (70 °C, 5 min) and centrifugation (10000 × *g*, 1 min). A 100 μl portion of the cadmium-replaced supernatant was applied to a high performance liquid chromatograph (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 °C) at a flow rate of 1 ml/min. The cadmium level of the eluate was monitored continuously with an atomic absorption spectrophotometer (Hitachi 170-50A).[25]

Results

As shown in Fig. 1, body weight increased with age up to 8 weeks, at first steeply and then slowly in both sexes. Although the absolute values of liver and kidney weights also showed a progressive increase up to 6 or 8 weeks, the relative values of the tissue to body weight were almost constant during the experimental period except for the latter half in the case of the liver. In contrast, the absolute spleen weight was almost constant throughout the experimental period despite the progressive increase of body weight. Therefore, the relative value with respect to body weight tended to decrease with age (Fig. 1).

To clarify the age- and sex-dependent responses of tissue weights and essential metal levels to dextran sulfate treatment, 4- and 12-week-old mice were injected with dextran sulfate intraperitoneally (40 mg/kg body weight). The changes of body weight and relative tissue weights are also represented in Fig. 1. Body weight tended to decrease slightly after the treatment in all experimental groups. The relative liver weight showed a tendency to increase (5—30%, absolute weight also increased except in the case of 12-week-old males), while dextran sulfate treatment hardly affected absolute kidney weight (data not shown). Increases in absolute and relative spleen weights[21] were observed consistently in all dextran sulfate-injected groups.

Essential metal concentrations in tissues changed in various manners with the change of tissue weight in the developmental period. The changes of hepatic essential metal concentrations are represented in Fig. 2. Zinc level showed a complicated pattern, namely the level increased up to 4 weeks, then decreased, and again increased. The pattern was just the same as that reported by Keen and Hurley.[26] Iron level decreased up to 4 weeks or 6 weeks and then increased. On the other hand, calcium concentration was substantially constant throughout the experimental period.

The injection of dextran sulfate produced increases of hepatic zinc and calcium levels, and both levels showed quite similar time-courses (transitory increase with a maximum at 16 h).[21] The changes of the two levels were well correlated even when compared among different ages and sexes (Fig. 2).

The changes of renal metal concentrations are given in Fig. 3. Zinc and calcium remained

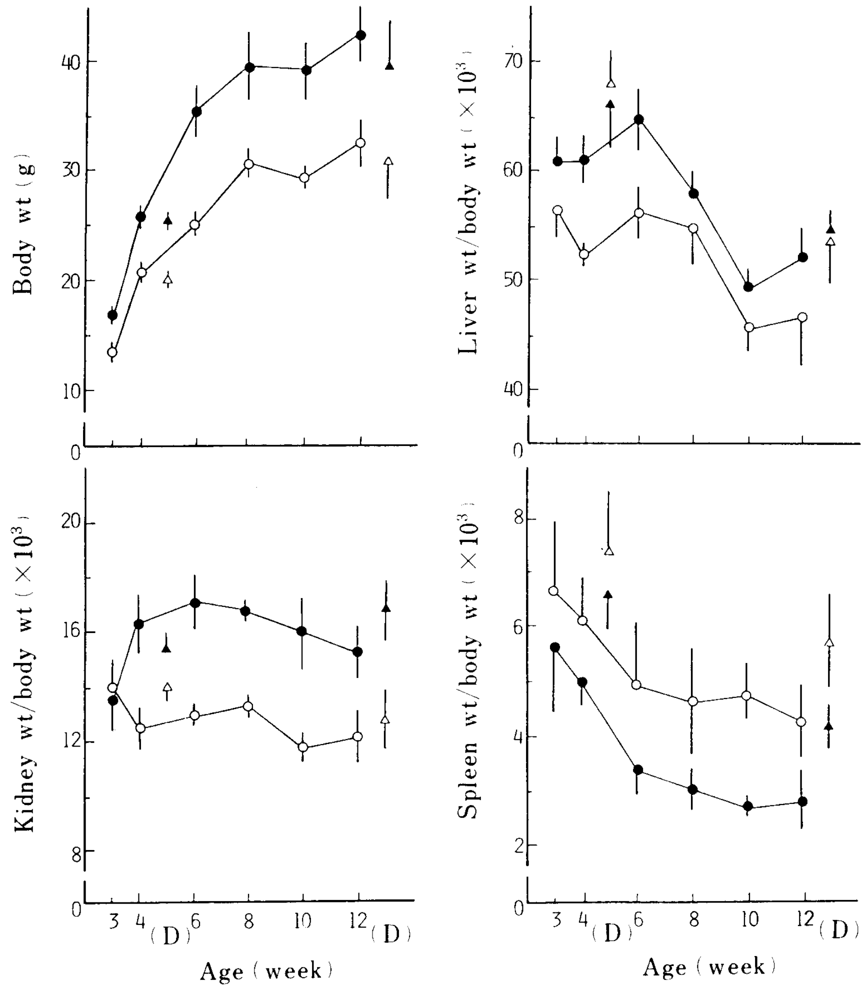

Fig. 1. Body Weight and Relative Tissue Weights at Different Ages

●, male; ○, female. Each point represents the mean of six mice ± standard deviation. D on the abscissa indicates a dextran sulfate-treated group (▲, male; △, female).

at a constant level throughout the experimental period, except for a somewhat higher calcium level observed at 3 weeks. On the other hand, iron concentration in both sexes increased progressively with age up to 8 or 10 weeks and then reached a plateau. The ratio of the value at 10 weeks to that at 3 weeks was 1.6 and 2.1 in male and female mice, respectively. Although the injection of dextran sulfate sometimes produced a small change in renal metal levels, the kidneys were least affected by the treatment among the three tissues tested in the present study.

As mentioned above, the absolute spleen weight hardly showed any age-dependant increase. Therefore, constant metal levels throughout the experimental period might be anticipated. However, the most striking change was observed in the iron level of spleen, as shown in Fig. 4. The level in both sexes increased with age. In females, especially, a linear increase was maintained even at 12 weeks. The ratio of the value at 12 weeks to that at 3 weeks was 5 and 8 for male and female mice, respectively. Splenic calcium and zinc concentrations were essentially constant (Fig. 5).

As well as the splenic iron concentration itself, its response to dextran sulfate treatment[21] also exhibited an age dependence (Fig. 4). In 4-week-old mice, the values fell to 74—76 and to 85—97% of the original values in concentration and in content, respectively, after the treatment, while in 12-week-old mice, they fell to 43—55 and to 58—71% in concentration

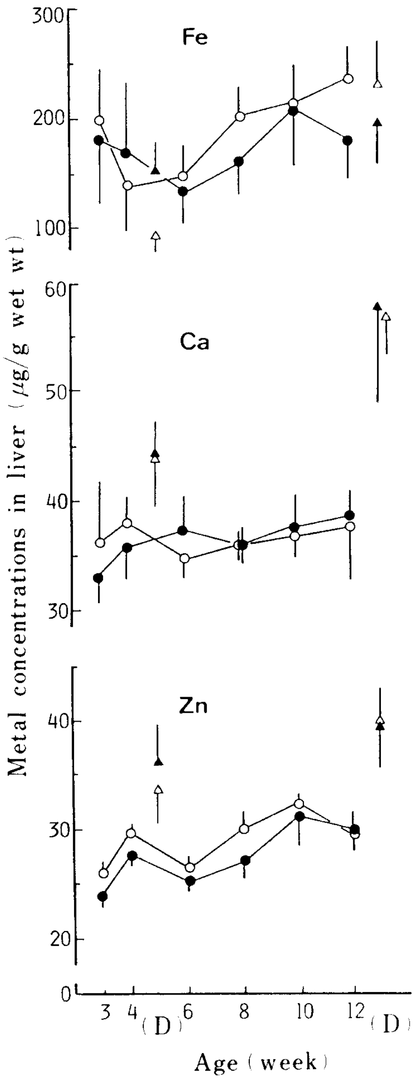

Fig. 2. Zinc, Calcium, and Iron Concentrations in the Liver at Different Ages

●, male; ○, female. Each point represents the mean of six mice ±standard deviation. D on the abscissa indicates a dextran sulfate-treated group (▲, male; △, female).

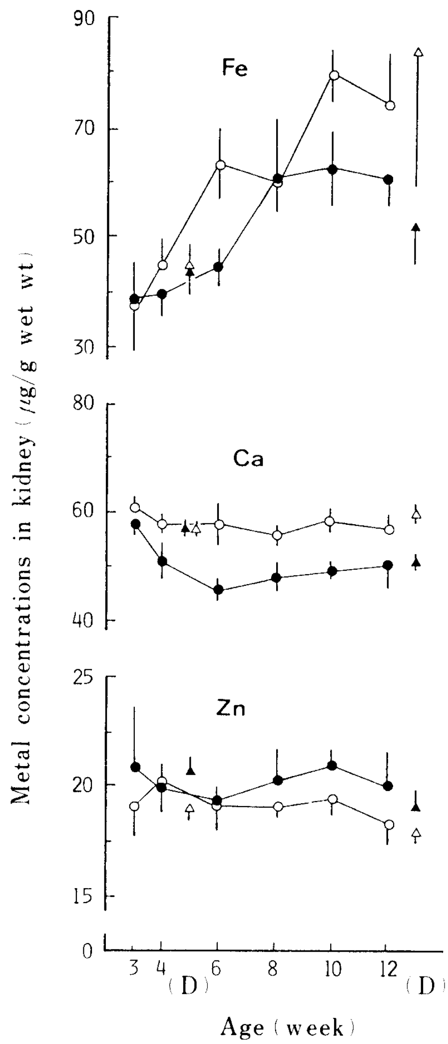

Fig. 3. Zinc, Calcium, and Iron Concentrations in the Kidney at Different Ages

●, male; ○, female. Each point represents the mean of six mice ±standard deviation. D on the abscissa indicates a dextran sulfate-treated group (▲, male; △, female).

and in content, respectively. A larger response in older (12-week-old) mice was also observed in the increase of splenic calcium concentration (Fig. 5). The lowered zinc concentration observed after dextran sulfate treatment may be explained by the increased spleen weight (see Fig. 1) with constant total content.

Concentrations of various metals in the liver and spleen homogenates and in their 170000 × g supernatant fractions obtained from 12-week-old males are summarized in Table I. Most of the hepatic zinc was detected in the supernatant fraction and an increase of the zinc level after dextran sulfate treatment was observed in the supernatant fraction, while the increase of hepatic and splenic calcium levels was ascribed to that in the precipitated fractions. On the other hand, both fractions contributed to the decrease of iron level in the spleen.

Figure 6 shows gel permeation-cadmium atomic absorption chromatograms of cadmium-replaced liver supernatants from dextran sulfate-treated and control mice. Cadmium was added to the original supernatants to detect induced Zn-Th as Cd-Th. Two peaks, which could hardly be seen in control supernatant, were observed at the retention times

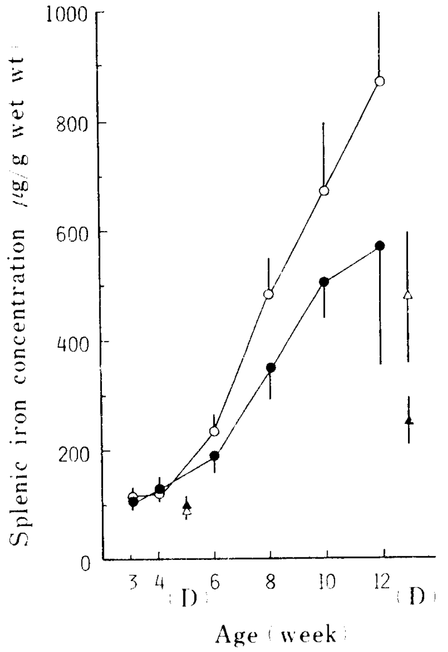

Fig. 4. Iron Concentration in the Spleen at Different Ages

●, male; ○, female. Each point represents the mean of six mice ±standard deviation. D on the abscissa indicates a dextran sulfate-treated group (▲, male; △, female).

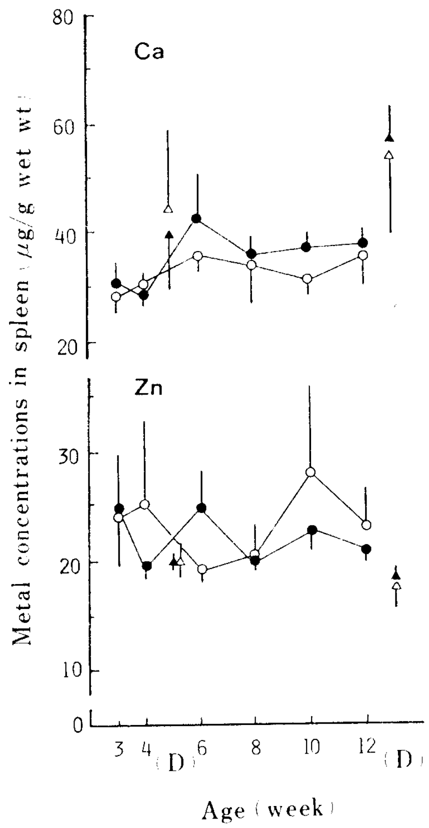

Fig. 5. Zinc and Calcium Concentrations in the Spleen at Different Ages

●, male; ○, female. Each point represents the mean of six mice ±standard deviation. D on the abscissa indicates a dextran sulfate-treated group (▲, male; △, female).

Table I. Concentrations (μg/ml) of Several Essential Metals in Tissue Homogenates and Supernatants from Control and Dextran Sulfate-Treated Male 12-Week-Old Mice[a)]

	Liver		Spleen	
	Zn	Ca	Ca	Fe
NT-homo	6.9	8.7	5.8	75.6
NT-sup	6.3	1.9	2.1	10.3
DS-homo	8.9	13.6	8.6	36.2
DS-sup	7.8	1.8	1.9	7.6

a) Male 12-week-old mice were killed without any treatment or 24 h after the injection of dextran sulfate (40 mg/kg body weight). A 0.4 g portion of liver and a half of the spleen from each mouse in each group were separately combined, and homogenized in 3 and 6 volumes of buffer solution, respectively. The homogenates were centrifuged at 170000 × *g* for 60 min. Homogenates and supernatants were digested and metal concentrations were determined by ICP-AES. NT and DS indicate control and dextran sulfate-treated groups, respectively.

of MT-I and -II.[20-23] As mentioned above, the increase of hepatic zinc level can be regarded as an increase in the supernatant fraction. Consequently, the increased hepatic zinc level observed after dextran sulfate treatment could be ascribed to the induction of Zn-Th[21] (when original supernatants (not cadmium-replaced) were subjected to the high performance liquid chromatography and the zinc level of the eluate was monitored, marked differences between

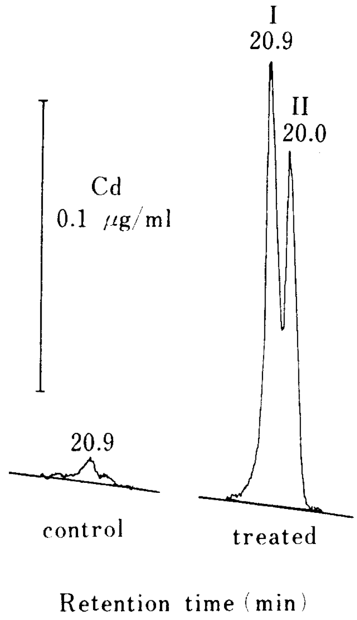

Fig. 6. Gel Permeation-Cadmium Atomic Absorption Chromatograms of Cadmium-Replaced Liver Supernatants from Control and Dextran Sulfate-Treated Male 12-Week-Old Mice

Mice were killed without any treatment or 24 h after the injection of dextran sulfate (40 mg/kg body weight). A 0.4 g portion of liver from each mouse was combined in each group, and homogenized in 3 volumes of buffer solution. The homogenates were centrifuged at $170000 \times g$ for 60 min. Cadmium solution was added to the original supernatants and the excess cadmium was removed by heat treatment and centrifugation. A 100 μl portion of the cadmium-replaced supernatants was subjected to HPLC-AAS. The detector level of AAS was set as indicated by the vertical bar. I and II indicate MT-I and -II, respectively.

dextran sulfate-treated and control groups were observed only in the MT fractions).

In this study, age and sex dependencies over the whole experimental period were studied in normal mice without any treatment, and the animals were used as control groups to dextran sulfate treatment. None of the changes observed could be ascribed to the injection of phosphate-buffered saline.

Discussion

In an investigation of the age dependence of essential metal levels in tissues, there may be several interesting periods: 1) neonatal period (this may cover the fetus), 2) growth period, 3) old age. Particular attention should be paid to the changes in the growth period, because most animal experiments are carried out in this period. We have reported a group of changes in tissue metal levels produced after the injection of several compounds that induce hepatic Zn-Th.[20-23] In those studies, the observed splenic iron level and its response to the injections exhibited a large variance, which might have been due not only to the diversity of compounds injected but also possibly to the differences in age and sex of animals used. The present study was undertaken to investigate whether the observed discrepancies could be ascribed to age and sex differences.

Iron is one of the most extensively investigated essential metals, and the sex and age differences in iron metabolism have been of interest for a long time.[27,28] Since most of the iron in tissues is non-heme iron (ferritin and hemosiderin) as storage iron,[29] the change of total iron level can also be seen as that of non-heme iron. Therefore, the age and sex dependences of hepatic iron concentration have been reported at several levels of iron (total iron, non-heme iron or ferritin iron) for various experimental periods.[26,29-37] In general, hepatic iron concentration shows a high level at birth, decreases during the lactation period due to the low iron concentration in milk, and then increases because of the high iron content in usual chows.[26,30,32-34] The results of our present study also showed the same tendency, especially in females. After weaning, females always show a higher iron level than males[33,35] because of their faster rate of synthesis of ferritin.[35]

Although liver and spleen are both storage organs of iron,[38] splenic iron level has not been as well examined as hepatic iron level.[26,29,34] However, it was the splenic iron level that showed the largest dependence on age and sex. Moreover, the level showed a dramatic decrease after dextran sulfate treatment.[21] Although both tissues (liver and spleen) store iron, Kaldor reported an interesting difference between them.[29] Namely, in old rats, the main storage form of iron was ferritin in both tissues, while in young (4-month-old) rats, hemosiderin (water-insoluble and not quickly utilizable) was the principal non-heme iron in the spleen. Leslie and Kaldor also reported that hepatic iron level changes in the neonatal period, not only with the change of ferritin level but also with that of iron/ferritin ratio.[34] From the above-mentioned viewpoints, there are several interesting problems left for further study: (i) why does only splenic iron decrease after dextran sulfate treatment, (ii) which type of non-heme iron (ferritin or hemosiderin) decreases, (iii) does the iron/ferritin ratio remain constant after the treatment.

The injection of an inflammatory drug induces Zn-Th in the liver.[39] We have confirmed this in the case of carrageenan and reported the concomitant changes of essential metal levels in tissues.[20] The changes were the same as those produced by dextran sulfate treatment. Recently, the metabolic change of storage iron on inflammation has become of interest.[40-43] The alteration of splenic iron level reported by us may be one of the responses in an inflammatory reaction.

In contrast to the long-lasting decrease in the splenic iron level, the alterations of other metal levels (transitory increases of hepatic zinc and calcium and splenic calcium) exhibit quite similar time-courses.[20-22] Moreover, the fact that both (hepatic and splenic) calcium levels changed to the same extent in the four dextran sulfate-treated groups in this study strongly suggests that a common cause exists for the two changes. Although this increase of calcium is not always accompanied by the induction of Zn-Th,[21] the increase has been observed after the injection of many compounds which induce MT.[44] Therefore, the similarity in the time-course of Zn-Th (hepatic zinc level) and both calcium levels may offer a clue to the mode of MT-induction by various substances.

As we have seen, the diversity in the splenic iron level and in its response to several treatments found in our previous studies[20-22] seems to be explained, to a large extent, by the differences in age and sex of the animals used. Although it is necessary to follow the time-course to determine the maximum response to dextran sulfate treatment, the marked dependence on animal age and sex is clear from the present study.

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