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THE SUBCELLULAR SITE AND NATURE OF INTRACELLULAR CADMIUM IN PLANTS

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DISCLAIMER

ABSTRACT

The mechanisms underlying heavy metal accumulation, toxicity and tolerance in higher plants are poorly understood. Since subcellular processes are undoubtedly involved in all these phenomena, it is of interest to study the extent of, subcellular site of and nature of intracellularly accumulated cadmium in higher plants. Whole plants supplied $^{109}CdCl_2$ or $^{112}CdSO_4$ accumulated Cd into roots and aerial tissues. Preparation of protoplasts from aerial tissue followed by subcellular fractionation of the protoplasts to obtain intact vacuoles, chloroplasts and cytosol revealed the presence of Cd in the cytosol but not in vacuoles or chloroplasts. Particulate materials containing other cell components were also labeled. Of the ^{109}Cd supplied to plants, 2 to 10% was recovered in both cytosol preparations and in particulate materials. Cytosol contained proteinaceous-Cd complexes, free metal and low molecular weight Cd complexes. Labeling of protoplasts gave similar results. No evidence was obtained for the production of volatile Cd complexes in tobacco.

INTRODUCTION

The main portal of entry of the toxic metal Cd in animals and man is through the consumption of vegetable foods. Inhalation of tobacco smoke constitutes a second major source of Cd intake for those who smoke (7). Perhaps the principal reason for these facts is that plants accumulate and concentrate many toxic metals (including Cd), often into tissues consumed by man. Despite these realities, for plants, the mechanisms of Cd accumulation, and also of toxicity, tolerance and growth inhibition are not well understood. While cell wall polymers undoubtedly play an important role in Cd accumulation in certain plants, the extent of, subcellular site of and nature of intracellular Cd accumulation in plants is not established. There exists in the literature much information on the uptake of Cd by various plants, the effects of soil composition and chemistry on uptake and the tissue distribution of this metal in whole plants (1,8,18) and references there in). Mechanisms of toxicity have been studied using isolated chloroplasts (9) and mitochondria (2), whole plants (4) tissue homogenates (11) and plant exudates (18). In relation to metal tolerance, the subcellular location of accumulated Zn (a geochemical relative of Cd) in root and shoot tissue has been studied in Zn-tolerant and non-tolerant clones of Agrostis (16,20). In these latter studies pectins of the cell wall were implicated in Zn tolerance. Although the vacuole has been suggested as a possible intracellular site for sequestration of heavy metals or organo-metal

chelates (18), there exists no direct evidence for this notion. To test this hypothesis, the possible involvement of the vacuole and other subcellular fractions in Cd accumulation was directly studied. Intact vacuoles, "enriched cytosol" and chloroplasts were isolated from protoplasts which had been prepared from aerial tissues contaminated with Cd and were analyzed. Results of these studies and of preliminary investigations into the nature of the cytosol Cd observed are reported.

MATERIALS AND METHODS

Tomato (c.v. New Yorker), pea (c.v. 1746), lettuce (c.v. oak leaf), barley (c.v. moore) tobacco (hybrid N. glauca x N. langsdorffii) and cabbage (c.v. red danish) were germinated in soil and transplanted as young seedlings to Hogland's solution. The concentration of Cd and Zn in the medium were <1ppb and 25 ppb, respectively. Iron was added (40 ppm) as Sequestrene 330 Fe (Geigy Agric. Co.). Cultures were then maintained in the greenhouse at 23°C. To label tissues with ^{109}Cd, approximately 2 to 3 week old plants were removed from Hogland's solution and held in water for 1 hour prior to exposure of the roots to 15 μCi, $^{109}CdCl_2$ in 10 ml of 0.2 mM HCl. Final Cd concentration was made 1ppm. For Hippeastrum, label was supplied through the stem of a detached flower. After uptake (3 to 7 hours), plants were returned to hydroponic growth. After 4 to 20 days, labeled leaves were removed and exposed to fungal enzymes to isolate protoplasts - essentially as previously described (22). For most tissues digestion was with 1.5 to 2% cellulysin (Calbiochem Co.), 0.5% pectinase (Sigma-from A. niger) in 0.7M mannitol for 10 to 15 hours at 19°C. Barley and pea leaves were digested with a 0.5%/0.5% enzyme mixture. Unlabeled protoplasts were prepared and mixed with labeled ones to achieve approximately 0.4 ml of packed cells. This material was washed twice with 80 ml of 0.7 M mannitol, 25 mM Na-citrate, pH 5.5 as previously described (3). The monitoring of washes indicated that media was removed. Washed protoplasts were fractionated to prepare intact vacuoles,

"enriched cytosol", chloroplasts and other fractions as previously described (3,22) and as diagramed in Figure 1. Leaf vacuoles were purified as described in (21). All glassware used in isotope experiments was silated, to reduce binding of Cd, by treatment with 0.5% dichlorodimethylsilane in $CHCl_3$. ^{109}Cd was counted in a Beckman Gamma 8000 counter at 40% efficiency. Solids were extracted with 2 N HCl and solutions were made 5% with HCl for counting. Extracts of plants supplied 6 or 60 ppm Cd as $CdSO_4$ were prepared by grinding leaves in 0.025 M KPO_4, pH 7.4 containing 2 mM dithiothreitol. Homogenates were centrifuged at 100,000 xg for 60 minutes and the supernatants fractionated on Sephadex G-50 fine columns (2.5 x 61 cm). Lysates from ^{109}Cd labeled protoplasts were similarly treated. Elution was at 4°C with 0.025 M KPO_4, pH 7.4 at 40 ml/hour. In ^{112}Cd experiments, fractions (4 ml) were analyzed for absorption at 280 and 255 nm, and then made 10% with HCl and directly analyzed by atomic absorption spectrophotometry. Paper electrophoresis was essentially as described by Tiffin (19). Uptake of Cd by protoplasts was studied using the following procedure. Pea or tobacco protoplasts (5×10^6) were labeled with 35 μCi of $^{109}CdCl_2$ (1-5 Ci/gm Cd) in 3.5 ml of M3 protoplast regeneration medium (15) lacking FeEDTA. To determine uptake from the medium, 100 μl aliquots were removed with time, centrifuged at 100 xg to pellet protoplasts and the supernatant sampled. To determine uptake into cytosol, 500 μl aliquots were removed and diluted with 12 ml of M3 medium and the protoplasts recovered by centrifugation. Protoplasts were lysed with water and the lysates centrifuged at 10,000 xg for 30 minutes.

RESULTS AND DISCUSSION

Possible roles in heavy metal accumulation of two major compartments within plant cells, the vacuole and cytosol, have been studied directly after isolation of these compartments from Cd laden leaf protoplasts. Whole plants labeled with ^{109}Cd accumulated 2 to 10 percent of the label supplied into leaf mesophyll cells. The label was equally distributed between "enriched cytosol" and particulate fractions (Table I). Intact vacuoles lacked label. Recent experiments (unpublished) indicate that the intracellular site of soluble Zn in leaf cells is, like Cd, the cytosol. Barley (not shown) gave similar results to that shown for lettuce. Recovery of washed mesophyll protoplasts from leaf tissue was estimated to be 50 percent and that of cytosol during protoplast fractionation to be about 95 percent. Thus, since half the protoplasts were lost during preparation for fractionation, cytosol and particulate values are estimated to be double those actually observed and shown. Data presented were obtained by analysis of plants 4 days after labeling. Similar results were obtained with pea and tobacco leaves fractionated 20 days after labeling. Therefore, no evidence for vacuolar accumulation was observed even where Cd was present in leaves for over 20 days. Control expeirments showed that subcellular fractionation methods did not leach organo-Cd from particulate materials into the cytosol fraction. Also, protoplast lysates - not prepared by treatment with 0.2 M K_2HPO_4 used to osmotically shock protoplasts to

release vacuoles and cytosol - gave elution patterns similar to cytosol patterns when separated by gel filtration. Particulate materials recovered during preparation of vacuoles contain unlysed protoplasts, broken cell debris and cell organelles other than vacuoles Fig. 1. The label recovered with the particulate materials may reside in organelles and membranes or may be associated with residual cell wall. Chloroplasts of pea and lettuce protoplasts recovered as described in (22), however, contained only a negligible amount of label (Table 1). The protoplast digestion medium is thought to contain solubilized cell wall oligosaccharides released during digestion of cell walls. About 80-90% of the label in the medium - after removal of protoplasts and debris - was precipitated by addition of EtOH to 80% (v/v). This label accounted for about 70% of the label in leaves. This result is consistent with cell wall being a major site of Cd accumulation in aerial tissues. Future expeiments using specific carbohydrases will examine the nature of the Cd complexes in this fraction. It should be noted that the "enriched cytosol" fraction may contain the leachates of other organelles (21). Future experiments will examine various organelles other than vacuoles and chloroplasts individually while assessing the retention of soluble contents in the isolated organelles.

Evidence in support of the purity and retention of contents of isolated vacuoles is given in Table II. These data were obtained using Hippeastrum and Tulipa petal and Tulipa leaf protolasts. Recent experiments (unpublished) indicate that vacuoles isolated from barley

and lettuce leaves retain nuclease, lack chlorophyll contamination and lack certain cytosol hydrolases. Also, the retention of K^+ and Ca^{++} in pea vacuoles isolated as described has been determined by proton induced x-ray emission spectroscopy (unpublished). Since Ca^{++}, Mg^{++} and other ions and small molecules are retained in isolated vacuoles Cd, if present, would be expected to be retained.

In view of the finding that plants form volatile Hg compounds from elemental Hg (6), we examined the possibility that volatile Cd compounds might also be formed. Tobacco plants were supplied $^{109}CdCl_2$ and allowed to accumulate the label for 10 days while being maintained in a closed system equiped with a cold finger trap (-70°C) and a 0.5M HCl gas-bubbler trap. No evidence was found for the production of volatile Cd. Digestion of labeled leaves to prepare protoplasts using a similar closed system indicated no release of volatile Cd during hydrolysis of cell walls to release protoplasts.

The nature of cytosol-^{109}Cd was studied using gel filtration on Sephadex G-50, fine. "Enriched cytosol" and soluble constitutents from protoplast lysates of tobacco had similar profiles (protoplast lysate shown in Fig. 2). Tobacco, pea and tomato all contained Cd complexes having apparent mol wts of >50,000 daltons and about 10,000 daltons. The large Cd peak in the region of small molecules was split (not shown) suggesting the presence of several components. With tomato, paper electrophoresis suggested the presence of free metal and several cationic complexes. Extracts of plants grown in the presence of 60 ppm ^{112}Cd were examined after gel filtration by atomic

absorption spectrophotometery, Fig. 3. These high levels of Cd were used to elevate levels of Cd complexes. Similar but less prominant organo-Cd complexes were found when plants were exposed to 6 ppm Cd. Profiles similar to that shown in Fig. 3 for tobacco were obtained with cabbage and tomato but not with lettuce and barley. Cadmium containing peaks occured at the void volume, at approximately 8 to 12,000 daltons and a split peak in the region of free metal and small molecules. Analysis of A_{280} revealed a strong absorbance at 255 nanometers associated with the peak (Fig. 3 - tube 54) eluting after free metal. The appoximately 8000 dalton peak was degraded by thermolysin at pH 7.5 indicating the protein nature of this material. Both the 8000 mol wt complex and the low mol wt fractions are being further studied.

The uptake of Cd by pea and tobacco protoplasts was studied to elucidate the mechanism of uptake at the cell level. Figure 4 shows the result of labeling pea protoplasts in M3 medium with ^{109}Cd at 1ppm Cd. Label was removed from the medium rapidly for the first few minutes and more slowly thereafter. Direct analysis of uptake into protoplast soluble (cytosol) materials showed a very rapid uptake after which some label appeared to return to the medium or accumulate into internal particulate materials. Internal particulate materials continued to increase in activity. The rapid but limited uptake into the cytosol was observed in various labeling media at pH 5.5 and 7.0 and is interpreted as a passive equilibration. Zinc is thought to be accumulated by a passive process not requiring metabolic energy (5).

Toabacco (GGLL) protoplasts prepared and cultured according to (17) showed similar kinetics when uptake was tested two days after isolation (prior to cell wall formation). After 4 days, some protoplasts in the culture began to divide and were therefore viable. However, these protoplasts were incapable of phosphate uptake when tested according to the procedure used by Mettler and Leonard (14) to measure phosphate uptake in tobacco suspension cell protoplasts. However,it should be pointed out that ion transport in leaf derived protoplasts has not yet been demonstrated. The activity in the particulate and cytosol fractions do not account for the Cd removed by protoplasts. This is undoubtedly due to the loss of protoplasts during washing to remove medium. Control experiments in which broken and intact protoplasts were treated alike showed that intact protoplast lysates contained the organo-Cd complexes described while the indentically treated broken protoplast control contained only free metal. Gel filtration profiles obtained for protoplasts treated for 30 minutes with 1ppm Cd containing ^{109}Cd were similar to that shown in Fig. 2 for protoplasts derived from labeled plants.

CONCLUSIONS

The data presented show that plants exposed to 1,6 or 60ppm Cd accumulate small amounts of the metal into the cytosol of leaf mesophyll cells. The vacuole, a major compartment for sequestering certain endogenously produced and exogenously acquired toxic metabolites in plants, does not appear to be involved in Cd accumulation. Cytosol Cd is in the form of low molecular weight complexes, free metal and protease-sensitive complexes of approximately 10,000 daltons. Metallothioneins are approximately this size and are thought to reside in the cytosol in animal cells (12). The preliminary nature of the characterization of the 10,000 dalton complexes makes speculation about their role difficult, however, several points may be made about their occurrence. First, finding these complexes in the cytosol of protoplasts exposed to ^{109}Cd and fractionated 30 minutes thereafter suggests a normal presence for these constitutents. It is possible that they serve a role in metal homeostasis and normally bind Zn, a required micronutrient in plants. Second, the complexes, like metallothioneins, occur in greater amounts where plants are exposed to higher concentrations of metal. Although extensive studies of induction have yet to be done, the results obtained are consistent with induction. It is emphasized, however, that the nature and role of the cytosol Cd complexes observed must await extensive chemical chaacterization and further study which will undoubtedly require the comparative analysis of Cd and Zn effects.

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Table 1

SUBCELLULAR DISTRIBUTION OF ^{109}Cd IN LEAF MESOPHYLL CELLS OF WHOLE PLANTS SUPPLIED LABEL

TISSUE	Tomato	Pea	Lettuce	Tobacco	Hippeastrum
	Percent of Label Supplied				
FRACTION					
"Enriched Cytosol"	0.91	0.47	0.68	2.4	2.0
Particulate	0.79	0.51	0.68	2.5	1.9
Vacuole	0	0	0	0	0
Chloroplast	-	negl.	negl.	-	-

Table II

EVIDENCE THAT ISOLATED VACUOLES LACK CONTAMINATION AND HAVE RETAINED THEIR CONTENTS.

Evidence for Purity			Retention of Contents		
Containment - Marker	Percent[1]	Reference	Vacuolar Constituent	Percent[1]	Reference
Chloroplasts - chlorophyll	<0.5	(21)	Anthocyanin	96-100	(21)
Mitochondria - Cyt. c oxidase	<0.2	(21)	Monasaccharides - D-glucose	80	(21)
"Microsomes", etc. -			Amino acids	≤ 50	(21)
NADH cyt. c reductase	<0.6	(21)	Certain proteins, i.e. RNase (16,000 daltons)	~ 50	(3)
DNA	1.0	(21)	Ions - K, Na, Mg, Ca, Cl	~ 100	(13)
RNA	1.0	(21)			
Cytosol - certain hydrolases	n.d.	(3)			
antho. synthetic enzymes	n.d.	(10)			

[1]percent of contents of 1 to 5 x 10^6 protoplasts or protoplast-cytosol equivalents found in the sap of 1 to 5 x 10^6 vacuoles. In all experiments protoplasts and vacuoles were counted using a 0.2 mm deep wel type slide. Error in counting is estimated to be ± 10 to 15%. n.d. - not detected.

FIGURE LEGENDS

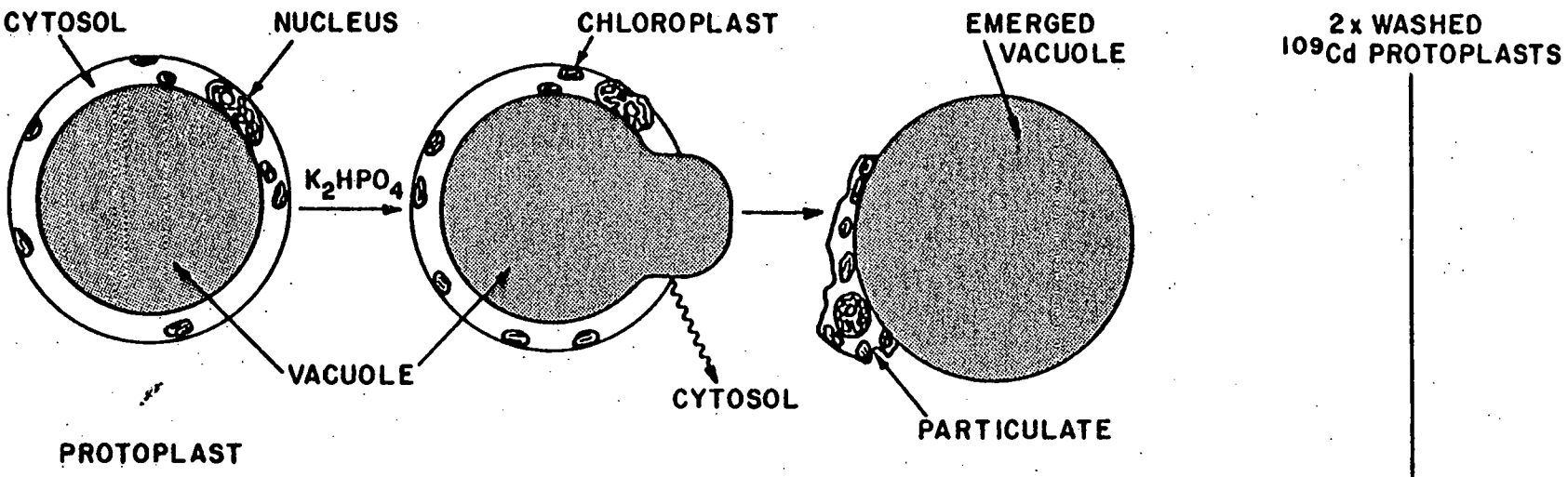
Fig. 1. Upon exposure to 0.17 M K_2HPO_4, pH 8.0 protoplasts undergo gentle osmotic shock. Intact vacuoles emerge from protoplasts at one point on the periphery while cytosol escapes into the medium. Particulate materials (composition noted on diagram) are shed largely as an aggregate which under preparative conditions is released from the emerged vacuole and aggregates with like material from other protoplasts, unlysed protoplasts and debris. The particulate material is then filtered (particulate fraction). Vacuoles are recovered by sedimentation (22) or flotation from sucrose-phosphate medium (21). Remaining soluble material comprises the "enriched cytosol" fraction.

Fig. 2. Sephadex G-50 profile (^{109}Cd vs. elution volume) of a tobacco protoplast lysate obtained from leaves of plants supplied ^{109}Cd (1ppm Cd). The molecular weight of the organo-Cd complex following the void volume was estimated by calibrating the column with chymotrypsinogen (25,000 daltons), myoglobin (17,800 daltons), RNAse A (13,700 daltons), cytochrome c (12,400 daltons) Bacitracin (1400 daltons) and $^{109}CdCl_2$.

Fig. 3. Sephadex G-50 profile of a leaf extract from tobacco grown in the presence of 60ppm ^{112}Cd. Molecular weight estimation of the 8100 dalton peak was determined as described in the legend of Fig. 2. Fraction volumes were 4 ml.

Fig. 4. Uptake of ^{109}Cd (1ppm Cd) by pea protoplasts in M3 medium. See Methods and Results.

SUBCELLULAR FRACTIONATION OF PROTOPLASTS



PARTICULATE

UNLYSED PROTOPLASTS
UNDIGESTED CELLS
CHLOROPLASTS
NUCLEI
MITO.
CYTOSOL
ETC.

CYTOSOL

&
LYSED VACUOLES
& ?

VACUOLE

Fig 1

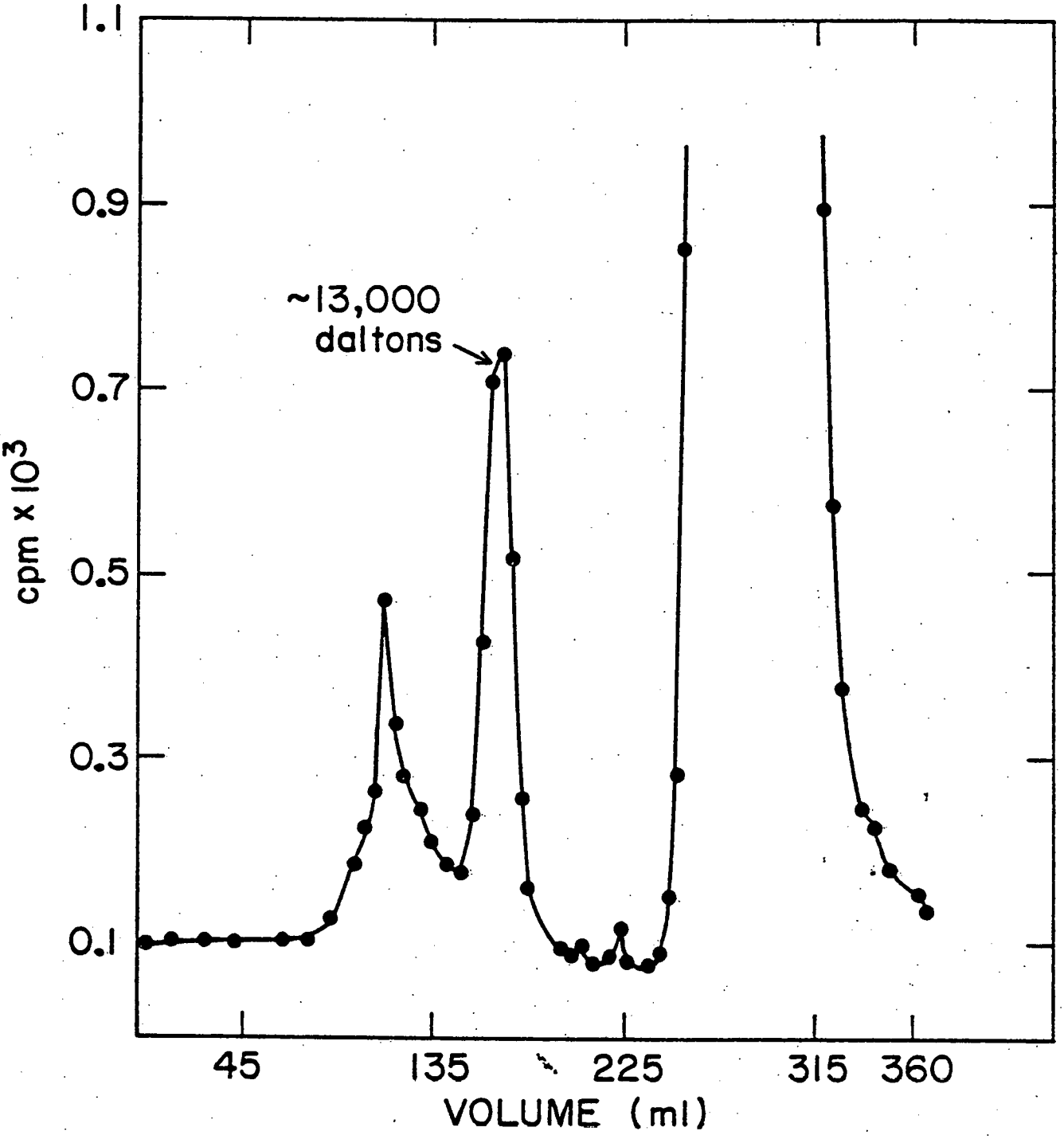

1.1
0.9
0.7
0.5
0.3
0.1
cpm x 10³
~13,000
daltons
45
135
225
315
360
VOLUME (ml)

Fig 2

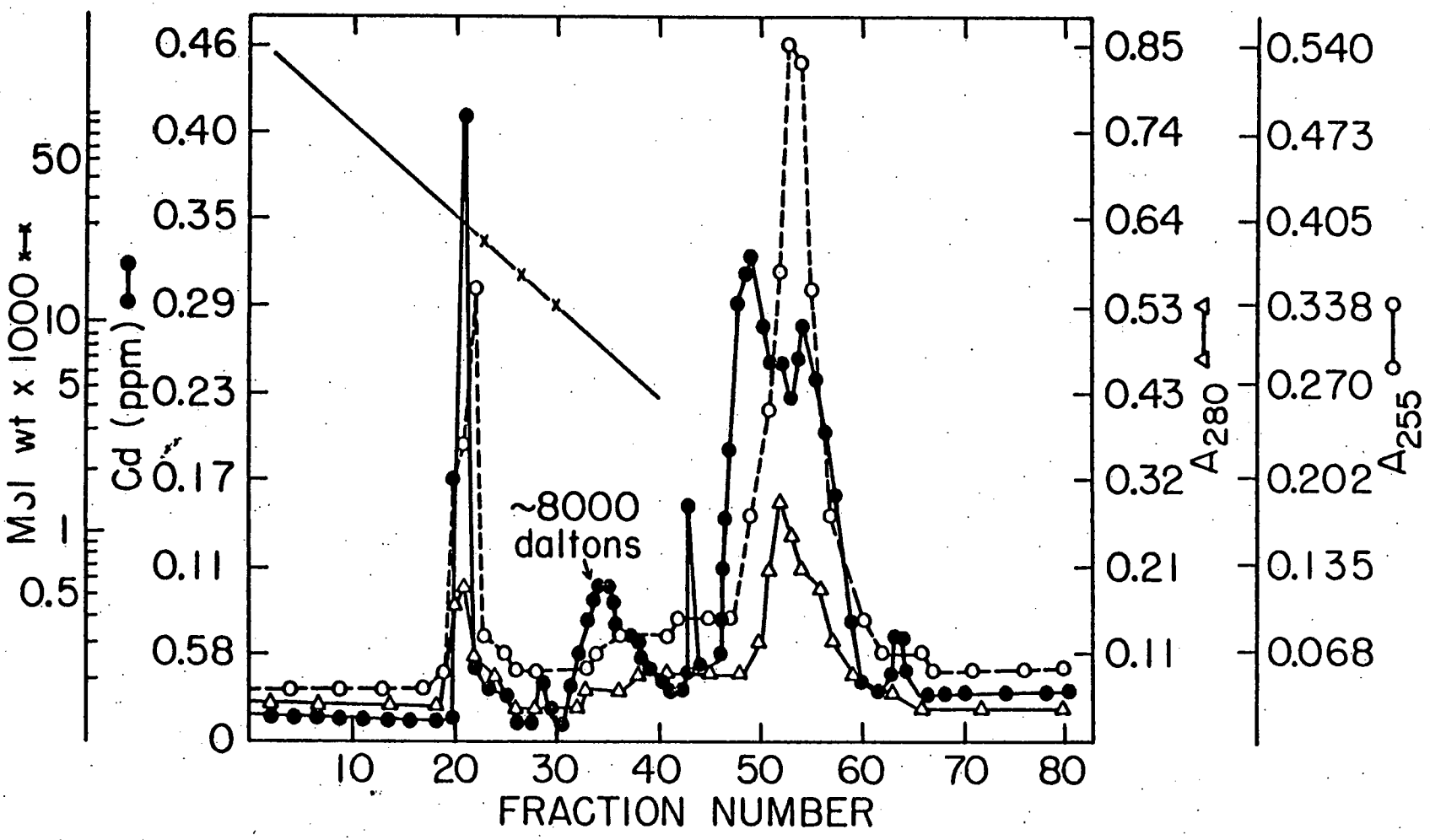

Mol wt x 1000
50
10
5
1
0.5
Cd (ppm)
0.46
0.40
0.35
0.29
0.23
0.17
0.11
0.58
0
~8000
daltons
0.85
0.74
0.64
0.53
0.43
0.32
0.21
0.11
A280
0.540
0.473
0.405
0.338
0.270
0.202
0.135
0.068
A255
10
20
30
40
50
60
70
80
FRACTION NUMBER

Fig 3

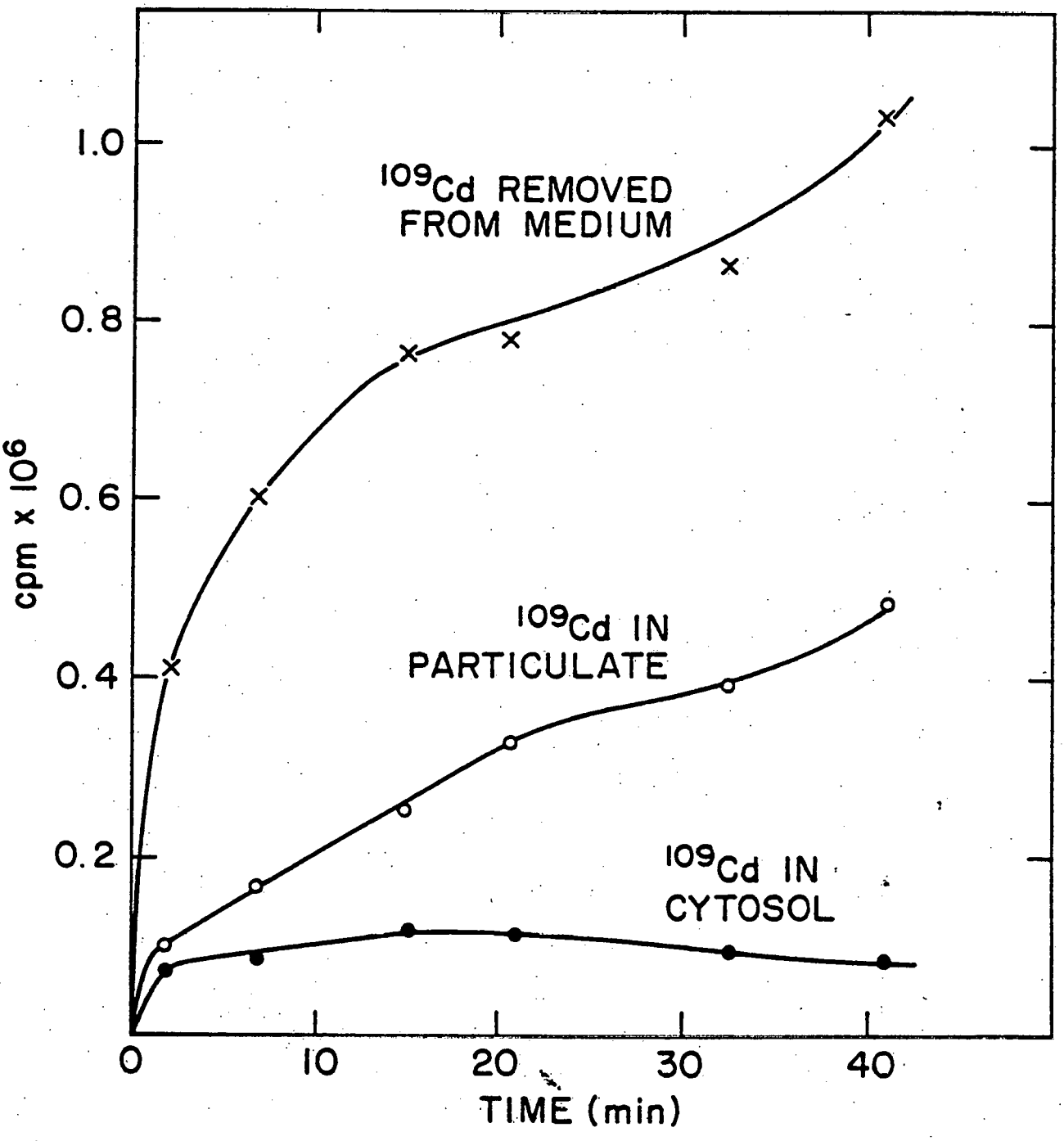

1.0
0.8
0.6
0.4
0.2
cpm x 10⁶
¹⁰⁹Cd REMOVED FROM MEDIUM
¹⁰⁹Cd IN PARTICULATE
¹⁰⁹Cd IN CYTOSOL
0
10
20
30
40
TIME (min)

Fig 4