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# THE INTERACTION BETWEEN Cd-ABSORPTION AND Cd-COMPARTMENTATION IN WOLFFIELLA GLADIATA

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SUMMARY

Following Cd absorption from a nutrient medium *Wolffiella gladiata* (Lemnaceae) plantlets were subjected to compartmental analysis. A plot of the quantity of easily exchangeable cadmium in the cytoplasm (EECd-cyt) versus time showed a sigmoid curve after absorption from weakly concentrated media (0.001 mM Cd and more) and a hyperbolic curve after absorption from highly concentrated media (up to 0.4 mM Cd). A plot of EECd-cyt versus the Cd-concentration in the medium showed sigmoid curves after absorption during short periods (10–20–30 min) and hyperbolic curves after long-term absorption (up to 4 hours). An increase in EECd-cyt resulted in a decrease in net absorption.

The amounts of Cd in the three distinguished intracellular (chemical) compartments increased partly simultaneously. The Cd that was retained until the last fraction was to be washed out of the plants is supposed to be strongly complexed. This fraction of complexed Cd is the last to be completed during absorption.

## 1. INTRODUCTION

Apart from experiments concerning the toxic effects of cadmium on plants (PAGE et al. 1972, NASU et al. 1984), many investigators were interested in absorption processes for this element.

CUTLER & RAINS (1974) and FRANÇOIS et al. (1979) found that Cd was mainly present in extracellular material, whereas the remaining quantity of Cd entered the cell by diffusion. SMEYERS-VERBEKE et al. (1978) concluded from their work that a fraction of Cd absorbed by wheat plants is taken up by metabolically mediated processes.

The fact that each time large quantities of Cd were found to be bound to the cell exterior led to studies about binding of Cd towards pectin (MALOVÍKOVÁ & KOHN 1980) and cuticles (CHAMEL et al. 1984) or on binding of heavy metals to cellulose (LIESER & GLEITSMANN 1983). On the other hand experiments were carried out on the effect that Cd-retaining substances in the absorption solution have on absorption (NASU et al. 1983) and on the effects of Cd-absorption on Cd-binding substances within the cells (RAUSER 1984a, RAUSER & GLOVER 1984).

Apart from the importance of characterization of these so-called metallothioneins, as studied by e.g. RAUSER (1984b) and WAGNER (1984), and influences of medium solution factors on binding capacities of these substances (O'KEEFFE & HARDY 1984), the point of localization of absorbed Cd, as previously examined by WAGNER (1979) and KHAN et al. (1984), deserves attention.

We considered it important to study the effect of absorption of Cd on the intracellular Cd-complexation and Cd-localization. In the present work, therefore, special attention has been given to the relation between localization and chemical speciation of Cd, and on the other hand Cd absorption. For that purpose *Wolffiella gladiata* (Lemnaceae) was used, a very suitable, submerged growing plant with fronds which mainly consist of two cell layers only. Following Cd absorption the plantlets were subjected to a washing procedure in order to distinguish several cellular Cd fractions using compartmental analysis as described by WALKER & PITMAN (1976).

## 2. MATERIALS AND METHODS

*Wolffiella gladiata* (Hegelm.) Hegelmaier is a non-flowering and rootless plant (DAUBS 1965), for many years in axenic culture at the laboratory. It was grown as described for *Spirodela polyrhiza* in a previous paper (SCHREINEMAKERS 1984). During the experiments the plants were kept at 28 °C and illuminated by a 400 W mercury discharge lamp at a distance of 75–80 cm.

In each experiment the number of plants used had a final dry weight of about 50 mg per sample. Prior to each experiment all the plants (about 4 g dry weight) were rinsed for 30 min. in 1.5 l of demineralized water. After the removal of adhering water by centrifuging for 15 s at 100 × g the plants were placed in absorption solutions containing per $m^3$ 55.5 moles of sucrose (also present in the growth media and added to avoid an osmotic shock), 0.21 moles of $Na_2HPO_4$, 6.5 moles of $KH_2PO_4$ (together buffering the solution at pH 5.3) and $CdCl_2$. The $CdCl_2$-concentrations were varied between 0.001 and 0.4 moles.$m^{-3}$.

In one experiment the plants absorbed Cd for 10, 20, 30, 45, 60, 90, 120, 180 and 240 min and were centrifuged again as described above. The plants were subsequently transferred to solutions containing 0.45 moles.$m^{-3}$ $Na_2EDTA$ and were buffered at pH 5.3 by the same amounts of $Na_2HPO_4$ and $KH_2PO_4$ as were present in the absorption solutions. After each absorption period Cd was washed out of the samples for 10, 20, 30, 45, 60, 90, 120, 180 and 240 min at a temperature of 28 °C. Subsequently the samples were centrifuged for 15 sec at 250 g, dried for two days at 80 °C, weighed and ashed at 550 °C. The ash was dissolved in 1 ml 3N $HNO_3$ and diluted to 25.0 ml with demineralized water. The cadmium content of the solutions obtained was determined using atomic absorption spectrophotometry.

In another experiment the absorption periods lasted for 0.5, 1, 2, 3 and 4 h. Thereafter the cadmium was washed out for several periods lasting 10, 20, 30, 45, 60, 90 min and 2, 3, 4, 5, 6, 8, 10, 12, 14, 16, 18, 20, 22 and 24 h.

In the compartmental analysis procedure described by WALKER & PITMAN (1976) efflux is assumed to be diffusional and therefore exponential.

The original procedure of having labeled ions washed out into an unlabeled solution (MACROBBIE & DAINTY 1958, GODBOLD et al. 1983, FRANCOIS et al. 1979) could not be used, because the shortest absorption periods lasted for 10 min, after which the ionic flux from most absorption solutions was still a net influx. We wanted to show the distribution of Cd among the cell compartments, following absorption during many absorption periods from a concentration range. Therefore absorption had to be stopped instantaneously. The use of EDTA prevents that free Cd is available in the efflux solution. An EDTA-concentration of 10 moles.m$^{-3}$, as used by VELTRUP & AUSTENFELD (1981) to wash Cu out of the free space of *Phaseolus* cells, appeared to be toxic to *W. gladiata*. Therefore the EDTA-concentration that was present in the growth medium of the plantlets (0.45 moles.m$^{-3}$) was also used for efflux of the absorbed Cd.

The second compartment may consist of Cd that is weakly complexed and might diffuse relatively easily across the plasmalemma. The Cd-concentration in this compartment has been described on a molar basis, to make it comparable with the external Cd-concentration. Doing so the results of compartmental analysis (in $\mu$g Cd/g dry weight) of the first experiment (*fig. 3* and *4:* efflux lasting for up to 4 h) were converted so that they were expressed on a molar basis (the dry weight of *W. gladiata* was 8% of the fresh weight, and cytoplasm fresh weight was assumed to be 5% of the plant's fresh weight).

## 3. RESULTS AND DISCUSSION

### 3.1. 4 h Cd-efflux

Three examples of compartmental analysis, lasting up to 4 h, are given in *fig. 1*. Each graph shows two intersections with the ordinate. The upper one refers to all cellular Cd except the "free space"-part. This amount of cadmium is assumed to be the net absorbed quantity of cadmium. The difference in the height of the two intersections is due to the fact that one Cd-fraction is easily exchangeable, though not as readily as the "free space"-part. It is assumed that the second Cd-fraction is washed out from the compartment bordering on the "free space", i.e. the cytoplasmic Cd that is readily availabe (not strongly complexed). This fraction will be referred to as the cytoplasmic Cd.

*Fig. 2* shows the quantity of net absorbed Cd versus the absorption period. In the lower graphs the amount of Cd increases more or less linearly versus time. Absorption solutions of higher concentrations result in a steady state after a shorter period of absorption.

*Fig. 3* shows the concentration of cytoplasmic cadmium versus the absorption time. The curves representing absorption from weakly concentrated media are slightly sigmoid. This observation can probably be related to the fact that short-term absorption from these media does not bring in sufficient Cd to saturate the strongly complexing agents within the cytoplasm. The absorption period is not long enough to let the plant synthesize enough metallothionein for a strong

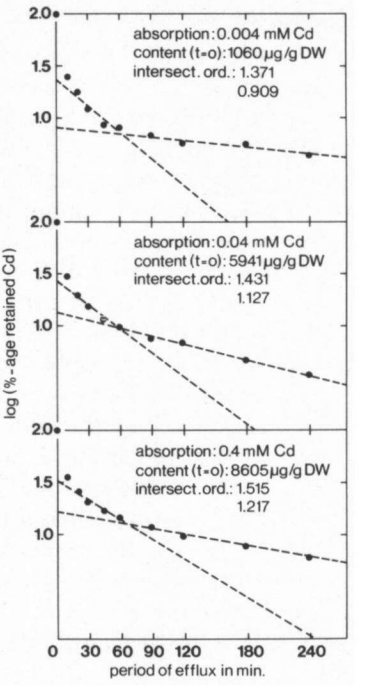


Fig. 1. Three examples of campartmental analysis. The dashed lines show the calculated lines. The upper intersection with the ordinate indicates the net absorbed Cd in the cells. The difference in the height of the two intersections is a measure for the Cd-content of the cytoplasm. The lower intersection represents the amount of Cd that is present in the vacuole, plus the amount of complexed Cd. The Cd-concentration of the absorption medium and the Cd-content of the plants before the efflux-period are mentioned at the top of each graph.

complexation of Cd. Many thiol-group-containing amino acids and regular proteins may bind Cd in a very strong manner (VALLEE & ULMER 1972). The data in *fig. 3* represent only the quantity of Cd, that is the first to be washed out of the plants after efflux of the 'free space". Cd that is more strongly bound to organic material (including possibly induced metallothioneins and/or thiol-compounds) was washed out in the last fraction (*fig. 1*).

The induceability of Cd-binding substances has been highlighted in the introduction. There are however many regularly occurring cellular materials that can complex Cd in vivo, as shown by CASTERLINE & BARNETT (1982), PETIT & VAN DE GEYN (1978) and VALLEE & ULMER (1972). For the possible complexation of cations in general the induction of organic acid synthesis was described by TORII & LATIES (1966).

Since the formation constants of heavy metal-chelator complexes are large, small quantities of Cd that enter the cytoplasm will be complexed immediately. It is assumed that the concentration within the cytoplasm of easily exchangeable Cd increases very slowly until the strongest Cd-complexes are formed. The complexed Cd within the cytoplasm belongs to the last fraction shown in *fig. 1*.

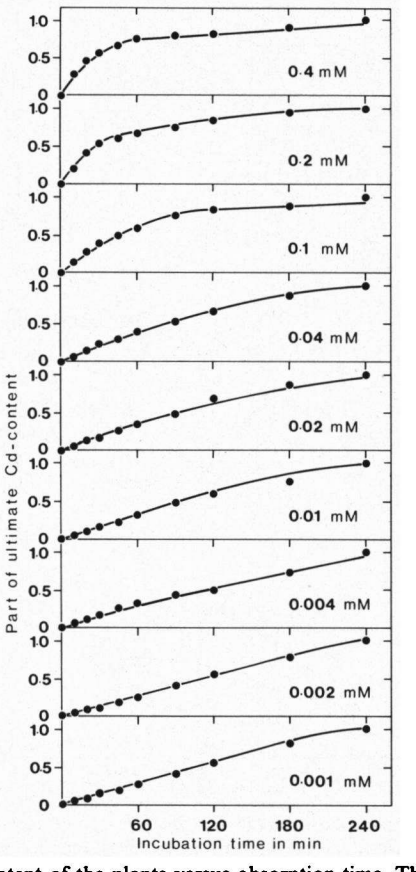


Fig. 2. Relative Cd-content of the plants versus absorption-time. The Cd-content after 240 min absorption is taken to be 100%. The contents are total Cd minus the "free space"-part. The numbers below each curve indicate the Cd-concentration of the absorption medium.

This fraction may also include vacuolar cadmium.

Absorption from media with higher Cd-concentrations produces the upper curves of *fig. 3*. These solutions are concentrated enough to ensure that even short-term absorption brings a saturating quantity of Cd into the cell. It is clear that the curve is sigmoid for the absorption form media with low Cd-concentrations, but gradually becomes hyperbolic after absorption from higher Cd-concentrations up to 0.4 moles.m$^{-3}$. After absorption from media with a low Cd-concentration the cytoplasmic Cd-concentration needs about 4 h to reach a steady state. Absorption from media with a high Cd-concentration results in a steady state after about 30 min. This development in time also occurs for the curves of net absorbed Cd (*fig. 2*). The simultaneous changes indicate that an increasing concentration of Cd in the cytoplasm causes a decrease in Cd-absorption.

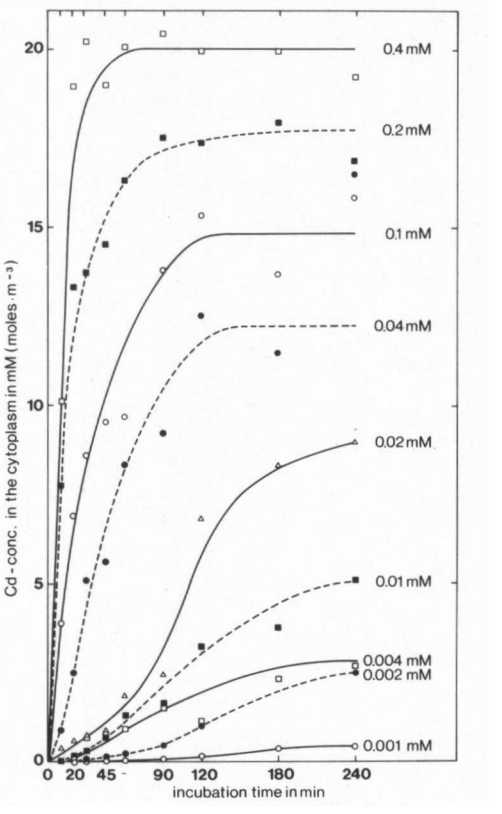


Fig. 3. Cd-concentration in the cytoplasm versus the absorption-time. The data refer to the concentration of easily exchangeable Cd in the cytoplasm. The complexed part is not shown. The numbers at the end of each curve indicate the Cd-concentration in the absorption medium.

According to the saturation-theory, after long-term absorption the curves are not hyperbolic but are sigmoid with a very small "lag"-phase. This phase is necessary because shorter absorption periods require a more concentrated medium , if enough Cd is to be brought in to saturate the complexans (*fig. 3* shows the quantity of Cd that is weakly bound, therefore not including strong Cd-complexes).

The same results for cytoplasmic Cd have been diagrammed in *fig. 4*. This representation shows that short absorption times give curves that are slightly sigmoid. According to the earlier mentioned saturation-theory only small amounts of Cd are needed per unit time to saturate the initially present complexing agents with Cd during long-term absorption. This implies that the longer the absorption period the shorter is the saturation- (lag-) phase of the curves for cytoplasmic Cd versus the external Cd-concentration. Therefore after long-term absorption the concentrations, such as those used in these experiments, give curves that have a hyperbolic shape.

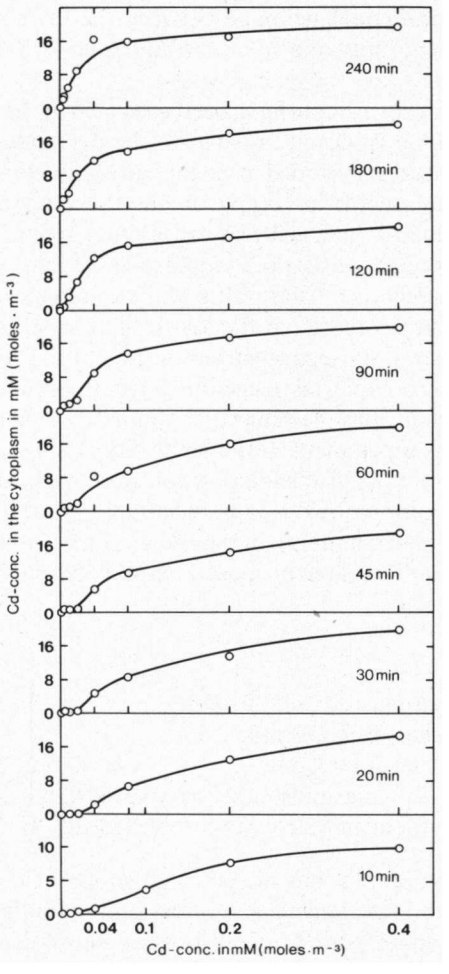


Fig. 4. Cd-concentration in the cytoplasm versus the Cd-concentration in the absorption medium. The data refer to the concentration of easily exchangeable Cd in the cytoplasm. The complexed part is not shown. The numbers below each curve indicate the absorption-time.

## 3.2. 24 h Cd-efflux

*Fig. 5* gives data on the compartmental analysis after Cd-efflux lasting up to 24 h. In each graph the efflux analysis results are shown after Cd has been absorbed from three media, each with a different Cd-concentration. The calculated linear regressions, representing the first (apoplasmatic) compartment, have not been drawn. They all intersect the ordinate at a value 2.0 (= 100%). The 2nd, 3rd and 4th compartments have been analyzed for all data and are shown in the figure.

After 0.5 h of absorption from 0.004 moles $Cd.m^{-3}$ the last four samples contained such small quantities of Cd that they could not be detected properly.

Since no clear distinction could be made between the Cd contents of the 3rd and the 4th compartment, only one value for both compartments together has been given.

All intersections with the ordinate have been indicated in the upper right-hand part of each graph. These values are related to the percentage of total Cd retained. The upper number, mentioned after each medium concentration, represents all the Cd that is present in compartments that are spatially separated from the first compartment which is the so-called outer space.

The second compartment consists of cytoplasmic Cd that is easily exchangeable (as in the previous section). After efflux of the third compartment the final fraction was washed out slowly (from the 4th until at least the 24th hour) and is assumed to be a chemically separated compartment. Cd from this fraction is thought to be strongly complexed to chelating agents within the cell.

The third compartment may be separated spatially or chemically from the second and the fourth compartment. If the separation is spatial this Cd-fraction may be present in the vacuole. A chemical separation on the other hand would result from the complexation of Cd to organic material that binds the Cd more weakly than does the organic material in the fourth compartment.

The list given below indicates the proposed cellular compartments, their location and their properties:

| | | |
|---|---|---|
| compartment 1 | | apoplasmatic Cd |
| compartment 2 | cytoplasmic soluble and easily exchangeable Cd | symplasmatic Cd |
| compartment 3 | a) vacuolar Cd or b) cytoplasmic weakly complexed Cd | |
| compartment 4 | cytoplasmic strongly complexed Cd | |

Compartmental analysis shows that the percentage of all Cd, belonging to the apoplast, decreases with increasing time of absorption (see *fig. 5* for the results of this calculation). Longer periods of absorption show that this percentage reaches a steady level. The absolute quantity of Cd in this compartment continues to increase until a maximum level is reached (*table 1*). It is plausible that the percentage of Cd belonging to the first compartment descreases with time. Under such conditions initial absorption means initial adsorption. This will result in an initial apoplasmatic Cd-fraction of approximately 100% of the Cd in the plant. Since the number of adsorption sites within the apoplast is limited, the quantity of Cd in this compartment shows a maximum level (about 5500 $\mu$g Cd/g dry duckweed). When Cd enters the cell itself the apoplasmatic percentage of all Cd will decrease.

From the results in *table 1* it is obvious that symplasmatic Cd is distributed over at least three (chemically and/or spatially separated ) compartments. The ratios of the contents of these compartments vary with increasing time of absorption (depending on the Cd-concentration in the absorption solution). This is very clear from the ratio after absorption from a medium containing 0.4 moles

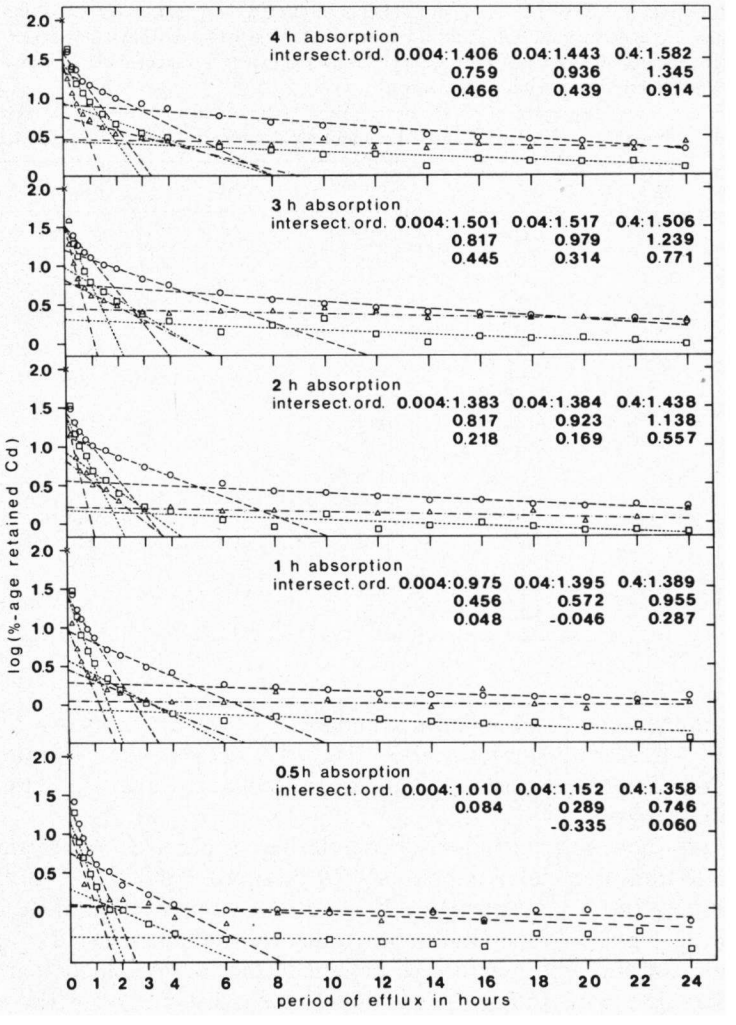


Fig. 5. Compartmental analysis of data after absorption of Cd by *Wolffiella gladiata*. After absorption for the period mentioned in each graph, Cd was washed out into EDTA-containing solutions for the period, given under the X-axis.
During each absorption period Cd was absorbed from solutions containing 0.004, 0.04 or 0.4 moles $Cd.m^{-3}$. Efflux of Cd is represented by △–△ after absorption from 0.004 mol $Cd.m^{-3}$, by □-----□ after absorption from 0.04 mol $Cd.m^{-3}$ and by ○ ---○ after absorption from 0.4 mol $Cd.m^{-3}$.
The numbers in each graph indicate the absorption period and the intersections of the calculated lines with the ordinate. The Cd-content in the plantlets before efflux was taken as 100% after each absorption and has been indicated by X on the ordinate. The first calculated line (the one representing the outer space), which intersects with the ordinate at this point, has not been drawn.

$Cd.m^{-3}$. The bottom section of *table 1* shows that after saturation with Cd of the first symplasmatic compartment (the 2nd in *table 1*), the increase of the Cd-

Table 1. Amounts of Cd in the four compartments of *Wolffiella gladiata*. The total amounts of Cd indicated are experimental data, and the amounts for each compartment have been derived from compartmental analysis. NA (not applicable) means that insufficient data were available to make a clear distinction between compartments.

| Cd-conc in the medium ($mol.m^{-3}$) | absorption period (in hours) | total Cd and Cd-content per compartment (in $\mu g$ Cd/g dry weight) | | | | |
|---|---|---|---|---|---|---|
| | | total | 1rst | 2nd | 3rd | 4th |
| 0.004 | 0.5 | 867 | 779 | NA | NA | NA |
| | 1 | 1196 | 1083 | 79 | 21 | 13 |
| | 2 | 1765 | 1339 | 310 | 87 | 29 |
| | 3 | 1631 | 1114 | 410 | 62 | 45 |
| | 4 | 2018 | 1504 | 398 | 17 | 99 |
| 0.04 | 0.5 | 3301 | 2833 | 404 | 49 | 15 |
| | 1 | 5048 | 3795 | 1065 | 143 | 45 |
| | 2 | 4766 | 3612 | 755 | 329 | 70 |
| | 3 | 6405 | 4299 | 1496 | 478 | 132 |
| | 4 | 7162 | 5176 | 1368 | 421 | 197 |
| 0.4 | 0.5 | 6463 | 4989 | 1114 | 286 | 74 |
| | 1 | 7366 | 5562 | 1140 | 521 | 143 |
| | 2 | 7865 | 5709 | 1075 | 797 | 284 |
| | 3 | 8300 | 5639 | 1324 | 847 | 490 |
| | 4 | 8618 | 5326 | 1385 | 1200 | 707 |

contents in the second and the third symplasmatic compartments may proceed even until 4 h after absorption started. The ratio between the Cd-contents in the second and third symplasmatic compartments changes in favour of the latter during absorption.

Many metabolic intermediates are complexing agents to di- or trivalent cations and to transition metals in particular (VALLEE & ULMER 1972; O'SULLIVAN 1969; DISILVESTRO & COUSINS 1983). If any strong complexation of Cd occurs *in vivo* in the absence of metallothioneins, this Cd-fraction wil be the latest to be washed out and will therefore be present in the fourth cell-compartment. A comparable complexation occurs during long distance transport of Cd through the plant (e.g. CASTERLINE & BARNETT 1982).

Because this relatively large intracellular sink (4th compart.) contains a spectrum of complexing agents with a spectrum of binding affinities for Cd (VALLEE & ULMER 1972; O'SULLIVAN 1969), it is unlikely that the third compartment consists of very weakly complexed vacuolar Cd, because its saturation occurs prior to that of the fourth compartment. Since all Cd that enters the vacuole must pass through the cytoplasm, the vacuole is unlikely to reach equilibrium before strongly complexing agents within the cytoplasm are "neutralized". WAGNER (1979) showed that Cd, absorbed during growth, did not enter the vacuole. The third compartment is therefore thought to consist of a quantity of complexing agents which are weaker, but are easier for the Cd to reach than those in the fourth compartment.

## 4. CONCLUSION

Assuming that during short-term absorption no substantial amount of metallothioneins will be synthesized the following conclusions may be drawn:
- The amount of easily exchangeable symplasmatic Cd, in the 2nd cell compartment, depends on the combination of the duration of absorption and the Cd-concentration in the medium.
- The degree of saturation of complexing agents (metabolic intermediates) within the cell by Cd is determined by the combination of the duration of absorption and the Cd-concentration in the medium.
- Complexing agents within the cell may probably be divided into three main groups, each with its own capacity for Cd and with its own mean strength of Cd-complexation.
- The concentration of easily exchangeable Cd in the cytoplasm influences to a great extent the absorption of this element; absorption is therefore dependent on 1) the combination of the duration of absorption and the concentration of an ion in the medium, on 2) the quantity of the intracellular organic material and on 3) the chemical nature of intracellular organic material (besides the well-known dependence on membrane-qualities, metabolic activity, etc.).
- The degree of absorption may very well be altered, when conditions of growth, prior to absorption, have been changed (not including the metal-induced synthesis of metallothioneins).
- It seems important to create a model that describes absorption, its dependence on both time and concentration and consequently its dependence on the intracellular concentration of easily exchangeable Cd. Using such a model it is easier to handle difficulties like:
1) detection limits for absorbed material after initial absorption.
2) efflux of absorbed material in the period of "initial" absorption, because the internal concentration exceeds the external one (see *fig. 3*).

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