Influence of lead on the chlorophyll content and on initial steps of its synthesis in greening cucumber seedlings

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Abstract

Lead uptake by young cucumber (*Cucumis sativus* L.) seedlings growing in 10^{-4} and 10^{-3} M $PbCl_2$ solution caused δ-aminolevulinic acid synthesis inhibition, reduced the activity of δ-aminolevulinic acid dehydratase and the chlorophyll centent in the cotyledons. Lead mainly accumulated in the roots (ca 93-96% of Pb uptake) and hypocotyls (ca 4-6%), whereas only trace Pb amounts were found in the cotyledons when 10^{-3} M solution was used. It is supposed that one of the causes of the reduction of chlorophyll synthesis by lead is its influence on tissue hydration which diminished to about 50 per cent as compared with the control. The direct effect of lead on the examined steps of chlorophyll synthesis cannot, however, be ruled out.

Key words: lead, chlorophyll, δ-aminolevulinic acid, cucumber seedlings

INTRODUCTION

In connection with the increasing atmosphere, soil and water bodies pollution with lead, numerous papers have appeared lately on the toxic action of Pb on plants, animals and humans. Particularly noteworthy is the influence of lead on hemoglobin biosynthesis. Lead inhibits several steps in the process of heme synthesis in animal tissues. As demonstrated by Sordo et al. (1982), δ-aminolevulinic acid dehydratase (ALAD) catalysing porphobilinogen condensation from two δ-aminolevulinic acid (ALA) molecules is an enzyme with high sensitivity to the toxic action of lead. Similarly, δ-aminolevulinic acid synthetase catalysing ALA synthesis and ferrochelatase building iron into the protoporphyrin IX system are inhibited by lead (for review see Mahaffey 1981).

There is no information, whether lead affects similarly biosynthesis of the porphyrin system in plant tissues, what would be associated with the action of Pb on chrophyll synthesis It is the general belief that chlorosis

due to heavy metals is caused by disturbance of iron metabolism indispensable in chlorophyll synthesis (Foy 1978). It is also known that lead accumulates in the membranes of thylakoid chloroplasts (Sabinis et al. 1969). Rebechini and Hanzely (1974) ascertained that lead disturbs the synthesis and structure of chloroplast membranes in *Ceratophyllum demersum*. Lead accumulated in chloroplasts also affects the processes of photosynthesis. Miles et al. (1972) noted disorders in the functioning of photosystem II, evoked by lead in experiments with isolated chloroplasts from spinach leaves and Hampp et al. (1973) demonstrated the influence of Pb on enzymes of the pentose cycle.

In the present study investigations were undertaken to find an answer whether lead taken up by plants affects the chlorophyll content and the initial steps of its biosynthesis.

MATERIAL AND METHODS

PLANT MATERIAL

Dark-germinated cucumber seeds (*Cucumis sativus* L. var. Wisconsin SMR 18) were transferred to 250-cm^3 beakers filled with salt solution containing (in mmols per dm^3): K_2SO_4 — 3, $Ca(H_2PO_4)_2$ — 1, $CaSO_4$ — 2, $MgSO_4$ — 1 with basic microelements added (Buczek 1979). pH of the solution was adjusted to 5.5. In this solution the plants grew for 24 h in a dark thermostat (27°C). Then they were transferred either to 10^{-3} or 10^{-4} M $PbCl_2$ solution or to a solution of NaCl (control) with chlorine concentration corresponding to Cl^- in $PbCl_2$ solutions. The plants were kept in these solutions for 18 h in darkness. They were subsequently transferred to solutions containing 5.5 mM KNO_3, 1 mM $Ca(NO_3)_2$ and $PbCl_2$ (10^{-3} or 10^{-4} M) or else a solution containing corresponding NaCl amounts (control) and placed in a photothermostat under continuous light (5.83 W·m^{-2}) at 27°C for 72 h. The above described preparation procedure of the plants was applied because lead phosphates or sulphates were precipitated when the full medium was used. At 6, 24, 48, and 72 h intervals after placing the plants under light the chlorophyll content was analysed and δ-amino-levulinic acid dehydratase activity, ability of synthesis of δ-aminolevulinic acid and soluble protein content as well as Pb accumulation were determined.

DETERMINATION OF ABILITY OF ALA SYNTHESIS

The ability of ALA synthesis by cucumber cotyledons during their greening was checked according to the method described by Beale and

Castelfranco (1974). Cut cucumber cotyledons (ca 1 g) were placed on Petri dishes and incubated in 5 cm^3 of 100 mM levulinic acid (competitive ALAD inhibitor) with dimethylsulphoxide (10% final concentration) added. The cotyledons were illuminated for one hour (5.83 $W \cdot m^{-2}$) and the amount of accumulated ALA was determined in the cotyledons according to Mauzerall and Granick (1956). The cotyledons were ground in a mortar with 5 cm^3 of 0.5 M phosphate buffer, pH 6.8. The extract was centrifuged for 15 min at 15 000 × g. ALA was determined in the supernatant by condensation with ethyl acetoacetate. After condensation the reaction mixture was mixed (1:1) with modified Ehrlich's reagent (Mauzerall and Granick 1956). Extinction was measured after 10 min at 553 nm. The amount of accumulated ALA was expressed in $\mu g \times mg^{-1}$ of protein $\times h^{-1}$.

ALAD ACTIVITY DETERMINATION

The enzyme was determined after Shemin (1962) with slight modifications. The cotyledons (2 g) were ground in a mortar with 10 cm^3 of 0.05 M Tris-HCl, pH 7.8, with dithiothreitol added. The supernatant obtained after centrifugation at 15 000 × g was used as crude enzymatic extract. The incubation mixture in its final volume (3 cm^3) contained 100 μmol Tris-HCl, pH 8.0, 1.5 μmol dithiothreitol, 10 μmol $MgCl_2$, 10 μmol ALA and 0.4 cm^3 enzymatic extract. The mixture was incubated for 1 h at 30 C. The reaction was interrupted with 3 cm^3 of 8 per cent TCA containing 10 mM $HgCl_2$. After centrifugation at 10 000 × g the amount of porphobilinogen (PBG) was determined in the supernatant by mixing it with modified Erlich's reagent (Mauzerall and Granick 1956) in a 1:1 ratio. Extinction was measured at 553 nm. For calculating the amount of product (PBG) formed, the molar extinction coefficient was applied: $6.2 \times 10^4 M^{-1} \times cm^{-1}$ (Shemin 1962). Since in crude enzymatic extracts part of the PBG produced during incubation is transformed to porphyrin, in calculation of ALAD activity this fact was taken into account: porphyrin was determined by mixing the postincubation supernatant with 5 N HCl in a 1:1 ratio. Extinction was read for porphyrin at 406 nm with the use of the extinction coefficient calculated: $53 \times 10^4 M^{-1} \times cm^{-1}$. The total PBG amount was calculated with the assumption that 1 mol porphyrin = 4 mol PBG (Shemin 1962). ALAD activity was expressed in nm of PBG $\times mg^{-1}$ protein $\times hour^{-1}$.

Protein content in the supernatants used for ALAD activity measurements and ALA synthesis ability were determined after Lowry et al. (1951) with BSA as standard.

CHLOROPHYLL CONTENT DETERMINATION

Chlorophyll was extracted with 90 per cent acetone and determined according

to Jeffrey and Humphrey (1975) with the use of the equation given
by these authors for chlorophyll of higher plants and *Chlorophyceae*:
$(11.93\ E_{664} - 1.93\ E_{647}) + (20.36\ E_{647} - 5.50\ E_{664})$ $(\mu g \times cm^{-1})$. Chorop-
hyll was converted to one dry weight unit, not to fresh weight, because
in the present work, like in the preceding one, it was demonstrated
(Burzyński and Grabowski 1984) that the presence of lead in the
solution in which the cucumber seedlings grew caused a marked diminution
of water content in the cotyledons and reduced their fresh weight. Changes
in dry weight were, however, slight, not exceeding a dozen or so per cent.
Soluble protein content calculated to dry weight unit was similar to that
in the cotyledons of the control plants (Table 1).

<div align="center">DETERMINATION OF Pb ACCUMULATION</div>

Pb content in the dry weight was determined with the use of an
AAS 1 N spectrophotometre of atomic absorption (Carl Zeiss, Jena). The
roots were washed with a strong current of distilled water and the particular
plant organs were separated and dried after weighing at 65°C to constant
weight. The material, after drying, was homogenised in a mortar. Dry
mass samples were burned in a mixture of nitric and perchloric acids
in a 4:1 proportion. The burned material was poured into 25-cm³
measuring flasks and made up with water. In thus prepared solutions
Pb content was read and converted to 100 mg dry weight.

<div align="center">RESULTS</div>

The changes in fresh and dry mass of the cucumber seedling cotyledons
growing in the tested $PbCl_2$ concentrations on the particular days of
measurement are shown in Table 1. It was demonstrated that the taken
up lead, independently of concentration in the external solution, caused
a distinct diminution of fresh weight of the cotyledons. With time a more
and more pronounced loss of water content was noted in these organs.
The dry weight of cotyledons, however, and the soluble protein content
in the dry mass did not change.

It is seen in Fig. 1 that the chlorophyll content in the cotyledons
of control seedlings increased up to 48 h of plant growth under light.
In the next period of growth the chlorophyll amount in the cotyledons
of control plants did not change. In the same period the chlorophyll
content in the cotyledons of plants treated with lead was markedly depressed
and, in dependence on $PbCl_2$ concentration, 10^{-4} or 10^{-3} M amounted
to 40 and 50 per cent of that in the control. In the cotyledons of

plants treated with 10^{-4} M $PbCl_2$ chlorophyll content still increased after 48 h, so that after 72 h the chlorophyll content was equal in the control plants and those treated with the latter Pb concentration. At the ten times higher $PbCl_2$ concentration the pigment content was by 40 per cent lower as compared with that in the control. In the cotyledons of seedlings treated with 10^{-3} M $PbCl_2$, the chlorophyll content was, thus, much lower. and in plants treated with 10^{-4} $PbCl_2$ retardation was observed in reaching a chlorophyll content equalling the pigment contained in control seedlings.

Table 1

Influence of lead on fresh and dry weight and soluble protein content in cucumber cotyledons

Time of growth under light, h	Pb concentration, M	Fresh weight, mg	Dry weight, mg	Protein amount, milligrammes		
				g^{-1}fr.wt.	per cotyledon	$100\ mg^{-1}$ dr.wt.
6	0	37 a	6.7 a	145 a	2.9 a	44.8 a
	10^{-4}	26 b	6.6 a	145 a	3.1 a	47.1 a
	10^{-3}	24 b	6.2 a	145 a	2.9 a	46.2 a
24	0	53 a	7.2 a	49 a	2.5 a	34.3 a
	10^{-4}	26 b	7.1 a	65 b	2.5 a	35.0 a
	10^{-3}	22 b	6.9 a	90 c	2.5 a	36.1 a
48	0	79 a	7.1 a	12 a	1.1 a	15.7 a
	10^{-4}	49 b	7.0 a	22 b	1.1 a	16.0 a
	10^{-3}	43 c	7.0 a	29 c	1.1 a	15.9 a
72	0	153 a	8.5 a	14 a	1.6 a	18.3 a
	10^{-4}	66 b	7.3 b	21 b	1.4 a	19.1 a
	10^{-3}	34 c	7.1 b	27 c	1.3 a	18.9 a

Means denoted by different letters in column at various times of growth differ significantly at the 5% level.

The influence of lead *in vivo* on δ-aminolevulinic acid dehydratase activity is shown in Fig. 2. An intensive increase in the activity of the enzyme was noted up to 48 h of illumination of the seedlings. Thereafter, the enzyme activity diminished in all combinations. Seedlings treated with 10^{-4} M $PbCl_2$ showed a small but significant decrease of ALAD activity as compared with control seedlings. Plants treated with $PbCl_2$ solution in a 10^{-3} M concentration reduced ALAD activity by 40 and 50 per cent after 48 and 72 h, respectively, of plant growth under light.

The *in vivo* influence of lead on the ability of ALA synthesis is shown in Fig. 3. $PbCl_2$ in both concentrations distinctly diminished the ability of synthesis. The negative influence of lead could be noticed as early as after 6 h of growth under light, and after 48 h ALA synthesis
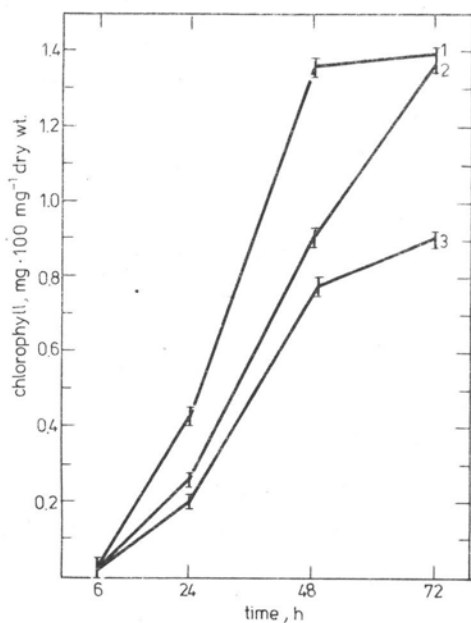
Fig. 1. Changes in chlorophyll content in the course of greening of cucumber seedlings growing in $PbCl_2$ solutions. 1 — control, 2 — 10^{-4} M $PbCl_2$, 3 — 10^{-3} M $PbCl_2$. vertical bars denote standard errors of mean values
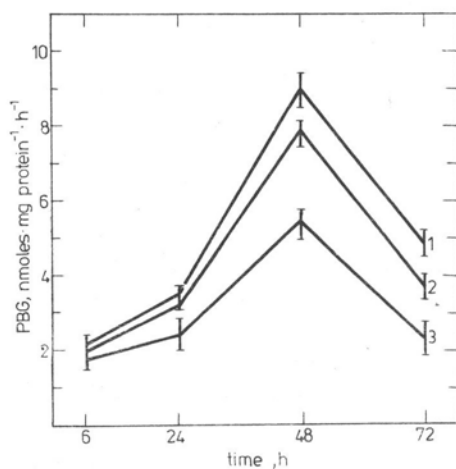

Fig. 2. Influence of $PbCl_2$ on δ-aminolevulinic acid dehydratase activity. 1 — control, 2 — 10^{-4} M $PbCl_2$, 3 — 10^{-3} M $PbCl_2$. Vertical bars denote standard errors of mean values

ability in the cotyledons of cucumbers treated with 10^{-4} M $PbCl_2$ was reduced by almost 50 per cent and by about 80 per cent in the plants treated with 10^{-3} M $PbCl_2$. After 72 h of growth under light the ALA synthesis ability in the cotyledons of the control seedlings and those treated with 10^{-4} M $PbCl_2$ decreased, but the negative influence of Pb persisted.
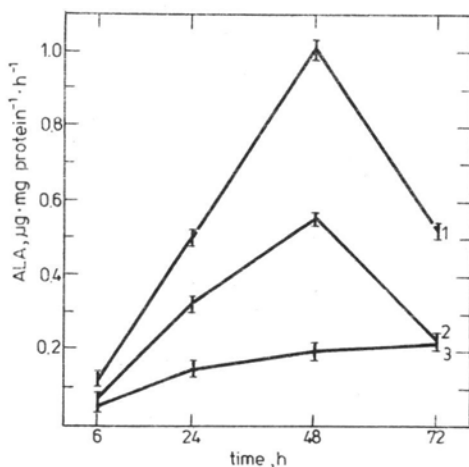

Fig. 3. Influence of lead on ability of δ-aminolevulinic acid synthesis in greening cucumber seedlings. 1 — control, 2 — 10^{-4} M $PbCl_2$, 3 — 10^{-3} M $PbCl_2$. Vertical bars denote standard errors of mean values

Table 2

Pb content (μg per plant), in cucumber seedlings growing for 72 h under light in the presence of $PbCl_2$

Pb concentration in solution, M	Whole plant		Roots		Hypocotyls		Cotyledons	
	μg	%	μg	%	μg	%	μg	%
10^{-4}	150.1	100	144.0	96	6.0	4	0.0	0
10^{-3}	452.0	100	420.4	93	27.1	6	4.5	1

Analysis of lead content in the plant as a whole and lead concentration in the particular organs demonstrated that after 72 h of growth under light, 93-96 per cent fell to the roots, about 4-6 per cent to the hypocotyls and the least about 1 per cent of the lead accumulated in the whole plant, fell to the cotyledons (Table 2). Changes in lead content in cucumber seedlings calculated to dry weight of the particular plant organs are shown in Table 3. Lead concentration in the root dry mass, hypocotyls and cotyledons depended on the $PbCl_2$ concentration in the external solution and on the lenght of growth time in the presence of lead. Lead concen-

tration in the roots of seedlings growing in a 10^{-3} M PbCl$_2$ solution exceeded on the average 2.0-2.5 times that in the roots of plants growing in the 10^{-4} M PbCl$_2$ solution. In the hypocytyls of plants growing in the higher Pb concentration for 48 h an about sevenfold increase of Pb content was noted, after 72 h of growth the increase was only fourfold. In the cotyledons, however, only slight Pb amounts were found as late as after 48 h in plants growing in the 10^{-3} M Pb solution, whereas in cotyledons of seedlings treated with 10^{-4} M PbCl$_2$ no lead was found.

Table 3

Changes in Pb content in particular cucumber organs

Time of growth under light, h	Pb concentration in solution, M	Lead concentration, mg $\cdot$ 100 mg^{-1} dr.wt.		
		roots	hypocotyls	cotyledons
0	10^{-4}	0.85	0.0	0.0
	10^{-3}	4.00	0.05	0.0
6	10^{-4}	3.80	0.01	0.0
	10^{-3}	9.87	0.11	0.0
24	10^{-4}	6.25	0.03	0.0
	10^{-3}	15.00	0.22	0.0
48	10^{-4}	6.67	0.04	0.0
	10^{-3}	17.55	0.33	0.01
72	10^{-4}	8.10	0.10	0.0
	10^{-3}	18.25	0.44	0.65

DISCUSSION

The results reported indicate that lead taken up by young cucumber seedlings disturbs the process of chlorophyll biosynthesis during greening of the plants. It appeared that the first step of this synthesis, namely. reaction leading to δ-aminolevulinic acid synthesis was particularly sensitive to the action of lead. The fact that lead in 10^{-4} M and 10^{-3} M concentrations reduced by 50 and 80 per cent, respectively, the ability of ALA synthesis after 48 h of growth under light points to the action of this metal on the early stage of chlorophyll synthesis. Beale and Castelfranco (1974) demonstrated that the chlorophyll centent in plant tissues is directly dependent on the amount of ALA produced.

It would seem, however, that the influence of Pb on chlorophyll synthesis is more complex and involves other steps of its synthesis as well. The reduced δ-aminolevulinic acid hydratase activity noted in the present experiments indicates that the next step of chlorophyll synthesis sensitive to the action of Pb is ALAD activity. Many investigators found a reduced ALAD activity in humans (Sordo et al. 1982) and in animals (Damstra

1977) exposed to the influence of lead. Thus the present experiments demonstrated that ALAD activity in plant tissues is also sensitive to the action of lead.

The mechanism of Pb action on ALAD activity is unknown. It may be supposed that the reduction of enzyme activity is the result of limitation of ALA biosynthesis, as demonstrated by the present author. The fact, however, that a raised ALA level has been found in the urine of humans exposed to the action of lead (Labreche and P'an 1982), and that the ALAD activity was inhibited (Sordo et al. 1982) may suggest a direct influence on ALAD synthesis or activity. It cannot, therefore, be ruled out that in plant tissues as well lead acts directly on the ALAD synthesis or activity level, notwithstanding inhibition of δ-aminolevulinic acid synthesis.

As consequence of the diminished ALA synthesis and depressed ALAD activity in cucumber cotyledons of plants taking up lead, a reduced chlorophyll synthesis is observed. However, the fact that in the presence of 10^{-4} M Pb synthesis took place for a longer time than in the control may suggest that plants are relatively resistant to the action of lead, or else that lead transport to the chlorophyll synthesis centres (cotyledons) occurs very slowly.

In the present experiments lead accumulated mainly in the roots. After 72 h of growth of the plants under light about 93-96 per cent of the taken up Pb was accumulated in the roots. The remaining Pb was stored in the hypocotyls, whereas in the cotyledons trace amounts of lead were only found in the presence of 10^{-3} M $PbCl_2$. If we disregard the too low sensitivity of the method of Pb determination, it may be supposed that the noted small influence of 10^{-4} M Pb on chlorophyll synthesis was rather the result of slow Pb transport to the cotyledons. On the other hand, the presence of lead in the hypocotyls of plants growing in the presence of 10^{-4} M $PbCl_2$ may explain the influence of the lower lead concentration of ALA synthesis and ALAD activity. The investigations of Hardy et al. (1970), namely, demonstrated that an unidentified factor indispensable for chlorophyll synthesis is produced in the cucumber hypocotyls.

The influence of lead on the plant water regime may be one of the causes of reduction of the activity of enzymes participating in chlorophyll synthesis. It was, namely, found that water stress due to the influence of lead on isolated plant tissue fragments (Burzyński and Jakób 1983) or young seedlings (Burzyński and Grabowski 1984) diminished hydration of plants. Similar results have been obtained in the present investigations: hydration of cucumber seedlings diminished by about 50 per cent in the presence of $PbCl_2$. It seems, therefore, that the influence of lead

on tissue hydration cannot be excluded as a factor influencing the biochemical processes occurring in plant tissues.

The present results thus suggest that lead uptake by cucumber seedlings markedly reduces the ability of δ-aminolevulinic acid synthesis by cucumber seedlings and δ-aminolevulinic acid dehydratase acitivity, this leading to. a diminution of the chlorophyll content in the cotyledons. Lead accumulation in plant tissues also affects negatively tissue hydration and this may be one of the causes of reduced chlorophyll synthesis.

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Wpływ ołowiu na poziom chlorofilu i na niektóre etapy jego syntezy w zieleniących się siewkach ogórka

Streszczenie

Pobieranie ołowiu przez młode siewki ogórka (*Cucumis sativus* L.) rosnące w roztworach 10^{-4} i 10^{-3} M $PbCl_2$ powodowało hamowanie syntezy kwasu δ-aminolewulinowego, zmniejszenie aktywności dehydratazy kwasu δ-aminolewulinowego oraz zmniejszenie zawartości chlorofilu w liścieniach. Ołów akumulował się głównie w korzeniach (około 93-96% pobranego Pb) i hypokotylach (około 4-6%) natomiast w liścieniach stwierdzono śladowe ilości Pb jedynie w kombinacji z 10^{-3} M $PbCl_2$. Przypuszcza się, że jedną z przyczyn zmniejszonej syntezy chlorofilu pod wpływem ołowiu jest wpływ tego metalu na uwodnienie tkanek, które zmniejszyło się o około 50 procent w porównaniu z kontrolą. Nie wykluczony jest również bezpośredni wpływ ołowiu na badane etapy syntezy chlorofilu.