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ESSENTIAL METALS AND METALLOTHIONEIN IN CADMIUM-INDUCED THYMIC ATROPHY AND SPLENOMEGALY

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Abstract—Cadmium chloride was injected intraperitoneally into mice and the animals were sacrificed 1, 3, and 9 days after the injection. The concentrations and contents of cadmium and six essential metals in the thymus, spleen, kidney, and liver were determined by inductively coupled plasma–atomic emission spectrometry. Although the accumulation of cadmium in the thymus and spleen was as low as 0.05 and 0.2–0.4% of the injected dose, respectively, compared to liver (60%) and kidney (6.5–9.0%), the two immune organs showed dramatic changes of weights; namely severe atrophy of thymus and enlargement of spleen. The concentrations of zinc in the two immune organs did not change during the atrophy or enlargement, though the contents showed significant decrease or increase dependent upon the changes in weights. The concentrations and/or contents of other essential metals also changed with time and the changes depended on organs. The distributions of cadmium in the supernatant fractions of thymus and spleen were determined by high speed liquid chromatography with a flame atomic absorption spectrophotometer. Two cadmium peaks which correspond to the two isometallothioneins were eluted exactly at the same retention times as those of liver metallothioneins.

Growth retardation is one of the most striking effects of zinc deficiency [1]. Recently, Beach *et al.* [2] and Jardieu and Fraker [3] demonstrated that mice deprived of zinc manifest a dramatic retardation of thymic growth and development. The atrophy was characterized by the preferential involution of thymic cortex and was accompanied by subsequent reduction of the humoral immune capacity [3].

Administration of a sublethal dose of cadmium was also found to cause a transitory thymic atrophy characterized by the similar preferential involution to that of zinc deprivation [4]. The accumulation of cadmium results in a concomitant increase of zinc and copper in liver and kidneys through the induction of metallothionein [5], and thus cadmium affects not only absorption of zinc [6] but also serum and tissue concentrations of zinc [7]. Zinc deficiency may underlie the involution of the thymus induced by both zinc depletion and cadmium administration.

Although the induction of metallothionein by cadmium loading is well documented in liver and kidneys of experimental animals [5], there have been only limited reports for the induction of metallothionein in immune organs; Amacher and Ewing [8] and Suzuki *et al.* [9] reported the induction in spleen, but there have been no reports for the induction in thymus.

The present study was intended to find the changes of zinc and cadmium concentrations during transitory atrophy of thymus and enlargement of spleen after cadmium loading. Although the primary concern was to determine the concentrations of the two metals, metal concentrations were determined by inductively coupled plasma–atomic emission spectrometry (ICP–AES) to find the effects of cadmium loading to typical essential metals such as iron, magnesium, calcium, manganese, and copper. It was also challenging in this study to detect metallothionein in organs small in size and low in accumulation of cadmium such as the thymus and spleen of mice by high speed liquid chromatography with a flame atomic absorption spectrophotometer (HLC–AAS) as a detector of cadmium [10].

MATERIALS AND METHODS

Experimental animals. Male BALB/C mice of 6 weeks old were purchased from Japan Charles River Co. (Atsugi, Japan) and were fed on a standard laboratory chow (Clea Japan, Tokyo) and distilled water *ad libitum*. Cadmium chloride solution in physiological saline at a concentration of 0.1 mg Cd/ml was intraperitoneally injected once into fifty-five 8 weeks old mice at a dose of 1.8 mg Cd/kg body weight. Forty mice were sacrificed by exsanguination under chloroform anaesthesia at 1 (10 mice), 3 (20 mice) and 9 days (10 mice) after the injection of cadmium. Fifteen mice were killed at 3 days after treatment for the analysis of metallothionein.

Determination of metal concentrations. Thymuses and spleens of two mice (four thymuses for 3rd day mice of cadmium-injected group) were pooled in each group, weighed, digested with 0.2 and 0.5 ml of mixed acid (HNO_3:$HClO_4$ = 5:1 v/v), and then diluted to 1 and 3 ml with doubly distilled water, respectively. Kidneys and largest liver lobes of two mice were pooled in each group, weighed, digested with 2 ml of mixed acid, and then diluted to 5 ml with doubly distilled water. Concentrations of metals in acid-digested solution were determined by ICP-AES (Jarrell-Ash Model 975 Plasma Atomcomp).

Preparation of tissue supernatants. Fifteen thymuses and spleens, obtained by sacrificing mice 3 days after the injection of cadmium (1.8 mg/kg body weight), were homogenized in three volumes of

Table 1. Body and organ weights*

	Saline‖ 3 days	Cadmium** 1 day	3 days	9 days
Body wt (g)	26.0 ± 1.6	26.2 ± 1.1	26.7 ± 1.1	27.0 ± 1.3
Thymus (mg)	51.2 ± 9.4	30.9 ± 6.6¶	23.5 ± 8.9¶	46.1 ± 4.6
Spleen (mg)	107 ± 11	104 ± 11	198 ± 34¶	130 ± 11¶
Kidney (mg)	384 ± 23	377 ± 28	411 ± 45	426 ± 29
Liver (g)	1.51 ± 0.07	1.34 ± 0.11¶	1.57 ± 0.18	1.63 ± 0.13†

* Values were expressed as mean ± S.D. of 10 samples and were significantly different from the control as follows: † $P < 0.05$ and ¶ $P < 0.001$.

‖ Mice were injected i.p. with physiological saline and sacrificed 3 days after the injection. These animals were used as controls.

** Mice were injected i.p. with cadmium chloride at a dose of 1.8 mg Cd/kg body weight and sacrificed 1, 3, and 9 days after the injection.

Table 2. Concentration and content of metals in thymus*

		Saline	Cadmium		
		3 days‖	1 day‖	3 days**	9 days‖
Zn	μg/g††	17.9 ± 0.9	18.0 ± 0.3	17.9 ± 0.4	18.9 ± 0.7
	μg/organs‡‡	1.82 ± 0.20	1.03 ± 0.08¶	0.79 ± 0.09¶	1.75 ± 0.14
Cd	μg/g	0.13 ± 0.07	0.81 ± 0.12¶	1.10 ± 0.29¶	0.59 ± 0.11¶
	ng/organs	13.5 ± 7.0	45.3 ± 2.5¶	51.3 ± 8.8¶	55.0 ± 12.4¶
Fe	μg/g	29.2 ± 0.6	35.9 ± 4.4†	40.6 ± 4.4¶	32.5 ± 8.4
	μg/organs	2.99 ± 0.40	2.01 ± 0.23‡	1.84 ± 0.08¶	3.02 ± 0.92
Mg	μg/g	225 ± 9	218 ± 4	207 ± 8†	237 ± 9
	μg/organs	22.9 ± 2.9	12.3 ± 1.3¶	9.41 ± 1.25¶	21.9 ± 1.87
Ca	μg/g	32.9 ± 3.9	50.4 ± 11.6†	48.1 ± 5.4‡	33.7 ± 2.9
	μg/organs	3.41 ± 0.89	2.78 ± 0.34	2.17 ± 0.03†	3.11 ± 0.35
Mn	ng/g	237 ± 12	375 ± 18¶	314 ± 38‡	240 ± 29
	ng/organs	24.2 ± 2.5	21.1 ± 2.2	14.0 ± 0.3¶	22.2 ± 3.3
Cu	μg/g	1.16 ± 0.17	1.54 ± 0.16†	1.40 ± 0.16	1.15 ± 0.07
	ng/organs	118 ± 19.1	75.8 ± 17.2†	63.9 ± 3.5¶	106 ± 10

* Values were expressed as mean ± S.D. of 5 samples and were significantly different from the controls as follows: † $P < 0.05$, ‡ $P < 0.01$, and ¶ $P < 0.001$.

‖ Whole thymuses of two mice were combined and digested with mixed acid.

** Whole thymuses of four mice were combined and digested with mixed acid.

†† Concentration of metal, μg/g wet weight.

‡‡ Content of metal, μg/thymuses of two mice.

Table 3. Concentration and content of metals in spleen*

		Saline	Cadmium		
		3 days	1 day	3 days	9 days
Zn	μg/g‖	19.6 ± 0.5	19.1 ± 0.3	19.7 ± 0.7	19.8 ± 0.2
	μg/organs**	4.17 ± 0.26	3.91 ± 0.32	8.16 ± 0.63¶	5.12 ± 0.29‡
Cd	μg/g	−0.045 ± 0.016††	1.28 ± 0.68¶	0.90 ± 0.18¶	1.36 ± 0.13¶
	ng/organs	7.8 ± 4.7	338 ± 33¶	406 ± 52¶	351 ± 41¶
Fe	μg/g	289 ± 24	219 ± 25‡	158 ± 13¶	240 ± 9‡
	μg/organs	62.2 ± 9.8	43.7 ± 9.2†	63.0 ± 3.1	62.3 ± 4.9
Mg	μg/g	234 ± 5	223 ± 5†	243 ± 10	238 ± 3
	μg/organs	49.9 ± 3.0	46.1 ± 3.9	101 ± 9¶	61.6 ± 3.2¶
Ca	μg/g	36.5 ± 2.5	32.9 ± 1.7	32.7 ± 2.3	41.6 ± 3.6†
	μg/organs	7.80 ± 0.75	6.69 ± 0.70	13.2 ± 1.0¶	10.7 ± 0.6¶
Mn	ng/g	227 ± 17	232 ± 21	160 ± 18‡	233 ± 26
	ng/organs	48.2 ± 0.1	48.0 ± 0	62.2 ± 2.8¶	60.2 ± 6.3¶
Cu	μg/g	1.41 ± 0.13	1.22 ± 0.08	1.50 ± 0.30	1.27 ± 0.08
	ng/organs	291 ± 29	242 ± 24†	634 ± 165¶	329 ± 29

* Whole spleens of two mice were combined and digested with mixed acid. Values were expressed as mean ± S.D. of 5 samples and were significantly different from the controls as follows: † $P < 0.05$, ‡ $P < 0.01$, and ¶ $P < 0.001$.

‖ Concentration of metal, μg/g wet weight.

** Content of metal, μg/spleens of two mice.

†† Negative value was due to excess residual acid in the sample compared to blank solution.

0.1 M Tris–HCl buffer solution (pH 7.4, 0.25 M glucose) using a teflon homogenizer under ice-water cooling and in an atmosphere of nitrogen. The homogenates were centrifuged at 105,000 *g* for 60 min to give supernatant fractions. Concentrations of cadmium in the supernatant fractions were determined by a flame atomic absorption spectrophotometer (Hitachi AA 170-50 A) after 10-fold dilution with doubly distilled water.

Detection of metallothionein by HLC–AAS. The outlet of a high speed liquid chromatograph (HLC 803 A, Toyo Soda, Tokyo) equipped with a gel permeation column (TSK GEL SW 3000, Toyo Soda, 7.5 × 600 mm with a precolumn of 7.5 × 100 mm) was directly connected to the nebulizer tube of a flame atomic absorption spectrophotometer (Hitachi AA 170-50 A). The supernatant fractions of thymus and spleen were applied in a 200 μl portion and the column was eluted with 50 mM Tris–HCl buffer solution (pH 8.0 at 25°, dissolved gas was removed at 80° under reduced pressure) containing 0.1% sodium azide at a flow rate of 1 ml/min. Molecular absorbances at 254 and 280 nm, and atomic absorbance of cadmium were recorded on a three-pen recorder (Rikadenki Model PG-3 with pen gap adjustment memory).

RESULTS

Body and organ weights after the injection of cadmium are summarized in Table 1. Although body weights sometimes decreased and a few mice died between 1 and 3 days after the injection in the former studies [4], no decreases of body weights and death of mice were observed in the present experiment. The weights of thymuses decreased significantly after the injection, the severest decrease being observed 3 days after the injection (decreased to 46% of the control), and then almost recovered to control level 9 days after the injection. In contrast to thymus, the weights of spleens increased significantly 3 days after the injection (increased to 185% of the control), and then started to decrease, but did not return to the control weight within 9 days after the injection. Liver showed a significant shrinkage 1 day after the injection and the weight increased to a significant level 9 days after the injection.

Tables 2–5 show the concentration (μg/g wet weight) and contents of metals (μg/organs or two mice) in the thymus, spleen, kidney and liver of the mouse after treatment with physiological saline (control) or cadmium. Zinc in the thymus and spleen remained at a constant concentration throughout despite the dramatic changes of organ weights, which resulted in a dramatic decrease or increase in zinc content in the thymus or spleen at 3 days after the injection. Iron content in the spleen remained at a constant value during the splenomegaly. Other essential metals in the two immune organs changed in the concentrations and/or contents with the changes of organ weights. Essential metals in the kidney and liver also changed in the concentrations and/or contents after treatment with cadmium as shown in Tables 4 and 5, though the changes were not so dramatical compared with those in the two immune organs.

Cadmium was mainly accumulated in the liver (60% of the injected dose) and kidneys (6.5–9.0% of the injected dose), and the metal in both organs was found to be associated with metallothionein on an SW column (data not shown). On the other hand, accumulation of cadmium in the immune organs was low as shown in Tables 2 and 3; as low as 0.05 per cent in the thymus and 0.2–0.4 per cent in the spleen of the administered dose. Moreover, the sizes of thymus and spleen were small; 0.2 per cent for thymus and 0.4 per cent for spleen of the body weight. The concentrations of cadmium were 280 ng/ml in both supernatants. Figures 1 and 2 show the distributions of cadmium and proteins (254 and 280 nm, arbitrary unit) in the supernatant fractions of thymus and spleen, respectively, 200 μl portions of each

Table 4. Concentration and content of metals in kidney*

		Saline	Cadmium		
		3 days	1 day	3 days	9 days
Zn	μg/g‖	21.1 ± 0.6	21.6 ± 0.3	23.5 ± 0.6¶	24.0 ± 0.6¶
	μg/organs**	16.2 ± 0.7	16.0 ± 0.3	18.8 ± 2.0†	20.5 ± 1.1¶
Cd	μg/g	0.120 ± 0.034	8.14 ± 0.25¶	9.90 ± 1.22¶	9.80 ± 1.67¶
	μg/organs	0.0765 ± 0.0293	6.05 ± 0.28¶	8.22 ± 1.31¶	8.38 ± 1.58¶
Fe	μg/g	68.4 ± 5.1	85.3 ± 4.3‡	71.4 ± 3.0	75.1 ± 4.4
	μg/organs	52.7 ± 5.9	63.4 ± 2.2†	59.5 ± 7.7	64.0 ± 4.5†
Mg	μg/g	218 ± 7	213 ± 4	205 ± 5†	216 ± 1
	μg/organs	168 ± 6	158 ± 1†	170 ± 17	182 ± 9†
Ca	μg/g	53.5 ± 1.7	52.1 ± 0.8	55.4 ± 1.7	55.6 ± 0.9
	μg/organs	41.2 ± 2.4	38.7 ± 1.4	46.1 ± 5.6	47.4 ± 2.8†
Mn	μg/g	1.51 ± 0.05	1.50 ± 0.04	1.20 ± 0.04¶	1.42 ± 0.04†
	μg/organs	1.16 ± 0.05	1.12 ± 0.02	0.993 ± 0.096†	1.21 ± 0.07
Cu	μg/g	4.64 ± 0.14	4.70 ± 0.04	4.48 ± 0.13	4.59 ± 0.10
	μg/organs	3.56 ± 0.13	3.24 ± 0.50	3.72 ± 0.39	3.90 ± 0.20†

* Whole kidneys of two mice were combined and digested with mixed acid. Values were expressed as mean ± S.D. of 5 samples and were significantly different from the controls as follows: † $P < 0.05$, ‡ $P < 0.01$, and ¶ $P < 0.001$.

‖ Concentration of metal, μg/g wet weight.

** Content of metal, μg/kidneys of two mice.

Table 5. Concentration and content of metals in liver*

		Saline	Cadmium		
		3 days	1 day	3 days	9 days
Zn	μg/g‖	32.6 ± 0.5	46.2 ± 0.7¶	42.0 ± 2.2¶	39.8 ± 1.8¶
	μg/organs**	98.5 ± 3.5	123 ± 11‡	131 ± 11¶	130 ± 7¶
Cd	μg/g	0.106 ± 0.024	21.1 ± 0.5¶	17.1 ± 2.2¶	17.5 ± 1.7¶
	μg/organs	0.322 ± 0.092	51.4 ± 3.5¶	52.7 ± 3.8¶	57.1 ± 5.8¶
Fe	μg/g	82.2 ± 1.4	117 ± 9¶	72.7 ± 3.5‡	71.6 ± 4.8‡
	μg/organs	248 ± 11	320 ± 36‡	227 ± 25	234 ± 14
Mg	μg/g	240 ± 4	240 ± 4	216 ± 7¶	243 ± 3
	μg/organs	725 ± 29	642 ± 48¶	674 ± 73	792 ± 29‡
Ca	μg/g	35.9 ± 1.3	51.5 ± 4.4¶	51.1 ± 9.7†	35.6 ± 2.9
	μg/organs	109 ± 7.1	135 ± 16†	159 ± 28†	116 ± 10
Mn	μg/g	1.15 ± 0.62	1.10 ± 0.05	1.06 ± 0.09	1.10 ± 0.03
	μg/organs	3.46 ± 0.12	2.96 ± 0.19‡	3.28 ± 0.24	3.60 ± 0.13
Cu	μg/g	8.18 ± 0.33	8.75 ± 0.09†	6.86 ± 0.27¶	8.25 ± 0.17
	μg/organs	24.7 ± 1.4	23.4 ± 2.0	21.5 ± 3.1	26.9 ± 1.5

* The largest lobes of two mice were combined and digested with mixed acid. Values were expressed as mean ± S.D. of 5 samples and were significantly different from the controls as follows: † $P < 0.05$, ‡ $P < 0.01$, and ¶ $P < 0.001$.

‖ Concentration of metal, μg/g wet weight.

** Content of metal, μg/livers of two mice.

supernatant fraction being applied to an analytical column of SW 3000. Cadmium peaks were detected at retention times of 11.4, 20.1 and 21.0 min in both chromatograms. Those peaks correspond to the void volume of the column, and metallothionein-II and -I in the eluted order. The three peaks were eluted exactly at the same retention times as those in the liver supernatant and the two cadmium peaks at retention times of 20.1 and 21.0 min were assigned to metallothionein-II and -I, respectively. This is the first demonstration for the detection of metallothionein in thymus at isometallothionein level. A protein peak at a retention time of 16.7 min was assigned to hemoglobin, the peak being highest in the spleen supernatant reflecting the highest iron concentration among the four organs.

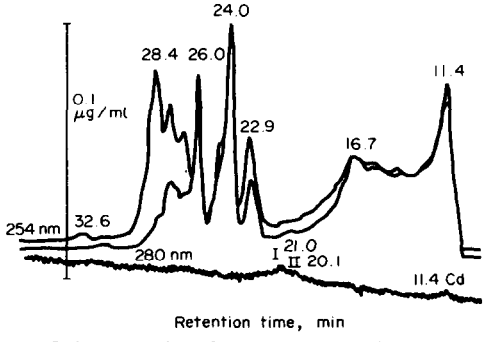

Fig. 1. Gel permeation chromatogram of thymus supernatant. Fifteen mice were injected with cadmium and sacrificed 3 days after the injection. Fifteen thymuses were combined and homogenized in five volumes of buffer solution. The homogenate was centrifuged at 105,000 *g* for 60 min. A 0.2 ml portion of the supernatant was applied to an SW column and the column was eluted with 50 mM Tris–HCl buffer solution. The detector level of a flame atomic absorption spectrophotometer was set as indicated by a vertical bar. I (21.0 min) and II (20.1 min) indicate metallothionein-I and -II, respectively.

DISCUSSION

Injection of cadmium into mice induced the dramatic decrease of thymus weight which was characterized by the preferential involution of cortex [4]. A similar atrophy of thymus has also been observed for a zinc deficient mouse and characterized by the same preferential involution [3]. Although thymus showed a similar atrophy upon both cadmium loading and zinc deprivation, spleen showed completely different responses: increased weight upon cadmium loading [4] and decreased weight upon zinc deprivation [2]. The atrophy of thymus during zinc deficiency was supposed to occur due to the elevated corticosterone levels [3]. The elevated level of glucocorticoid hormones can also be observed by various stresses which are known to cause a reduced zinc concentration in serum and to induce hepatic metallothionein synthesis [11, 12]. Zinc concentration in serum also decreases upon cadmium loading

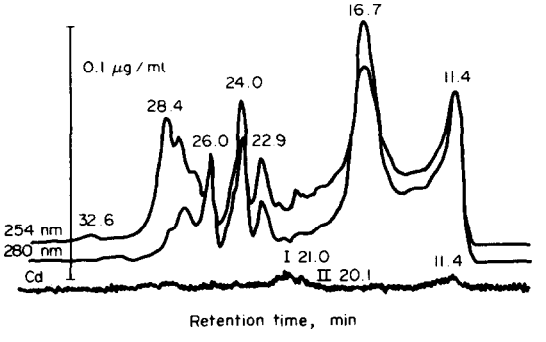

Fig. 2. Gel permeation chromatogram of spleen supernatant. Fifteen spleens were obtained from the same animals as shown in Fig. 1 and homogenized in five volumes of buffer solution. A 0.2 ml portion of the supernatant was applied in the same way as shown in Fig. 1. I (21.0 min) and II (20.1 min) correspond to metallothionein-I, and -II, respectively.

to rats [7]. Regulation of metallothionein synthesis by glucocorticoids has been actively investigated using cell cultures in relation to zinc metabolism [13, 14] and primary induction of metallothionein by a glucocorticoid, dexamethasone, was suggested [15]. Therefore, although more detailed data such as zinc concentrations in thymus and spleen during zinc deprivation, and glucocorticoid levels after cadmium loading are required to estimate the relations, some relations may probably be expected from the atrophy of thymus caused by zinc deprivation and cadmium loading via zinc and/or glucocorticoids.

The present study revealed the changes of essential metals not only in the thymus and spleen but also in the liver and kidney after cadmium loading, the former organs being affected more severely and cadmium being accumulated most heavily in the latter organs. The essential metals in the two immune organs showed dramatic changes in their concentrations and/or contents reflecting the dramatic decrease or increase of the organ weight. On the other hand, although the essential metals in the liver and kidney changed significantly from controls, the changes were not so dramatic as in the two immune organs showing more selective effects to the immune organs despite the low accumulation of cadmium.

Among the essential metals in the liver and kidneys, zinc showed most significant increase both in the concentration and content. Most of the increased zinc was bound to metallothionein with cadmium. On the other hand, zinc in the thymus and spleen was not affected in concentration, but severely affected in content by cadmium loading due to the dramatic decrease or increase of the organ weight. The proportional changes of zinc contents with the changes of the organ weight suggested the overall change of zinc in both organs and not due to the changes of zinc in special chemical forms. Magnesium in the supernatant fractions of liver, kidney, and spleen of rats is mainly present in the lower molecular weight fraction which includes free metallic ions, and is completely different in subcellular distributions from zinc [9]. However, the change of magnesium in the thymus and spleen of mice in the present experiment was almost the same as that of zinc.

The changes of iron in the thymus were not proportional to the decrease of the weight and may suggest a different distribution of iron from zinc between cortex and meddull. In the spleen, one of the most important organs for iron metabolism, iron may be handled quite differently from zinc and magnesium upon cadmium loading. Spleen maintained the iron content at the same level as control despite a dramatic increase of the weight. Liver also maintained the iron content as spleen did, though the content changed differently in the two groups one day after the injection of cadmium. Although the chemical forms and roles of calcium were not investigated in this study, calcium seems to increase in concentration and/or content with acute changes of organ weights by cadmium loading. As observed previously in a different mouse strain [5], the copper content of the kidneys did not increase with the accumulation of cadmium.

The chemical forms of cadmium in the supernatant fractions of the thymus and spleen were investigated by HLC–AAS. As the sizes of the two organs, especially thymus, were small and the accumulation of cadmium was low, it was challenging to detect the chemical forms in the two immune organs. Cadmium present in the applied supernatant fractions was only 56 ng and the samples were applied without any preliminary fractionations. Although an application of pre-fractionated and concentrated metallothionein fraction of the thymus extract may certainly give a better chromatogram, Figs 1 and 2 demonstrated that the chemical forms of cadmium in small organs of low accumulation can be investigated routinely by HLC–AAS. In contrast, the detection of isometallothioneins by a combination of the usual gel filtration and ion exchange chromatographic procedures would require at least several hundred μg of protein-bound cadmium. Thus, as the accumulated amount of cadmium in the thymus was as low as 26 ng, several hundred mice would be necessary for the identification of isometallothioneins in this organ by these conventional techniques.

The distribution profiles of cadmium in Fig. 2 indicated the presence of the two isometallothioneins in the spleen of singly cadmium-injected mouse as also observed in the spleen of repeatedly cadmium-injected rats [9]. The presence of a large amount of haemoglobin (retention time 16.7 min; Fig. 2) may suggest a certain extent of contamination of the spleen supernatant with blood and hence a possibility of an exogenous origin of metallothionein (metallothionein in erythrocytes). This seems improbable, however, since the cadmium concentration in blood usually is less than 0.1 μg/ml. Appreciably higher concentrations (e.g. 1.7 μg/ml blood) have been observed only after repeated injections of cadmium when damage to the liver and kidneys has occurred [16].

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