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The effects of lead and kinetin on greening barley leaves

A. WOŹNY, J. SCHNEIDER and E.A. GWÓŹDŹ

Adam Mickiewicz University, Faculty of Biology, Institute of Experimental Biology, PL-61-713 Poznań, Poland

Abstract

The content of lead in greening etiolated barley leaves remained the same, regardless the time of incubation of excised leaves in the presence of lead ions (8 - 24 h). The lead deposits have not been detected within mesophyll cells, but were found in intercellular spaces of mesophyll, in guard cells and in cuticle covering stomata. This suggests that lead may be transported in the leaves *via* transpiration stream. Lead reduced the content of chlorophyll, especially chlorophyll *b* content and the average number of grana, whereas in the presence of kinetin the content of chlorophyll increased. In the combined treatment (lead + kinetin) kinetin diminished the inhibitory effect of lead on the chlorophyll content. The number of chloroplasts in mesophyll cells remained unchanged after lead treatment, whereas kinetin alone or applied together with lead increased the average chloroplasts number. The thylakoids system in chloroplasts of kinetin and kinetin + lead treated plants was similar to that observed in control, although the grana number was smaller. Both lead and kinetin increased the content of condensed chromatin in nuclei.

Key words: chlorophyll, chloroplast, growth regulators, heavy metal, *Hordeum vulgare*, ultrastructure.

Introduction

The influence of lead as well as other environmental pollutants have been analyzed at different level of plant organization. The leaves obtaining only a few percent of lead absorbed by roots (Burzyński 1985) were so far seldom used as a model system. However it is known that lead induces both structural and functional changes within the leaves. The development of leaves growing in the presence of lead is delayed and the sizes of their leaf blades are reduced. Despite the changes observed in leaves the negative effect of lead on cotyledons has not been detected (Woźny and Jerczyńska 1991).

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One of the characteristic symptoms of lead toxicity is impaired chlorophyll (Chl) synthesis, which is in turn responsible for the decreased net photosynthetic rate (Burzyński 1985, 1987, Sharma and Chopra 1987, Stiborová *et al.* 1987). The lead-induced inhibition of both photosynthesis and transpiration were shown for leaves of sunflower and maize (Bazzaz *et al.* 1975) and seedlings of pea and maize (Poskuta *et al.* 1987, 1988). Besides the reduction of Chl synthesis, lead may affect other processes regulating photosynthesis, such as gas exchange, stomatal closure (Bazzaz *et al.* 1974, Burzyński 1987, Poskuta *et al.* 1987) and water uptake (Burzyński 1987). The lead-induced reduction of activity of two key photosynthetic enzymes, ribulose-1,5-bisphosphate carboxylase/oxygenase and phosphoenolpyruvate carboxylase, were shown in barley and maize seedlings (Stiborová *et al.* 1987). In etiolated wheat leaves exposed to light lead inhibits the transformation of etioplasts into chloroplasts (Wrischer and Meglaj 1980). The studies on isolated chloroplasts showed that lead affects the photosynthetic activity on the level of both photosystem 1 (PS 1) and photosystem 2 (PS 2), however the PS 2 seems to be more sensitive to Pb than PS 1 (Miles *et al.* 1972, Bazzaz and Govindjee 1974, Wong and Govindjee 1976, Wrischer and Meglaj 1980, Becerril *et al.* 1988).

Limited data are available about the effect of lead on plants in combination with exogenously applied cytokinins. The aim of the present study was to establish the influence of lead on Chl content and chloroplast ultrastructure of greening barley leaves. Attempt has also been made to answer the question whether kinetin modifies the toxic effect of this metal.

Materials and methods

The study was performed on etiolated barley (*Hordeum vulgare* L. cv. Grit) leaves, excised from 7- and 12-d-old seedlings growing in dark at 20 °C on filter paper moistened with distilled water. Thirty excised primary leaves were placed in 15 cm^3 of following solutions: *a*) water (control), *b*) 0.05 or 0.5 μM of kinetin, *c*) $PbCl_2$ ranging from 0.04 to 4.0 mM, *d*) kinetin (0.5 μM) and $PbCl_2$ (0.4 mM). After 12 or 24 h of dark preincubation the leaves were exposed to continuous white fluorescent light (50 μmol m^{-2} s^{-1}) and incubated for further 12 or 24 h. The each experiment was repeated three times. Chloroplast pigments were isolated according to Hiscox and Israelstam (1979). This method allows complete extraction of chl, without tissue maceration and the dimethylsulfoxide (DMSO) based pigment extracts are much more stable than acetone or ethanol extracts. The chlorophyll content was estimated by the method of Arnon (1949).

The lead content in the leaves was estimated after sample mineralization, using an atomic absorption spectroscope *Spectra AA* (*Varian,* Canada).

Chloroplasts: Chloroplast number per cell was determined in light microscopy on semi-thin sections. Number of grana per chloroplast and thylakoids per granum were determined in transmission electron microscopy on ultrathin sections, both of them were obtained from 30 leaves.

Stomata: The stomata size was measured by ocular micrometer *OK15 KM* on adaxial epidermis of 7-d-old leaves. The epidermis samples were collected from the middle part of the leaves, adjacent to vascular bundles. The length and the width of the guard cells were measured as well as the pore size of stomata (at half of their length). Thirty stomata were measured in each experimental variant.

Lead detection and localization: Lead has been detected by a cytochemical method using sodium rhodizonate (Glater and Hernandez 1972) and by transmission electron microscopy on transverse sections collected from *1*) parts of the leaves submerged in incubation solutions and *2*) those located about 1 cm above the incubation media. The aim was to check transport of lead ions in the leaves.

Electron microscopy: The fragments of the 12-d-old leaves were collected, and the central part of the leaf blades, located about 5 cm from the tip, were used for experiments. The material was firstly fixed in formaldehyde and glutaraldehyde mixture (Karnovsky 1965) and after washing with the buffer, fixed in OsO_4. The samples were contrasted in 2 % uranyl acetate, dehydrated in an acetone series of increasing concentration and embedded in a low viscosity epoxy resin (Spurr 1969). The semi- and ultrathin sections were cut out by ultramicrotome (*Ultrotome III, LKB,* Sweden) and examined by *JEM 7A* (*JEOLCo*, Japan) transmission electron microscope at 50 or 80 kV.

For the statistical analysis Student *t*-test, standard deviation, coefficient of variation and confidence interval was determined.

Results

Macroscopical observations: Freshly harvested, etiolated leaves were yellowish and have the leaf blades which were tubularly curled up at the base and were straightened at the tip, with full turgor.

The treatment of the excised leaves with a wide range of $PbCl_2$ concentrations (0.04 - 4.0 mM) showed that leaves tolerated rather high lead concentration. The leaves survived for several days even at as high external concentration of $PbCl_2$ as 1 mM. However, 2 mM $PbCl_2$ was lethal after 2 or 3 d of treatment. An external symptom of lead toxicity in etiolated leaves exposed to light was inhibition of greening accompanied by the gradual loss of the turgor. The inhibition of greening was more marked at the tip of the leaves. The decrease of greening at the midblade and base was correlated with increasing concentration of $PbCl_2$. After 12 and 24 h of light the inhibitory effect on greening was already visible at 0.1 mM $PbCl_2$ and intensified with increasing lead concentration.

The kinetin (0.5 μM) treatment of the leaves caused the enhancement of greening process as compared to control. The leaf blades were straightened entirely and showed the full turgor.

The leaves treated with both kinetin (0.5 μM) and $PbCl_2$ (0.4 mM) had the same morphology as control leaves. The tips of the leaves remained yellowish, the basal part was tubularly curled up and the leaves had normal turgor.

Chlorophyll content: In 7- and 12-d-old leaves $PbCl_2$ inhibited mainly the synthesis of Chl *b*. In 7-d-old leaves $PbCl_2$ (0.4 mM) inhibited the synthesis of Chl *b* by 75 % whereas Chl *a* only by 56 % . The same was observed for 12-d-old leaves.

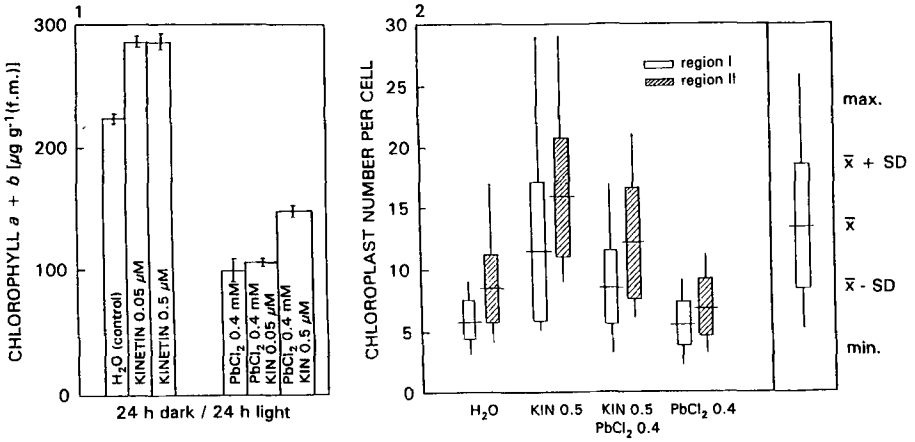


Fig. 1. The changes of chlorophyll *a+b* content in 7-d-old, detached barley leaves, incubated in analyzed solutions for 24 h in the darkness and 24 h on the light.

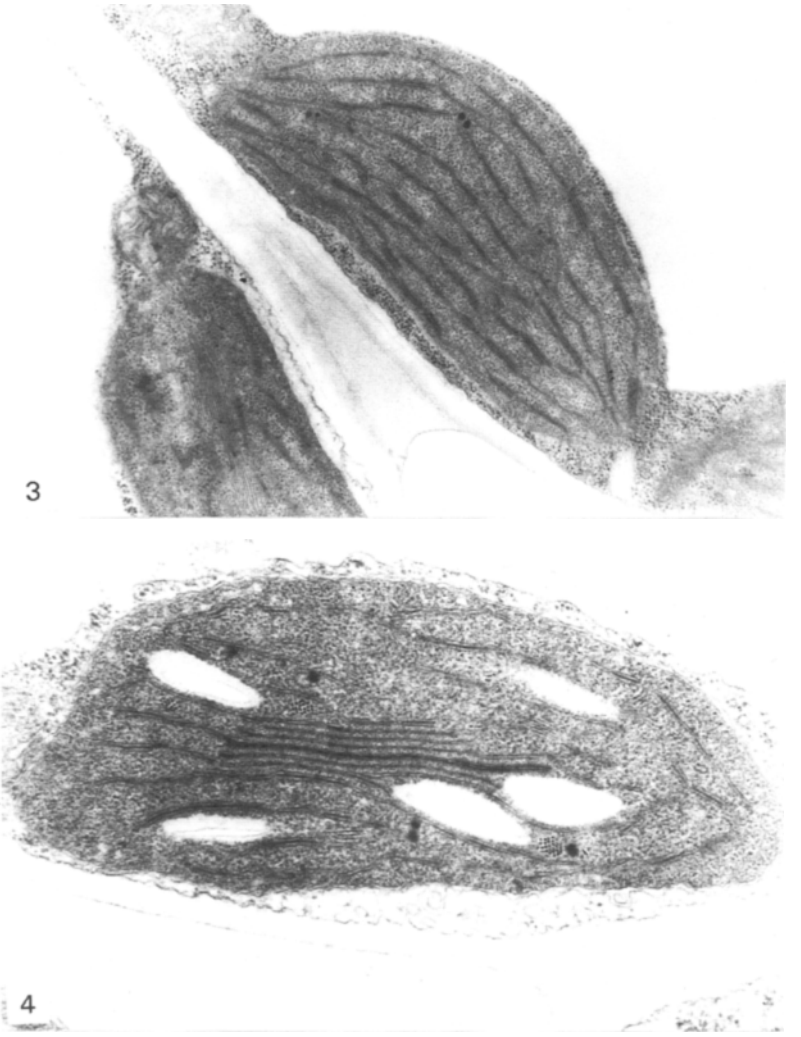
Fig. 2. The chloroplast numbers in the mesophyll cells of 12-d-old leaves, incubated for 72 h in the analyzed solutions (concentrations: kinetin in μM, lead in mM). The plastids were counted in the central part of leaf blade (region I) and between central part and vascular bundle (region II).
Inset: The graphic scheme of statistical analyses, min and max - the minimal and maximal values, $\bar{x}$ - mean value, $\bar{x}$ + SD and $\bar{x}$ - SD - mean value increased and decreased respectively by standard deviation.

The 7-d-old leaves incubated in the control conditions (24 h in darkness and 24 h in light) contained 294.47 μg of Chl *a+b* per g of fresh mass (f.m.). The Chl content in leaves growing in 0.4 mM $PbCl_2$ solution was reduced by 56 % compared to the control and amounted 129.69 μg g^{-1}(f.m.). The 12-d-old leaves incubated in 0.4 mM $PbCl_2$ contained 106.65 μg(chl *a+b*) g^{-1}(f.m.), which was 50 % less than control and 19 % less than 7-d-old, Pb-treated leaves. The light response of 12-d-old leaves was reduced compared to 7-d-old ones, whereas the lead-induced inhibition of Chl *a+b* synthesis remained at the same level. Most differences were statistically significant at $P = 0.01$, the others at $P = 0.05$.

Kinetin (0.5 μM) caused the increase in Chl *a+b* content by 27 % (Fig.1) as compared to control. The leaves treated with both kinetin (0.5 μM) and $PbCl_2$ (0.4 mM) had only a little lower Chl content in comparison with the control leaves. Thus kinetin (0.5 μM) probably decreased the inhibitory effect of lead on Chl *a+b* synthesis.

Number and ultrastructure of chloroplasts: The leaves treated with $PbCl_2$ alone contained similar chloroplast number per cell as the control (Fig. 2). The presence of kinetin in the incubation medium increased the average chloroplast number. The kinetin treated leaves contained 11 ± 5.7 chloroplasts per cell in region located at

central part of leaf blade and 16 ± 5.0 in region between the central part of leaf blade and the neighbouring vascular bundles. The chloroplasts number in control leaves cells was 6 ± 1.6 and 8 ± 2.7, respectively, in cells of leaves treated with kinetin + lead 8 ± 3.1 and 13 ± 4.6, respectively (Fig. 2).



Figs. 3 and 4. The chloroplasts ultrastructure of mesophyll cells in leaves incubated for 72 h in: H_2O (control) (Fig. 3, × 20 000), and $PbCl_2$ (0.4 mM) solution (Fig. 4, × 30 000).

The ultrastructural differences of leaves from various experimental variants concerned mainly the number of thylakoids per granum and number of grana per the chloroplast. The chloroplasts of control leaves showed the features of developing chloroplasts (Fig. 3). They contained the highest number of grana per chloroplast (32.3 ± 9.0, Fig. 5). In grana 2 - 5 thylakoids (2.6 ± 0.7 in average) were present

(Fig. 6). Phytoferritin and few, small plastoglobuli have been observed in stroma (Fig. 3). The thylakoids system in chloroplasts of kinetin-treated leaves was similar as in control, although the grana number was smaller (23.7 ± 9.3). The grana of certain chloroplasts were better developed than in control. The number of thylakoids reached 7, although the average number was similar to that observed in control (2.8 ± 0.9, Fig. 6).

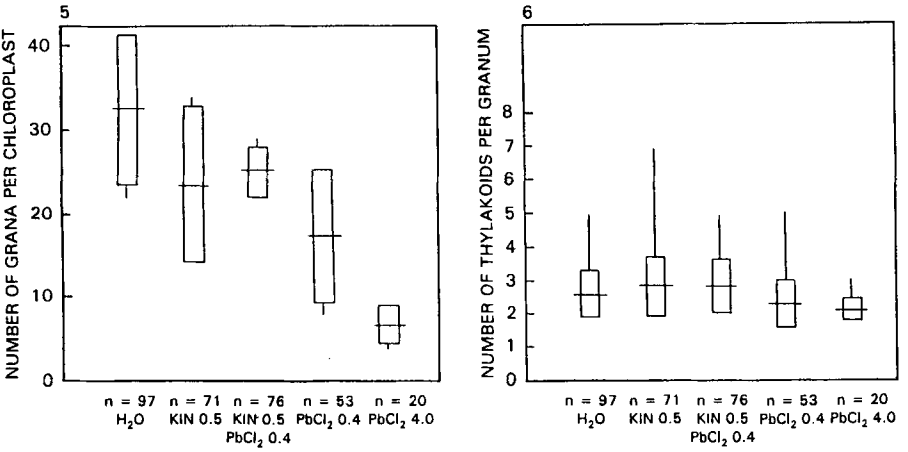


Fig. 5. The number of grana per chloroplast of 12-d-old leaves, following 72 h of incubation (concentrations: kinetin in μM, $PbCl_2$ in mM).

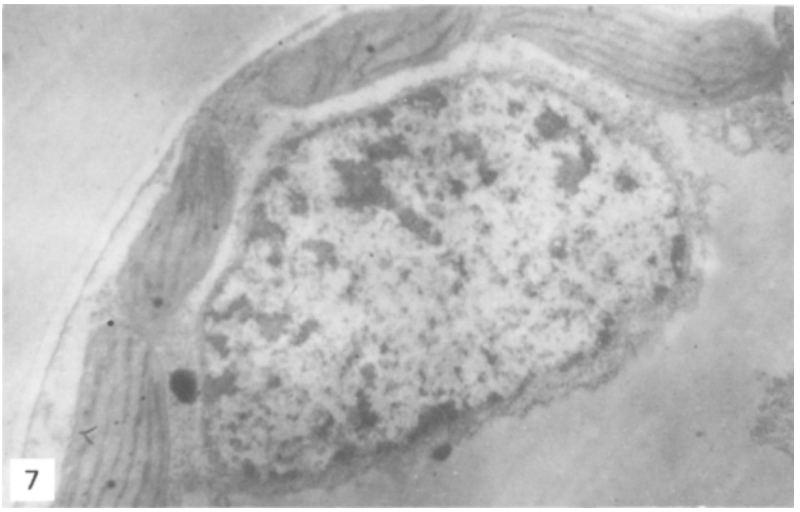
Fig. 6. The number of thylakoids per granum in chloroplasts of 12-d-old leaves, after 72 h of incubation (concentrations: kinetin in μM, $PbCl_2$ in mM).

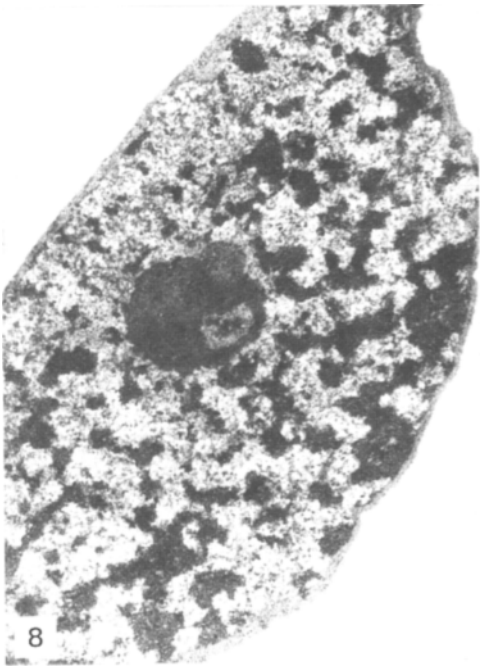
A similar chloroplast ultrastructure in leaves treated with kinetin + lead as compared to leaves treated with kinetin alone was found. The chloroplasts contained in average 25.3 ± 3 grana and 2.8 ± 0.8 thylakoids per granum. Differences were: the elongated shape of certain grana, lack of phytoferritin and the presence of small, single starch granules (Fig. 4). The plastids of leaves treated only with $PbCl_2$ (0.4 mM) contained elongated grana and relatively high number of slightly enlarged plastoglobuli. The starch granules were observed more frequently than in leaves treated with both kinetin and lead. The average number of grana was 17.7 ± 8.1, *i.e.* decreased by 54.8 % to the control. The mean thylakoids number per granum was 2.3 ± 0.7 (Fig. 5). The increase of $PbCl_2$ concentration in the medium to 4.0 mM caused the significant decrease of the thylakoids number in granum to 2.3 ± 0.3 (Fig. 6). Thylakoids were considerably elongated and occupied up to 2/3 of plastid section. Large and numerous plastoglobuli but no starch granules or phytoferritin were observed. In none of the chloroplasts of lead-treated leaves lead deposits were found.

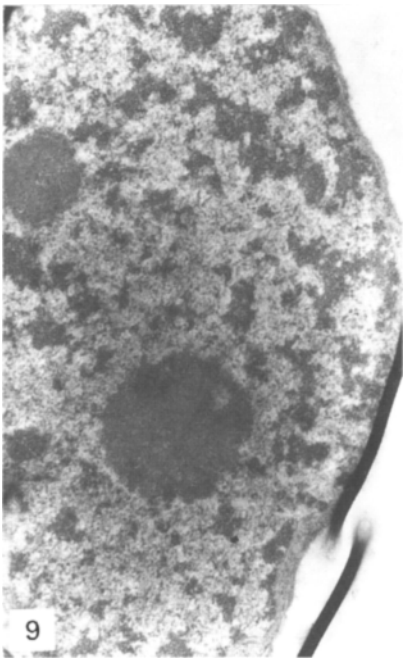
It is worth to notice that both $PbCl_2$ (0.4 mM) and kinetin caused the increase of compact chromatin content in nuclei as compared to control leaves (Figs. 7 - 9).

The effect on stomata: In the presence of lead alone (0.4 mM $PbCl_2$) or in combination with kinetin (kinetin 0.5 μM + $PbCl_2$ 0.4 mM) the percentage of open

stomata as well as pore size decreased in comparison with control (Fig. 10). Kinetin at concentration 0.5 μM caused the statistically significant increase of stomata opening. In the presence of kinetin 93.3 % of stomata was open whereas only 63.3 % in control.







Figs. 7 - 9. The nuclei of mesophyll cells of 12-d-old leaves incubated for 72 h in: H_2O (Fig. 7, × 10 000), kinetin (0.5 μM, Fig. 8, × 17 000), $PbCl_2$ (0.4 mM, Fig. 9, × 14 000).

Lead accumulation: Based on preliminary experiments the 12-d-old etiolated leaves were incubated for 48 h in water (control) or in solutions containing 0.04, 0.4 and 2.0 mM of $PbCl_2$. The increasing concentration of $PbCl_2$ in incubation medium was correlated with lead accumulation in leaf tissues (Fig. 11). Leaves incubated in 0.04 mM $PbCl_2$ contained 175 $\mu g(Pb^{2+})\ g^{-1}$(d.m.). Ten times higher $PbCl_2$ concentration caused the 5 times increase of lead content in leaves. When 2 mM $PbCl_2$ was used the leaves contained 2125 $\mu g(Pb^{2+})\ g^{-1}$(d.m.), which was 2.5 times more than observed in leaves incubated at lower concentration of $PbCl_2$ (0.4 mM) (Fig. 11).

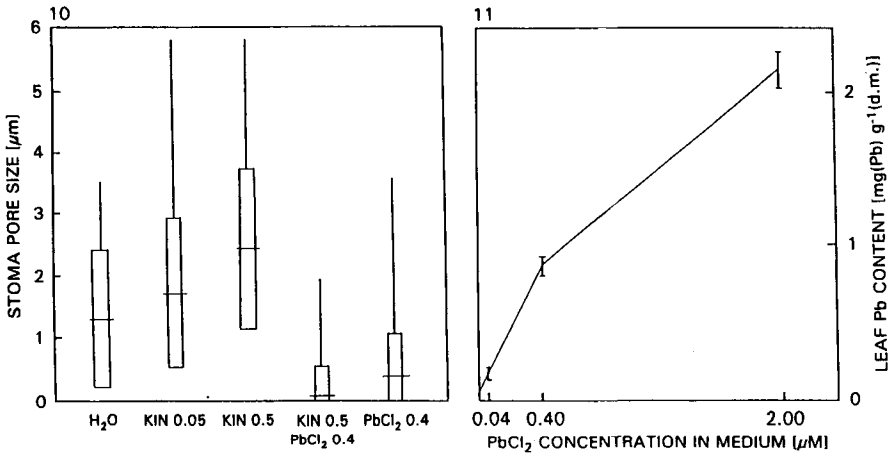


Fig. 10. The pore width of the stomata from adaxial epidermis of 7-d-old leaves, after 48 h of incubation (concentrations: kinetin in μM, $PbCl_2$ in mM).

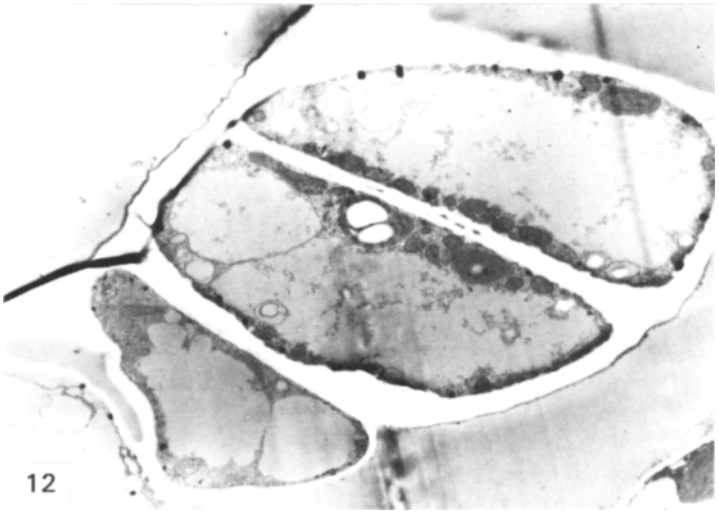
Fig. 11. The relation between the Pb content, accumulated in the detached 12-d-old leaves and the $PbCl_2$ concentration in the incubation medium. Leaves were incubated for 48 h in H_2O - control, or in solutions containing 0.04, 0.4 and 2 mM of $PbCl_2$.

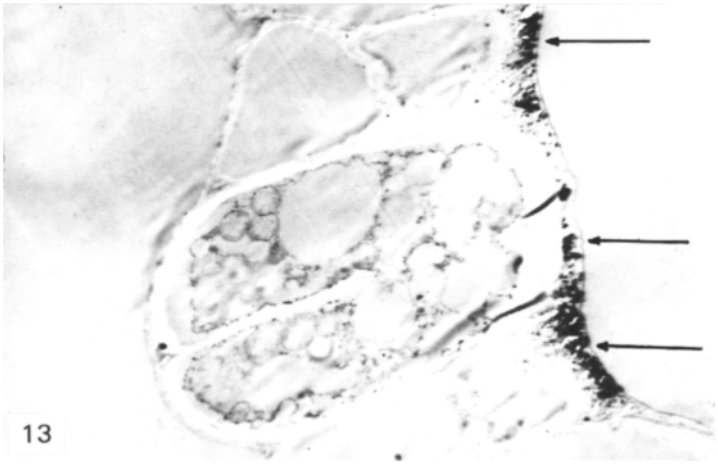
The time-dependent accumulation of lead was analyzed using $PbCl_2$ concentration (0.4 mM), which inhibited the chlorophyll synthesis by 50 %. Leaves were incubated for 8, 24 and 48 h and lead content was 760, 820 and 840 $\mu g\ g^{-1}$(d.m.), respectively. The results showed that main lead accumulation in barley leaves took place during the first 8 h of treatment.

Localization of lead within the leaf tissues: Lead was detected (by rhodizonate method) after 72 h of leaves incubation in solution containing kinetin (0.5 μM) + $PbCl_2$ (0.4 mM) or $PbCl_2$ (0.4 mM) only. In both experimental variants the lead was detected in vascular bundles of leaf fragments which were submerged in $PbCl_2$ solution. By using this method no lead was detected in the leaf fragments located during the incubation above the $PbCl_2$ solution.

Based on electron dispersion properties of lead, the presence of this metal was observed by electron microscopy in leaf segments located above $PbCl_2$ solution, whereas it was undetectable by cytochemical method. The electron microscopic

examination showed lead deposits within vascular bundles, in intercellular spaces of mesophyll, in stomata and in cuticle covering stomata.





Figs. 12 - 13. Sections through the basal part of stomata from 12-d-old leaves incubated for 72 h in: H_2O (Fig. 12, × 11 000), and $PbCl_2$ (0.4 mM) solution, arrows indicate lead deposits in cuticle (Fig. 13, × 20 000).

Within vascular bundles large deposits of lead were found in xylem elements, pits, plasmodesmata, wood parenchyma cells and intercellular spaces. In the xylem elements small lead deposits were located close to cell wall. Rarely and only at the highest $PbCl_2$ concentration used (2.0 mM), lead deposits were observed in the primary cell wall, but there were no in the secondary one. In the wood parenchyma cells and in phloem, lead occurred mostly along the cell walls. Lead deposits were

cells and in phloem, lead occurred mostly along the cell walls. Lead deposits were present at exoplasmic side of plasma membrane at characteristic "pockets" inclusions, in vacuole and small quantities in cytoplasm. No lead deposits were found in mesophyll cells, whereas in guard cells lead deposits were detected only when 4.0 mM $PbCl_2$ solution was applied. A considerable amount of lead deposits was found in cuticle covering the guard cells (Figs. 12 - 13), which suggest that lead might be removed *via* transpiration stream.

Discussion

The time-dependent study on the lead uptake by excised barley leaves indicates that lead is absorbed and accumulated mostly during the first 8 h of treatment. The similar pattern of lead uptake was described for onion roots (Wierzbicka 1987).

In 12-d-old leaves incubated either in water or in $PbCl_2$ solution the content of chlorophyll was lower than in 7-d-old, which results from the reduced sensitivity of their plastids to the light being among others ageing effect accelerated by darkness. It is suggested that the prolonged dark incubation of plant might be responsible for changing the ratio of prolamellar body to prothylakoids, in disadvantage of the latter ones (Wellburn *et al.* 1983). On the other hand the most of the photochemical functions, starting during the greening processes, take place within the prothylakoids (Wellburn and Hampp 1979). The decrease of the greening observed in the etiolated, lead-treated barley leaves resulted from altered chloroplasts ultrastructure. The quantity of this organelles was only slightly lower than in control, however, the number of the grana within the chloroplasts was decreased and the stacked thylakoids were elongated. Kinetin stimulated the greening process of excised barley leaves and applied in combination with lead diminished the negative effects caused by this metal. Cytokinins are known to modify the toxic effects of heavy metal ions and other poisons (Bessonova *et al.* 1984, Woźny 1987), however the mechanism of this process remains unknown. The kinetin-stimulated greening of leaves resulted from increased number of chloroplasts, since their ultrastructure was the same as in the control.

It is worth to mention that lead (Radecki *et al.* 1989) (Fig. 9) and, in larger extent, kinetin (Fig. 8) or benzylaminopurine (Kinoshita *et al.* 1991) were responsible for the increase of condensed chromatin content in nucleus. A similar effect was observed as a result of dehydratation of plant cells (Čiamporová 1989). The enhanced level of condensed chromatin is ultrastructural feature of increased DNA content, which is, however, transcriptionally non-active (Nagl and Peschke 1982).

An interesting finding is the detection of lead deposits in the cuticle covering guard and subsidiary cells. One of the possible explanation is that metal ions moving through xylem and via apoplastic pathway are concentrated in the cuticle of stomata. The barley leaves used in our experiments were incubated under controled conditions thus lead deposits in the cuticle covering stomata could not originated from atmospheric contaminations. The lack of such deposits onto cuticle of control plants additionally confirms this hypothesis.

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