and H2B genes of yeast are adjacent on the genome[23] whereas in calf thymus and other higher orgnism the two genes are repeated and present on different chromosomes.

H3 and H4 histones are the most conserved of all known proteins, and they also are the most similar as between calf thymus, *Physarum* and *Dictyostelium* as judged by electrophoretic mobility. However, even with these highly conserved histones, the mobility of H3 is slightly variable, being slower in SDS when derived from *Dictyostelium* than from *Physarum*. Both yeast and *Neurospora* have closely similar H3 and H4 histones, and in both organisms the single copy genes are known to be linked and, uniquely amongst histone genes, to possess introns[23].

Conclusion

Although the cellular and acellular slime moulds *Dictyostelium* and *Physarum* are probably not closely related, both are simple eukaryotes. Their histones, as judged by electrophoretic separation, are closely similar, only H1 showing any considerable divergence, and H2A rather less. Both slime moulds yield histones with substantially different migration patterns from calf thymus. H3+H4 are the most strongly conserved in evolution and, therefore, it is not surprising that they show minimal charge differences. H2A and H2B are somewhat less conserved and here differences from the calf thymus pattern are detectable, especially with H2A. The non-core particle histone H1 is the least conserved and shows considerable differences in charge both as between the two slime moulds, and between either slime mould and calf thymus.

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Uptake and toxic effects of heavy metal ions: Interactions among cadmium, copper and zinc in cultured cells

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Summary. Absorption of metal ions by KB, HeLa and L-59 cells has been analyzed by atomic absorption spectrophotometry in the course of culture. Ions of the elements of the fourth period in the periodic chart such as Fe(II), Cu(II), Zn(II), Mn(II) and Ni(II) were not taken up, but those of the higher periods, such as Cd(II), Pb(II), Hg(II) and Ag(I) were were taken up easily. The uptake behavior by the cultured cells was in accordance with the characteristic features of metals, that metals in the fourth period are essential elements, and most of the elements of the fifth and the sixth periods are non-essential or toxic elements.

The initial rate of Cd(II) uptake and the Cd(II) concentration has a sigmoidal relationship. Cd(II) was absorbed homotropically through cell membranes. The uptake of Cd(II) was specifically inhibited by Cu(II), but was affected little by Zn(II). The toxicity of Cd(II) to KB cells was greatly enhanced in the presence of Cu(II). On the contrary, the toxicity of Cd(II) was reduced by the addition of Zn(II) at several concentrations of Cd(II). The toxicity of Cd(II) did not depend on the amount of Cd(II) absorbed in the cells, but was determined by cofactors such as Cu(II). The interaction between Cd(II) and Cu(II) may be important for Itai-itai disease.

Key words. Cd uptake; heavy metal uptake; essential and toxic metal; metal interaction; heavy metal toxicity.

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Introduction

A lot of investigations have been carried out to reveal the essentiality and toxicity of heavy metals, using cells in culture [1-7]. Also, considerable attention has been given to the differences between essential and toxic elements[8-11]. Schroeder pointed out that the toxicity of abnormal trace metals in the body is associated only with their excess and accumulation in tissues due to aging or inadequacy of the excretory mechanism[12]. Essential trace metals are, of course, harmful when they are present in excess or are deficient. It was reported that the difference between toxic and essential elements lay in the log-normal and normal distribution of these elements respectively[8].

To study the distinction between essential and toxic metal ions on the cellular level in vitro, we have carried out uptake experiments on such metal ions as cadmium, lead, mercury, silver, iron, copper, zinc, manganese and nickel, using several kinds of cell lines.

Biological interactions between toxic and essential elements have been studied intensively[13-16]. Especially in relation to Itai-itai disease, the interactions between cadmium and copper, and cadmium and zinc have aroused considerable interest[17-19]. It has been shown that cadmium ions are taken up by cultured cells and fixed within the cells[20,21]. The uptake of cadmium was inhibited by the addition of copper ions in the culture medium, but was not affected by the addition of the other micro-essential metal ions[22,23]. Moreover, cadmium which had already been taken up by the cells was released to the culture medium on the addition of copper ions[23]. The mechanism of this specific interaction between cadmium and copper has not been revealed.

The present report describes the uptake of heavy metal ions and the interaction among cadmium, copper and zinc in the metal uptake by cultured cells, in addition to the toxicities of these metal ions.

Materials and methods

Human carcinoma cell lines, KB and HeLa, and a mouse carcinoma cell line, L-59, were grown as monolayer cultures. Eagle's minimum essential medium, supplemented with 100 units/ml of penicillin, 100 µg/ml of streptomycin and 2% newborn calf serum (Microbiological Associates, Maryland), was used as the growth medium. Cells (5×10^4 in 2 ml of growth medium) were distributed in tissue culture tubes 15 × 150 mm) and incubations were performed at 37 °C by the stationary culture method.

Forty eight hours after the inoculation of the cells into tissue culture tubes, growth medium was replaced by fresh medium containing one or two kinds of metal ions such as Cd(II), Pb(II), Hg(II), Ag(I), Fe(II), Cu(II), Zn(II), Mn(II), Co(II) and Ni(II). Two to four replicated cultures were harvested as indicated below for the measurement of cell growth and metal uptake into cells. The medium was transferred to a clean tube and cells attached to the wall of the tube were washed twice with 2 ml of phosphate-buffer saline without Ca(II) and Mg(II) [PBS(–)]. Washed cell monolayers were used for the measurement of the cell growth and the measurement of the content of metal ions. Growth of the cells was estimated by measuring the protein content by the method devel-

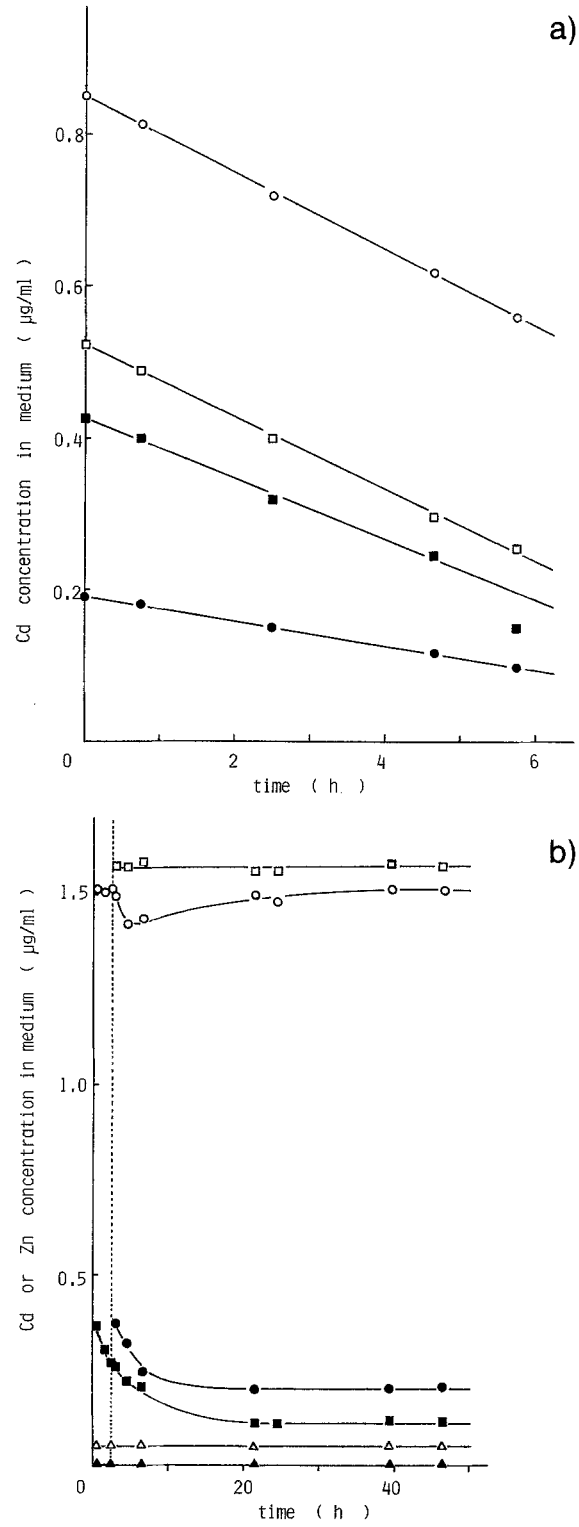

Figure 1. *a* Time course of changes of Cd(II) concentration in the culture medium due to the uptake by KB cells in the presence of Cu(II) (0.08 µg/ml). The initial concentrations of Cd(II) were 0.85 (—○—), 0.52 (—□—), 0.42 (—■—) and 0.19 µg/ml (—●—). Each plot was the mean of 3 replicate cultures. *b* Uptake of Cd(II) by KB cells in the presence of Zn(II) which was added to the culture medium before or after the addition of Cd(II). Zn(II) (1.5 µg/ml, —○—) was added 2.5 h before Cd(II) (0.4 µg/ml, —●—) addition. Zn(II) (1.6 µg/ml, —□—) was added 2.5 h after Cd(II) (0.4 µg/ml, —■—) addition. Zn(II) (—△—) and Cd(II) (—▲—) concentrations in the medium of the control culture were also shown.

oped by Lowry et al., using bovine serum albumin as the standard[24]. The concentration of the metal ions in the culture medium was determined directly using an atomic absorption spectrophotometer (AAS) (Hitachi 180; Nippon Jarrell-Ash 855). The initial rate of Cd(II) uptake was determined with and without Cu(II) addition at several concentrations of Cd(II) from 0.2 to 0.8 μg/ml. The amount of Cd(II) taken up by the cells was measured indirectly by the decrease of Cd(II) concentration in the culture medium[20].

The compounds used were $Cd(NO_3)_2 \cdot 4\ H_2O$, $Pb(NO_3)_2$, $Hg(NO_3)_2$, $AgNO_3$,$FeSO_4(NH_4)_2SO_4 \cdot 6\ H_2O$, $CuSO_4 \cdot 5\ H_2O$, $ZnSO_4 \cdot 7\ H_2O$, $MnSO_4 \cdot 4\ H_2O$, $Co(NO_3)_2 \cdot 6\ H_2O$, and $NiSO_4 \cdot 6\ H_2O$ (Wako Chemical, Osaka); the reagents were used without further purification. Each metal solution was added to the culture medium just prior to addition to the culture. The concentration of the stock solution of each metal ion was 1000 μg/ml in distilled water. The standard solution for the analyses by AAS was obtained from Wako Chemical, Osaka.

Results

Uptake of metal ions by cultured cells

The uptake experiments with Cd(II), Pb(II), Ag(I) and Hg(II) were carried out at several concentrations which were in the ranges of 0.2–1.2 μg/ml of Cd(II), 0.5–2.0 μg/ml of Pb(II), 0.5–5.0 μg/ml of Ag(I) and 0.3–1.0 μg/ml of Hg(II), respectively. In each of the experiments the absorption of these metal ions was observed. The uptake was initiated soon after the addition of the metal ions and reached a stationary state at about 10 h. The initial metal concentrations in the growth medium and the metal uptake are shown in the table for KB, HeLa and L-59 cells.

For KB cells, 50–60% of Cd ions which were added to the culture medium were taken up by the cells, and 20–40% of Pb, 15–18% of Ag and over 30% of Hg in the culture medium were taken up by the cells in replicate cultures. The uptake of these metal ions by HeLa and L-59 cells was similar to those by KB cells. No changes in the concentrations of these metal ions were observed in the culture medium without cells. The increase in the content of these elements in cells corresponded to the decrease in the concentration in the culture medium. It has already been reported that counter ions such as NO_3^-, SO_4^{2-} and Cl^- did not influence the absorption of metal ions[21].

On the other hand, the decrease of the concentrations of Fe(II), Cu(II), Zn(II), Mn(II), Co(II) and Ni(II) in the culture medium was not observed for any of the cultured cells at several concentrations from 0.2 to 5.0 μg/ml, as shown in the table. Standard deviations of the measurements on replicate cultures were within 0.05 μg/ml. The content of these elements in cells was so small that changes of the concentration of these metals in the culture medium due to the cell growth could not be observed. KB, HeLa and L-59 cells revealed distinct differences in the metal uptake between essential and toxic metal ions under these culture conditions.

Uptake of Cd in the presence of Cu and Zn

The Cd(II) concentration in the culture medium was measured for several hours in the presence of Cu(II) which was added simultaneously with Cd(II) as shown in figure 1a. The initial Cd(II) concentrations were 0.19, 0.41, 0.53 and 0.85 μg/ml. The initial rates of Cd(II) uptake were measured from the slopes of the decreases of the Cd(II) concentrations in the culture medium. When

Metal uptake by cultured cells

Metal ions	KB Initial metal concentration (μg/ml)	Uptake[a] (μg/tube)	HeLa Initial metal concentration (μg/ml)	Uptake[b] (μg/tube)	L-59 Initial metal concentration (μg/ml)	Uptake[a] (μg/tube)
Cd	0.46	0.52	0.47	0.50	0.41	0.18
	0.26	0.28	0.29	0.38	0.25	0.14
	0.12	0.14	0.13	0.08		
Pb	0.38	0.18	0.97	0.42	0.70	0.15
	0.19	0.16	0.50	0.30		
			0.19	0.12		
Hg	0.67	0.50	–	–	0.79	0.94
	0.41	0.26				
Ag	0.31	0.09	0.17	0.06	0.38	0.10
	0.14	0.05	0.11	0.04		
Fe	2.2	0.04	0.45	0.05	0.32	ND
			0.13	0.01	0.19	ND
Cu	6.0	ND	0.42	ND	0.80	ND
	1.5	0.04	0.15	ND	0.38	ND
Zn	1.0	ND[c]	0.51	0.03	0.84	ND
			0.22	0.01	0.41	ND
Mn	2.2	ND	0.49	ND	0.58	ND
			0.09	ND	0.11	ND
Co	2.3	ND	–	–	–	–
Ni	1.4	ND	0.42	0.02	0.49	ND
			0.11	0.01	0.27	ND

[a] Metal uptake in 10 h of incubation. The values were the means of 2 replicate cultures. [b] Metal uptake in 30 h of incubation. The values were the means of 2 replicate cultures. [c] The uptake was not detected within the sensitivity.

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the uptake rate was plotted against the initial Cd(II) concentration, sigmoidal relations were obtained in measurement both with and without Cu(II) addition. Transport of a metal ion through the cell membrane may be written as equation 1,

$$M + D \rightleftharpoons D \cdot M \rightarrow D + M' \quad (1)$$

where M is metal ion outside the cells, D is membrane and M' is metal ion inside the cells. When several metal ions are transported through a cell membrane homotropically, equation 2 is derived.

$$n\,M + D \rightleftharpoons D \cdot M_n \rightarrow D + n\,M' \quad (2)$$

where n is the number of metal ions which are transported simultaneously. The rate of the ion transport according to equation 2 is expressed as equation 3,

$$\log \frac{V}{V_{max} - V} = n \log[M] - \log K' \quad (3)$$

where V is a rate of metal uptake, V_{max} is a maximum rate, K' is a constant, and [M] is the concentration of ions outside the cells. In figure 2a $\log V/(V_{max} - V)$ was plotted against log [M]. The value of n was estimated as 2 from the slope of the line. The plots of reciprocal rate of Cd(II) uptake (V^{-1}) against $[Cd]^{-2}$ were approximately straight lines (figure 2b).

When Zn(II) was added to the culture medium 2.5 h after the addition of Cd(II), Cd(II) uptake was not affected, and the Zn(II) concentration in the culture medium was constant within the limits of experimental error. On the contrary, when Zn(II) was added prior to the Cd(II) addition, Cd(II) uptake was reduced to 68% and a small decrease of Zn(II) concentration in the culture medium was observed several hours after the addition of Cd(II), and in 20 h the Zn(II) concentration came back to the initial level (figure 1b).

Toxicity of Cd, Cu and Zn for KB cells

The effects of metal ions on the cells were determined in 48 h in contact with Cd(II) at several concentrations less than 1.0 µg/ml in the presence of Cu(II) and Zn(II) ions. A synergic interaction between Cd(II) and Cu(II) in toxicity for KB cells has been observed. In the presence of even a small amount of Cu(II) the toxicity of Cd(II) was greatly enhanced, as shown in figure 3a. On the contrary,

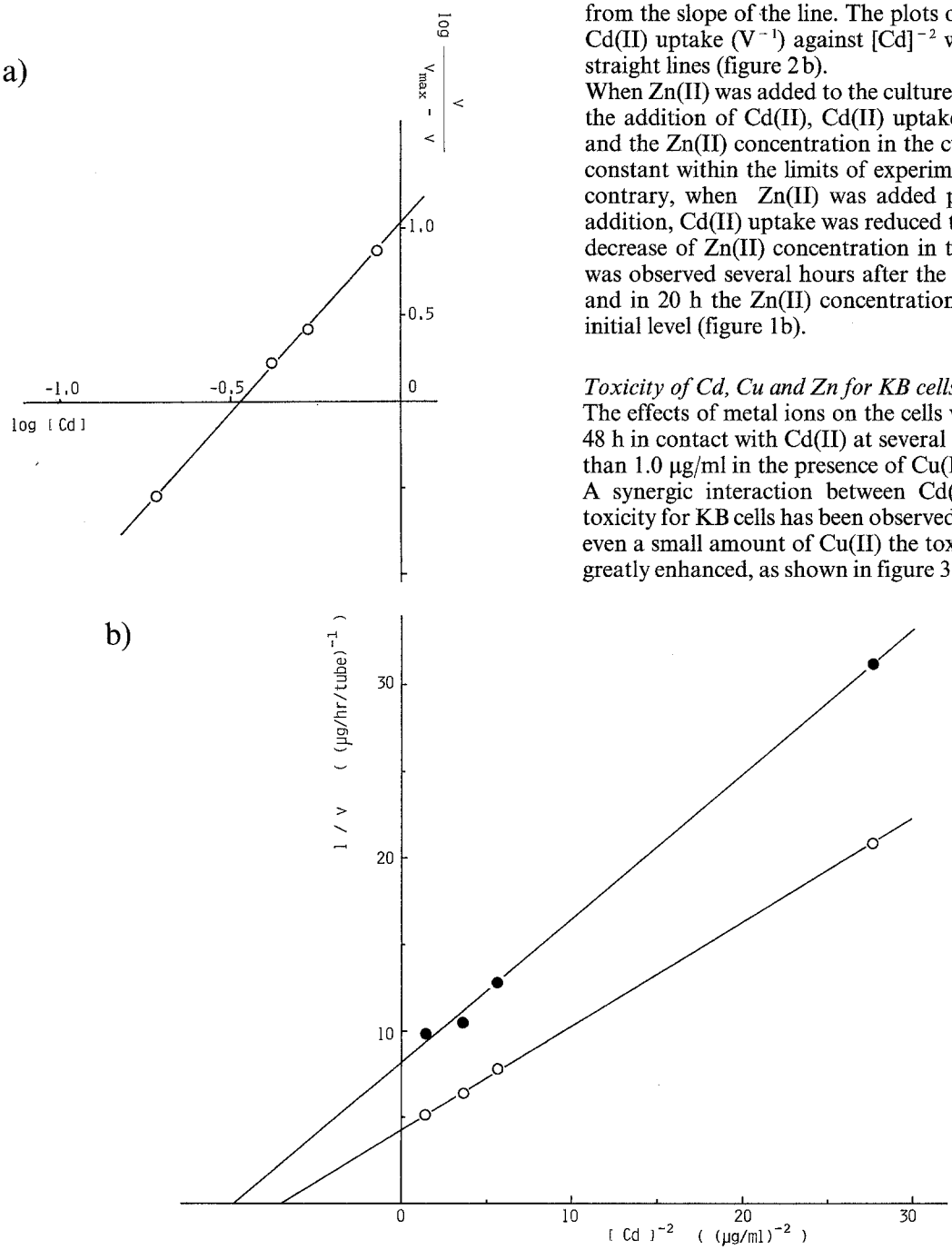

Figure 2. The relation between Cd(II) initial concentrations and initial rates of Cd(II) uptake. *a* Plots for the Cd(II) uptake according to equation 3. *b* Double reciprocal plots for the inhibition of Cd uptake with Cu(II) (0.08 µg/ml) (—●—), and without Cu(II) addition (—○—).

KB cells were almost completely damaged at the Cd(II) concentration of 1.0 μg/ml, but the proliferation of the cells was restored in the presence of 5 μg/ml of Zn(II) as shown in figure 3 b.

Discussion

The results presented in this report indicate that ions of elements such as Fe(II), Cu(II), Zn(II), Mn(II), Co(II) and Ni(II) in the fourth period in the periodic table were apparently not absorbed by the cultured cells, but that those in the fifth and sixth periods such as Cd(II), Pb(II), Hg(II) and Ag(I) were absorbed easily under these culture conditions. The uptake of Zn(II) and Cd(II) in animal experiments has been studied intensively[25-27]. Both Zn(II) and Cd(II) are absorbed, but Cd(II) was accumulated much more than Zn(II) in the liver, kidney and other organs. This fact is in good agreement with the observations of the uptake of Zn(II) and Cd(II) in vitro[20,21,28]. The uptake of Zn(II) by mouse 3T3 cells was observed by Schwarz et al.[29]. However the amount of Zn(II) taken up by the cells was much smaller than that of Cd(II) in our experiments. Zn(II) concentration seemed to be controlled strictly by the homeostatic mechanism. In the same way, essential ions such as Fe(II), Cu(II), Mn(II), Co(II) and Ni(II) may be protected by cell membranes. In contrast, there may be no homeostatic controls for the metal ions such as Cd(II), Pb(II), Hg(II) and Ag(I). It is interesting that these metals are toxic and are notorious elements for causing environmental pollution. Metallothionein is induced not only by several toxic metals but also by the several essential metals[30,31], so the distinctive difference in the metal uptake by cultured cells can probably not be explained by the induction of metallothionein. KB, HeLa and L-59 cells are all transformed cell lines which are quite different from normal cells in nature, and the culture conditions such as the serum concentration were chosen to be suitable for metal uptake experiments. Nevertheless, it is important that distinctive phenomena were observed in several cell lines. Uptake of Cd(II) by cultured cells has been studied intensively[20,21,32]. When KB cells which were cultured with Cd(II) were immersed in 10% EDTA (disodium ethylenediaminetetra-acetic acid) in PBS(–), the rate of release of Cd(II) was very low. However, when the cells containing Cd(II) were immersed in 5% EDTA in distilled water, half of the Cd(II) in the cells flowed out suddenly. In that case, the cell membrane was burst and the shape of the cell was changed. Therefore a greater part of the Cd(II) which was taken up by the cells was not adsorbed on the cell surface but absorbed into the cells[21]. It has been reported that enough RNA for the synthesis of metallothionein is produced within 2 h after contact with Cd(II) and that 90% of cellular Cd(II) is located in the cytosol as Cd-metallothionein[33]. It was also reported that the absorbed Pb(II) in cells was fixed in an insoluble complex[3].

A method of kinetic analysis of enzymatic reactions has

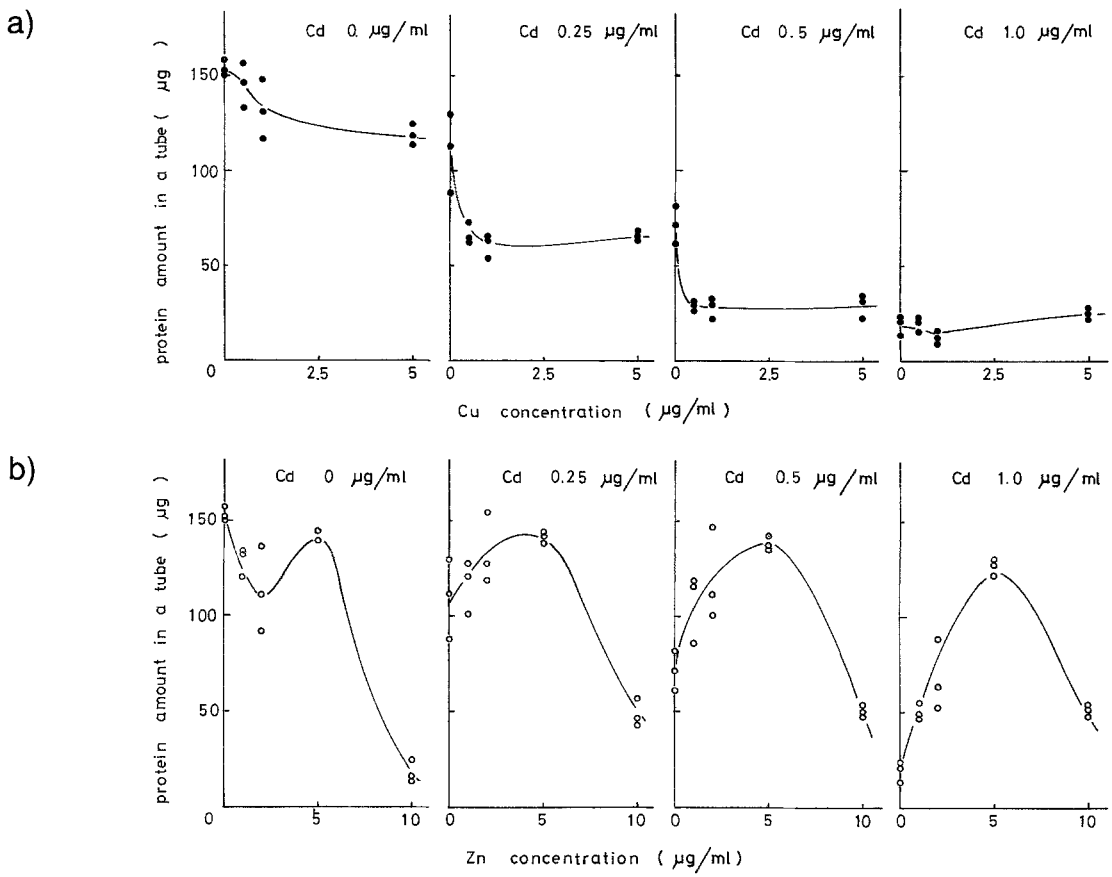

Figure 3. The toxicity of Cd(II) for KB cells in the presence of Cu(II) or Zn(II). Toxicities were estimated by the measurement of the cell growth by the protein content in a tube, 48 h after the addition of the metal ions. *a* Cd(II) and Cu(II), and *b* Cd(II) and Zn(II).

been applied to metal ion transport through a cell membrane[34, 35]. The metal transport process was explained successfully by the Michaelis-Menten kinetics of enzyme reactions which have hyperbolic relations between metal concentrations and rates of metal uptake. Although there has been no evidence so far to demonstrate that some proteins function as carriers of heavy metal ions through a cell membrane, a carrier (D) was assumed in this case, to derive equation 1[36, 37]. Cd(II) uptake by KB cells showed a sigmoidal type of relation between Cd(II) concentrations and initial rates of Cd(II) uptake. Michaelis-Menten kinetics cannot be applied to enzyme reactions in cases where there is a sigmoidal relation between the reaction rate and the substrate concentration. Equation 3 was therefore derived, assuming that several metal ions were transported simultaneously, as for equation 2. The value of n was estimated to be 2. It followed that the Cd(II) uptake might take place in a cooperative process in which some kind of carrier protein took part.

The maximum rate of uptake was reduced by the inhibition of Cu(II) (figure 2b). The effect of Cu(II) on Cd(II) uptake could not be explained by a competitive mechanism in which the maximum rate of reaction was maintained in the inhibitory process, by analogy with enzyme kinetics. Analysis of the inhibition kinetics provides no evidence for an un-competitive or non-competitive mechanism either. Therefore it was supposed that it might not be possible to explain the effect of Cu(II) on Cd(II) uptake as a simple process.

The effect of Zn(II) on Cd(II) uptake was small compared with that of Cu(II) on Cd(II)[23]. A slight change of Zn(II) concentration during the Cd(II) uptake and the small reduction of Cd(II) absorption by the pre-addition of an excess amount of Zn(II) showed a weaker interaction between Zn(II) and Cd(II).

The toxicity of Cd(II) was greatly enhanced in the presence of Cu(II). On the contrary, the toxicity of Cd(II) was reduced at a suitable concentration of Zn(II). The uptake of Cd(II) was specifically inhibited by the addition of Cu(II)[22]. In other words the toxicity of Cd(II) to the cells was very severe without the absorption of Cd(II), when Cd(II) was added in the presence of Cu(II). And the toxicity to the cells became weaker in spite of the absorption of Cd(II), when Cd(II) was added in the presence of Zn(II) (figure 1b)[23]. The toxicity of Cd(II) to cells was not determined by the amount of Cd(II) which had been taken up into the cells. It was supposed that Cd(II) might affect the function of the cell membrane to exert its toxic effects.

The toxicity of Cd(II) is of interest with respect to Itai-itai disease, which occurs in post-menopausal women with several deliveries[38]. It has been pointed out that the copper concentration in the serum of a pregnant woman increases more than twice as much as that of a non-pregnant woman[10]. Therefore, an enhancement of toxicity to the cells by the addition of Cd(II) and Cu(II) may be important for Itai-itai disease.

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