SOLUBILITY AND CELL TOXICITY OF CADMIUM

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Cadmium (Cd), Cd fume and cadmium oxide (CdO) were more easily dissolved in protein and amino acid solution such as fetal calf serum, albumin, glycine or neutralized cysteine monohydrate solution than in water or Tyrode's solution. But, there was no difference in the dissolution of cadmium sulphite (CdS) between in the former solutions and in the latter. The solubility of Cd, CdO or Cd fume in albumin and glycine solution increased with increasing their concentration.

When the Cd dissolved in protein or amino acid solution was added to the cultures, these dissolved Cd showed toxic action on the FM3A cells in proportion to the amount of Cd value irrespective of the sort of the solvent.

On the column chromatographs by gel filtration on Sephadex G-200, the Cd dissolved in albumin solution or fetal calf serum coexisted with albumin fractions. In the case of the culture medium containing the Cd dissolved in albumin solution or fetal calf serum, the Cd was also associated with albumin fractions. When, however, metallothionein-Cd was added to the cultures, the Cd in the culture medium was combined mainly with metallothionein fractions and partly with albumin fractions and the Cd bound with thionein did not show the toxic action on the FM3A cells.

In the previous papers¹⁾²⁾, it was observed that the solubility of Cd fume and CdO in Tyrode's solution or distilled water was remarkably enhanced by the addition of serum, albumin or amino acids such as glycine, alanine or cystein monohydrate and the dissolved Cd in protein and amino acid solutions showed toxic action on the macrophages and 3T6 fibroblasts in culture.

In this paper, the increased solubility of metallic Cd and some particulate Cd compounds in protein or amino acid solution was confirmed and then it was studied whether the Cd dissolved in protein or amino acid solution was combined with protein or not in the cultures. Furthermore, it was studied whether or not Cd-protein complex was less toxic than the equivalent amount of ionic Cd²⁺, if Cd was combined with protein in the cultures.

MATERIALS AND METHODS

Chemicals and biological materials

Cd fume: The Cd fume was generated from Cd metal with the high frequency

induction furnace which was described in the previous papers³⁾⁴⁾. The purity grade of the sample metal of Cd was 99.999%. The particle size (geometric) of Cd fume used in this experiment was 0.72–1.12 micron.

Cd powder: Metallic Cd powder was purchased from Wako Pure Chemical Industries Ltd. and the Cd powder was sieved below 325 mesh before use.

CdO, Cd chloride ($\text{CdCl}_2 \cdot 2\frac{1}{2}\text{H}_2\text{O}$) and Cd sulphide (CdS): The former two samples were purchased from Wako Pure Chemical Industries Ltd. (special grade) and the latter from Koso Chemical Co Ltd.

Metallothionein Cd: This was kindly presented by Dr. M. Kimura in our Institute. This metallothionein-Cd was prepared from rabbit liver and the preparing method was described in his paper⁵⁾.

Albumin: The crystalline bovine albumin was purchased from Sigma Chemical Company.

Fetal calf serum: Sterile fetal calf serum was purchased from Gibco Company.

Glycine and cysteine monohydrate: These were purchased from Wako Pure Chemical Company. (special grade)

The solubility experiment

Triple distilled water, Tyrode's solution, fetal calf serum and 2% albumin, 2% glycine or 2% neutralized cysteine monohydrate water or Tyrode's solution was used as the solvents.

Two milligrams of each Cd sample were suspended in 10 ml of each solution and then these suspensions were dispersed for 2 minutes by ultrasonic vibration (29 KHz. 45 W). These dispersed suspensions were centrifuged at 60,000 r.p.m. for 30 min to separate the supernatant. The Cd content of each supernatant was determined by an atomic absorption spectrometer with an acetylene flame (Hitachi Type 308).

Cell toxicity and the determination of the amount of Cd in the culture medium.

Effects of the Cd samples dissolved in various solvents on the cultured FM3A, (a C3H mouse mammary carcinoma cell line) were examined. The cells were cultured in minimal essential medium (MEM) supplemented with 10% fetal calf serum. Each sample of Cd dissolved in various solvents was added to the cultures after sterilization by filtration through Millipore membrane filter (0.22 micron pore size). The cell suspensions (1×10^5 cells/ml) containing various concentrations of each dissolved Cd sample were incubated at 37°C in an atmosphere of 5% CO_2 in air. In the control, the same amounts of the each solvent solution were added to the cultures. After incubation for 48 hours, the number of cells was counted in a hemocytometer.

After the determination of the cell toxicity the cultures were centrifuged at 1,500 r.p.m. for 5 min to remove cells. The amount of Cd in each supernatant was determined with an atomic absorption spectrometer.

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Column chromatography

This was done on columns of Sephadex G-200 obtained from Pharmacia Fine Chemicals which were packed in 2.5×50 cm column. Elution was performed with 0.1 M Tris-HCl buffer (pH 8.0) containing 0.02% sodium azide. The flow rate was kept at 20 ml/hr. The fractions were collected with a fraction collector (LKB Co.) and each fraction volume was kept at 5.1 ml. The protein concentration in the collected fractions was determined as absorbancy at 280 m μ or 250 m μ with a spectrophotometer and the Cd content in these fractions was determined with an atomic absorption spectrometer. The Cd dissolved from CdO in fetal calf serum and 2% albumin Tyrode's solution were used as samples. The supernatants of the culture medium were also used as samples. After determination of cell toxicity, the cultures were centrifuged at 1,500 r.p.m. for 5 min to remove the cell and the supernatant was submitted to gel filtration.

In this column, γ -globulin (molecular weight (M.W.) 160,000), albumin (M.W. 60,000), and myoglobulin (M.W. 16,000) could be separated as protein of known molecular weight and it was confirmed that the correlation between the elution volume and molecular weight was linear.

RESULTS

1. Dissolution of Cd samples

The results obtained in the solubility experiment of Cd, Cd fume, CdO and CdS are shown in Table 1. The supernatants from the suspensions of the various samples in water contained very small amounts of Cd. When the fetal calf serum was used as solvent, the amounts of Cd in the supernatants from Cd, Cd fume and CdO suspensions were about 5 to 20 times as much as those in supernatants in water. When the albumin, glycine or neutralized cysteine monohydrate solution was used as solvent, the amounts of Cd in the supernatants from Cd, Cd fume and CdO suspensions was higher than in the serum. While in the case of CdS, there was no significant difference in the amount of dissolved Cd between in water and in protein or amino acid solution. The dissolutions of Cd samples in Tyrode's solution were about equal to those in the water. When Tyrode's solution containing protein or amino acid was used as solvents, the dissolutions of above Cd samples were about equal to those in water containing amino acid or protein.

Table 1. The amounts of Cd dissolved in various solvents

Sample \ Solvent	Water ($\mu\text{g/ml}$)	Fetal calf serum ($\mu\text{g/ml}$)	2% albumin ($\mu\text{g/ml}$)	2% glycine ($\mu\text{g/ml}$)	2% neutralized cysteine monohydrate ($\mu\text{g/ml}$)	0.01% NaEDTA ($\mu\text{g/ml}$)
Cd	2.3	11.9	29.3	84.9	91.8	99.2
Cd fume	3.2	34.9	62.0	112.5	119.7	107.2
CdO	1.0	21.5	66.5	147.8	151.2	117.2
CdS	1.1	1.2	2.5	1.2	1.9	7.2

Fig. 1. Solubility and solvent concentration

Fig. 2. Solubility and solvent concentration

2. Relation between the solubility and solvent concentration

Water solution of various concentrations of albumin and glycine were used as solvent. The albumin or glycine concentrations were 0.001, 0.01, 0.1, 1 and 10%. The experimental results are shown in Figs. 1 to 4. As seen in the Figs. 1 to 3, the dissolution curves of Cd against concentration of glycine and albumin showed different patterns. In the glycine solution, the dissolution of Cd from Cd powder, Cd fume and CdO rapidly increased from 0.01% and was almost saturated over 1%. In albumin solution, however, the dissolutions

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Fig. 3. Solubility and solvent concentration

Fig. 4. Solubility and solvent concentration

were very rapid over 1%, while it was slow under 1% and the dissolution increased with increasing albumin concentration until 10%. The dissolution of CdS in albumin or glycine solution did not increase in albumin or glycine concentration from 0.01% to 10% as shown in Fig. 4.

3. Cell toxicity of the dissolved Cd

The results are shown in Fig. 5. As shown in the Figure, the Cd dissolved from

Fig. 5. The cell toxicity of dissolved Cd and metallothionein-Cd.

× CdO-10% FCS MEM	△ CdO-cysteine-TS
○ Cd fume-cysteine-TS	□ CdO-glycine-TS
● CdO-albumin-TS	■ CdO-FCS
◐ CdS-cysteine-TS	⊙ CdCl ₂ in 10% FCS MEM
∇ CdO-NaEDTA	▲ metallothionein-Cd

Fig. 6. Fractionation of Cd-albumin solution by gel filtration on Sephadex G-200.

---- Cd concentration — 280 m Optical density
(The sample applied to the column contained 55 μg Cd.)

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CdO in albumin-, glycine- and cysteine-Tyrodé's solution and fetal calf serum showed cell toxicity in proportion to Cd values irrespective of the sort of the solvent. When the Cd dissolved from CdO in MEM supplemented with 10% fetal calf serum was added to the cultures, the cell toxicity of this dissolved Cd also was as strong as those in the above cases. When CdCl₂ dissolved in MEM supplemented with 10% fetal calf serum (100 µg/ml) was added to the cultures, the toxic action of this dissolved Cd was as strong as the Cd dissolved in various solvents. In the cases of dissolved Cd from Cd fume and CdS in cysteine-Tyrodé's solution, the correlations between the Cd value and cell toxicity were the same as those in the above cases.

4. Column chromatographs of dissolved Cd

The Cd dissolved from CdO in 2% albumin-Tyrodé's solution or fetal calf serum was separated by gel filtration. In the case of dissolved Cd in 2% albumin solution, the Cd was completely associated with albumin fractions as shown in Fig. 6. In the case of the dissolved Cd in fetal calf serum, the Cd was almost associated with albumin fractions as shown in Fig. 7.

After the determination of the cell toxicity, the supernatants of the cultures were separated by gel filtration. In the case of the supernatants of the culture containing Cd dissolved in 2% albumin-Tyrodé's solution or in fetal calf serum, the dissolved Cd in

Fig. 7. Fractionation of Cd-serum by gel filtration on Sephadex G-200.

---- Cd concentration — 280 m Optical density
(The sample applied to the column contained 90 µg Cd)

Fig. 8. Chromatographs of the culture medium of FM3A cells mixed with dissolved Cd.
(TS: Tyrode's solution)

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both culture mediums exclusively coexisted with albumin fractions (This fractions contained mainly albumin.) as shown in Fig. 8-1, 2. While, when Cd dissolved in glycine-Tyrodé's solution was added to the cultures, the Cd in this culture medium was not associated with any protein fractions as shown in Fig. 8-3. When the CdCl₂ with a small dose (3.88 μg) was added to the cultures, the Cd in the culture medium coexisted with albumin fractions exclusively (Fig. 8-4), but with a larger dose (12.20 μg) the Cd was mainly associated with albumin fractions and partly with low molecular fractions. (Fig. 8-5)

5. *The cell toxicity and chromatograph of culture medium containing metallothionein-Cd.*

The cell toxicities of the cultures containing metallothionein-Cd were determined and the supernatants of these culture mediums were separated by gel filtration.

The metallothionein-Cd added to the cultures was less toxic than the equivalent

Fig. 9. Chromatograph of the culture medium of FM3A cells mixed with metallothionein-Cd.
(The sample applied to the column contained 6.27 μg Cd.)

Table 2. The amounts of Cd in albumin fractions and in metallothionein fractions and cell toxicity

Amounts of metallothionein-Cd added to the cultures ($\mu\text{g}/\text{ml}$)	Cd recovered from culture medium		Cell toxicity % for control (Cell No.) (%)
	Cd-albumin ($\mu\text{g}/\text{ml}$)	Cd-metallothionein ($\mu\text{g}/\text{ml}$)	
0.45	0.13	0.29	93.0
1.10	0.26	0.78	77.5
2.20	0.44	1.70	69.2

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amount of the other dissolved Cd as shown in Fig. 5.

One of the chromatographs of these culture mediums is shown in Fig. 9. As shown in the Figure, the Cd in the medium was partly associated with albumin fractions and mainly with the fractions which corresponded with metallothionein (M.W. about 10,000). The amounts of Cd in albumin fractions and metallothionein fractions were calculated separately from each chromatograph of the medium as shown in Table 2. As shown in the Table, it was found that the cell toxicity of metallothionein Cd was parallel to the amount of Cd which was combined with albumin fractions. Thus, it was considered that Cd bound with thionein in the culture medium did not exert toxic action on FM3A cells.

DISCUSSION

The present series of experiments indicate the following results:

- 1). Cd, CdO or Cd fume were more easily dissolved in protein or amino acid solution than in water or Tyrode's solution. But, there was no difference in the dissolution of CdS between in the former solutions and in the latter. The solubility of Cd, CdO or Cd fume in albumin and glycine solution increased with increasing their concentration.
- 2). When the Cd dissolved in protein or amino acid solutions was added to the cultures, these dissolved Cd was found to be combined with protein or amino acid in the culture medium when observed after a 48 hr-incubation, as judged on the results of gel filtration of Sephadex G-200.
- 3). When CdCl₂ dissolved in MEM supplemented with 10% fetal calf serum was added to the cultures, Cd was combined with albumin fractions in the case of adding a small dose of CdCl₂, and in the case of a large dose, it was mainly combined with albumin fractions and partly remained in a free state.
- 4). The Cd dissolved in various solvents showed toxic action on the FM3A cells in proportion to the amount of Cd irrespective of the sort of the solvent.
- 5). Metallothionein-Cd was less toxic than the other dissolved form of Cd when compared at the equivalent amount of Cd. In the culture medium metallothionein-Cd was partly combined with albumin fractions and mainly with metallothionein fractions and the toxic action of metallothionein-Cd was found in proportion to the amount of Cd combined with albumin fractions.

From the above results, it was assumed that the particulate Cd such as Cd powder, Cd fume or CdO was dissolved in culture medium containing many sorts of amino acid and further supplemented with serum. Thus, the dissolved Cd values depended on the amounts of amino acid and serum in culture medium. It was reported in the previous papers¹⁾²⁾⁶⁾ that the dissolved Cd was more toxic than particulate Cd itself. Therefore, it was quite reasonable to assume that the cellular toxicity of particulate Cd in the cultures might be varied by the composition of the culture medium.

It was reported⁷⁾ that the toxic action of metal on the cells was reduced by the addi-

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tion of serum in culture medium. But, in the present study, the results of the cell toxicity of Cd dissolved in various solvents on FM3A cells depended on dissolved Cd values irrespective of the sort of the solvent. This fact seemed to be explained by the fact that Cd dissolved in protein or amino acid solution was being released in culture medium. The present results on column chromatographs, however, showed that in the case of the culture containing Cd dissolved in fetal calf serum or albumin solution, the Cd in the medium coexisted with albumin fractions when examined after the determination of cell toxicity. In the case of the culture containing Cd dissolved in glycine-Tyrode's solution, the Cd was not combined with any protein fractions. In this case it was considered that Cd may be combined with glycine because in the case of CdCl_2 , the Cd was mainly combined with the albumin fractions. Therefore, it was assumed that in the case of glycine, if Cd were free, perhaps the Cd should be associated with albumin fractions. In the column chromatographic procedure the Cd dissolved in the various solvents was exposed to 0.1 M Tris HCl buffer on the column, it was, at present, obscure whether or not the Cd was combined with protein or amino acid on the column for above procedure. At least, if the results of column chromatograph were true, it was considered that the dissolved Cd combined with albumin fractions and dissolved Cd in free state had the same toxic action on the FM3A cells. These facts will be investigated for the other many metals.

As shown in the chromatograph of the culture medium containing metallothionein-Cd, the Cd was mainly combined with metallothionein fractions and partly with albumin fractions, and the cell toxicity of these cultures depended on the amount of Cd combined with albumin fractions. It was considered that there were differences in the binding properties of Cd between with albumin fractions and with metallothionein fractions.

Just before publication, the author was aware of Weinzierl's report about dissolution of Cd in serum in 1972. This report showed about 80% of Cd in Cd-serum was small diffusible molecules. That did not coincide with the present results.

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