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Response of intact cyanobacterial cells and their photosynthetic apparatus to Cd^{2+} ion treatment

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Abstract

Intact cells of *Synechococcus elongatus* were treated with different concentrations (0.1 and 1.0 mM = $Cd_{0.1}$, $Cd_{1.0}$) of $CdCl_2$ for 24 h. $Cd_{0.1}$ treatment stimulated growth of the cell culture and chlorophyll (Chl) *a* concentration in the culture. $Cd_{1.0}$ inhibited both the above mentioned parameters. The oxygen evolving activity of intact cells ($H_2O \rightarrow BQ$) as well as of isolated thylakoid membranes, TM ($H_2O \rightarrow$ DCPIP; $H_2O \rightarrow$ PBQ + FeCy) decreased after 24 h of $Cd_{1.0}$ cultivation to 7 %. Photosystem 1 (PS1) activity was less sensitive to the effect of Cd^{2+} than PS2 activity. $CdCl_2$ concentration in cultivation media after 24 h of cultivation proved that the cyanobacterium cells take up these ions to a large extent from the cultivation medium. After 24 h of the $Cd_{1.0}$ treatment only 12 % of the amount of Cd^{2+}originally added to the cultivation medium was found. The ratio of external-antenna pigments, phycocyanin, and allophycocyanin to Chl increased approximately twofold with growing Cd^{2+} concentration in the cultivation medium. This ratio was found in both TM and dodecylmaltoside extracts.

Additional key words: chlorophyll; dodecylmaltoside; heavy metals; photochemical activities; photosynthetic pigment content; photosystems 1 and 2; *Synechococcus*; toxic metals.

Introduction

At present, much attention is paid to the presence of heavy metals in the environment. The list of so-called heavy metals includes metals that are highly toxic to plants and animals (Pb, Cd, Hg, As) as well as metals that, in low concentrations, are essential for living organisms (Cu, Mn, Zn, Co). As the present study deals with the influence of toxic heavy metal ions, we shall further use the terms "toxic metals" and "toxic metal ions".

Cadmium, which causes substantial changes in human and animal organs and tissues, is one of the most intensively studied toxic metals. It also has cancerogenic effects (Chaney *et al.* 1999). Toxic metals enter the organism from soil and water along the food chain by means of autotrophs. Organisms develop various mechanisms to protect themselves against the harmful effect of toxic metal ions on biochemical processes.

Autotrophic organisms accumulate toxic metal ions in two ways. The first is rapid physicochemical sorption of the metal on cell surfaces. The other involves slow penetration of toxic ions into cells. In the case of cadmium, the size of polyphosphate bodies increases significantly in the course of this process and large amounts of cadmium are accumulated. This indicates that cadmium is probably localised in the polyphosphate bodies (Jensen *et al.* 1982, Fernandez-Piñas *et al.* 1995). On the other hand, cadmium has also been detected in cells that have no polyphosphate bodies (Jensen *et al.* 1982). Plant organisms can also react to an increased content of toxic metals in the environment by forming specific binding polypeptides, phytochelatins (Domažlická *et al.* 1994).

In phototrophs, Cd ions inhibit chlorophyll (Chl) biosynthesis by means of a reaction with the thiol groups of the enzymes of 5-aminolevulinic acid synthesis and the ternary complex of the enzyme protochlorophyllide reductase, followed by accumulation of free protochlorophyllide (Stobart *et al.* 1985). In vascular plants exposed to Cd^{2+}, total Chl content and the Chl *a/b* ratio decrease. Cadmium ions induce changes in the arrangement and structure of the light-harvesting Chl-protein complex 2 (Krupa 1988, Krupa *et al.* 1987, Šeršeň and Kráľová 2001).

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Abbreviations: APC – allophycocyanin, BQ – *p*-benzoquinone, Chl – chlorophyll, CPC – cyanobacterial phycocyanin; DCMU – 3-(3',4'-dichlorophenyl)-1,1-dimethylurea; DCPIP – 2,6-dichlorophenolindophenol; *n*DM – *n*-dodecyl-β,D-maltoside; FeCy – ferricyanide; HEPES – N-[2-hydroxyethyl]piperazine-N'-[2-ethanesulfonic acid]; MeV – methylviologen; OEA – oxygen evolving activity; PBQ – phenyl-*p*-benzoquinone; PS – photosystem; TM – thylakoid membranes.

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of the Chl molecule by a heavy metal atom (Hg, Cd, Cu, Ni, Zn, or Pb) in plants living in an environment containing heavy metal ions has also been observed. This substitution impedes efficient radiation of harvesting by the affected Chl molecules (Küpper *et al.* 1996). Photosystem 2 (PS2) is a multiprotein complex localised in the thylakoid membrane (TM). It is particularly in the PS2 region, on the donor as well as the acceptor side that many toxic heavy metals block electron transport in vascular plants (Clijsters and Van Assche 1985, Krupa and Baszyński 1995). The oxygen-evolving complex localised on the luminal side of TM, which supplies electrons from water to the PS2 reaction centre, is the main target of the toxic effect of heavy metals on vascular plants (Bernier *et al.* 1993, Ouzounidou *et al.* 1993, Skórzyńska and Baszyński 1993, Maksymiec *et al.* 1994, Krupa and Baszyński 1995).

Several studies on the influence of Cd^{2+} on cyanobacteria exist in the literature. Takatera and Watanabe (1992) investigated the synthesis of metallothioneins by the cyanobacterium *Anacystis nidulans*, grown for 2 weeks in the presence of 8.9 μM Cd^{2+} using the technique of gel permeation chromatography. Fernandez-Piñas *et al.* (1995) dealt with the interaction effect of cadmium and calcium on growth, oxygen and nitrogen evolution, and growth cycles in the cyanobacterium *Nostoc UAM 208*. Bekasova *et al.* (2000) investigated the mechanism of cadmium ion binding and the influence of Cd^{2+} on structure and function of *Nostoc muscorum* cells.

The aim of our study was the investigation of the influence of Cd^{2+} on the photosynthetic apparatus of the cyanobacterium *Synechococcus elongatus*, first of all on the subcellular and molecular levels. There were two reasons for this. Toxic metals evidently inhibit photosynthetic processes, which are essential for photoautotrophs. Moreover, cyanobacteria bind and accumulate toxic metal ions quickly and in large amounts (Kostyaev *et al.* 1980). These properties of cyanobacteria can be of substantial importance for renewal of land damaged by industrial production.

Materials and methods

Cyanobacteria cultivation: *Synechococcus elongatus* Nägeli form *thermalis* Geitler, strain Kovrov 1972/8, was grown at 57 °C in the medium of Kratz and Myers (1955) with addition of 10 mM $NaHCO_3$. Irradiated was with the 500 W incandescent lamp, aerated by a mixture of air with 2 % of CO_2 (Šetlíková *et al.* 1999).

TM isolation: Cyanobacterial intact cells were collected by centrifugation (2 200×*g*, 10 min, 4 °C) and washed with buffer PM (30 mM K_2HPO_4, 5 mM $MgCl_2$, pH 7.8). TM were obtained by hydrolysis of the cell wall using 0.2 % egg-white lysozyme (m/v) in MPM buffer (500 mM mannitol, 30 mM K_2HPO_4, 5 mM $MgCl_2$, pH 7.8 for 2 h, at 40 °C, shaking in the dark) and by osmolysis of the spheroplasts formed in hypotonic buffer solution (HM) containing 20 mM HEPES, 5 mM $MgCl_2$, pH 6.5. After centrifugation (2 200×*g*, 10 min, 4 °C), the sediment containing TM was washed several times with HM buffer to remove most of the biliproteins. Finally, the TM were re-suspended in MHM buffer (500 mM mannitol, 20 mM HEPES, 5 mM $MgCl_2$, pH 6.5), frozen in liquid nitrogen, and stored at –80 °C.

Preparation of detergent extracts: An amount of *n*-dodecyl-β,D-maltoside (*n*DM) was added to a suspension of TM containing 1 kg m^{-3} Chl such as to achieve its final concentration of 1 % (m/v). Incubation with the detergent was carried out at room temperature, 30 min under constant agitation. The mixture was then centrifuged (350 000×*g*, 60 min, 4 °C). The supernatant mainly contained solubilised PS2 particles.

Spectroscopic methods: So-called apparent optical density (A_{750}) measured at a wavelength of 750 nm in a 1 cm cell served as a measure of culture mass growth. The Chl *a* content was analysed by the Ogawa and Vernon (1971) method (50 mm^3 of the sample is extracted into 5 cm^3 of 100 % methanol and the absorbance of the filtrated solution is measured at 666 nm, $\varepsilon = 65.8\ m^3\ mol^{-1}\ cm^{-1}$). The content of phycobilins was analysed by the Bennett and Bogorad (1973) method. Cyanobacteria of the genus *Synechococcus* lack phycoerythrin. A sample containing 10 μg Chl was made up to 5 cm^3 with a solution (10 mM KCl, 40 mM HEPES, pH 6.5) and disintegrated using an ultrasonic homogeniser (*Cole Parmer*, type *CP 130 PB-1*, USA) at 5 W output and a vibration amplitude of the probe tip of 50, duration three times 1 min, cooling in an ice bath. Absorbance was then measured at 652 and 615 nm, and the pigment concentration [μg cm^{-3}(measured sample)] was calculated using the following equations:

$$c(CPC) = 187\ A_{615} - 89\ A_{652}$$

$$c(APC) = 196\ A_{652} - 41\ A_{615}$$

Photochemical activities: Oxygen evolution activity (OEA) in whole cells was measured using a Clark oxygen electrode in the arrangement described by Bartoš *et al.* (1975) [the cell suspension diluted with cultivation medium to a value of $A_{750} = 0.2$ contained a final concentration of 1.16 mM *p*-benzoquinone (BQ) as an electron acceptor, the surface irradiation of the reaction cell was 1 000 μmol $m^{-2}\ s^{-1}$ "white light"]. OEA of TM or dodecylmaltoside extracts was measured using a Clark oxygen electrode (*Hansatech*, U.K.). The reaction cell was irradiated by red radiation of approx. 720 μmol $m^{-2}\ s^{-1}$. For PS2

activity measurement, the electron acceptor mixture of 1.0 mM potassium ferricyanide (FeCy) and 0.3 mM phenyl-*p*-benzoquinone (PBQ) in a total volume of 1 cm^3 MHM medium containing 10 μg Chl was used. For PS1 activity measurement, a mixture of non-physiological electron donors and acceptors was used [10 mM ascorbate-Na, 0.15-0.20 mM DCPIP, 67 μM methylviologen (MeV), 10 μM DCMU, 5 mM NH_4Cl, a few crystals of catalase] in a total volume of 1 cm^3 HM medium containing 10 μg Chl. Water splitting activity of TM or of the detergent extract was likewise measured as the initial rate of photoreduction of a 30 μM 2,6-dichlorophenolindophenol (DCPIP) solution in 1 cm^3 of reaction medium (10 mM KCl, 40 mM HEPES) containing 20 μg Chl.

Analysis of metal content: Contents of Fe and Mg were analysed by means of atomic absorption spectrometry, using a *Varian Spectr AA-300A* spectrometer. For Cd and Mn, a *Varian Spectr AA 400* atomic absorption spectrometer with electrothermal atomisation in a graphite cell and dosage device was used. Atomisation was done in an acetylene-air flame.

Results

Cells were cultivated for 24 h in final concentrations of 0, 0.1, and 1.0 mM $CdCl_2$ (Cd_0, $Cd_{0.1}$, $Cd_{1.0}$). Samples of the cell culture were taken at intervals of 0, 6, 12, and 24 h and characterised on cell, thylakoid membrane, and *n*DM-extract levels. TM and *n*DM-extracts were only isolated after 24 h of cultivation in $CdCl_2$.

Cells

Increase in culture mass (A_{750}) during the experiment in the $Cd_{0.1}$ culture was similar to that in the Cd_0 culture (Fig. 1*A*), but in the $Cd_{1.0}$ culture it was lower than in the Cd_0 culture, especially after 6 h of cultivation.

Chl *a* content measured after 24 h of cultivation was slightly higher in the $Cd_{0.1}$ culture than in the control (Fig. 1*B*). Contrarily, the Chl content of the $Cd_{1.0}$ culture decreased by 50 % after 24 h of cultivation.

Oxygen evolving activity (OEA): Similar to cell culture density (A_{750}), OEA values of the control sample were near to those of the $Cd_{0.1}$ sample (Fig. 1*C*). The 1.0 mM $CdCl_2$ was again inhibitory: the OEA was approx. 95 % lower after 24 h of Cd^{2+} treatment. OEA, related to the amount of Chl in the culture (Fig. 1*D*), was similar to the OEA values in Fig. 1*C*.

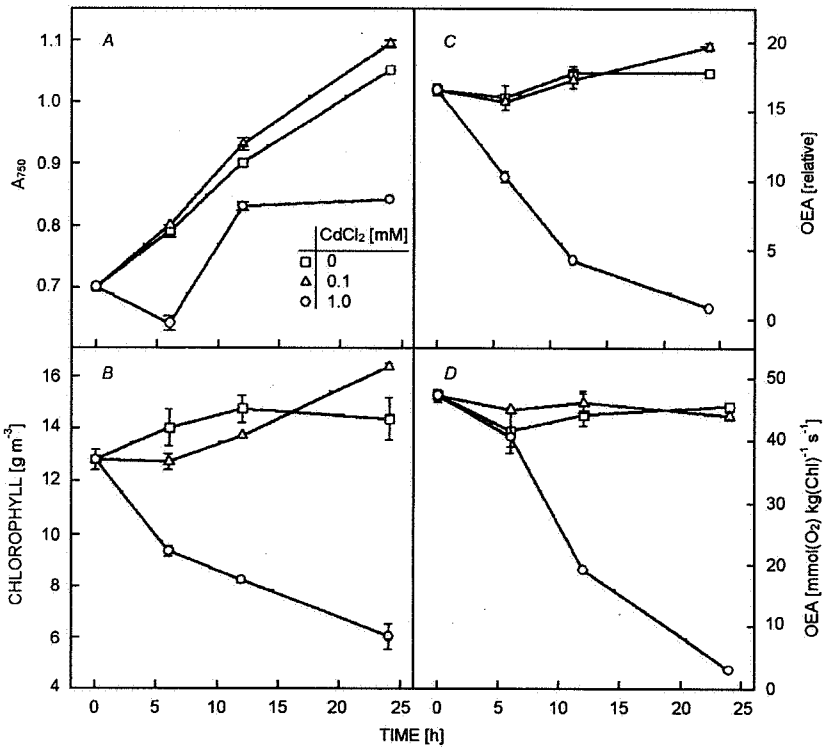

Fig. 1. Culture mass (A_{750}) (*A*), chlorophyll *a* concentration (*B*), and oxygen evolving activity (OEA) (*C*, *D*) in the cell culture during 24 h of cultivation of the cyanobacterium *Synechococcus elongatus* in different $CdCl_2$ concentrations. Arithmetic mean ± standard deviation ($n = 3$).

Table 1. Concentrations of Cd^{2+}, Mg^{2+}, Fe^{3+}, and Mn^{2+} in cultivation media after 24 h of cell cultivation in the presence of various $CdCl_2$ concentrations. Arithmetic mean ± standard deviation ($n = 3$).

$CdCl_2$ [mM]	Cd^{2+} [g m^{-3}]		Mg^{2+} [g m^{-3}]		Fe^{3+} [g m^{-3}]		Mn^{2+} [mg m^{-3}]	
	0 h	after 24 h	0 h	after 24 h	0 h	after 24 h	0 h	after 24 h
control	0	0.006±0.001	50.50	15.31±0.03	11.089	0.499±0.003	145.0	73.0±1.3
0.1	11.240	4.259±0.145	50.50	12.58±0.01	11.089	0.113±0.005	145.0	44.6±0.3
1.0	112.400	13.351±0.547	50.50	14.75±0.29	11.089	0.109±0.014	145.0	15.1±0.6

Table 2. Photochemical activities: DCPIP photoreduction [mmol($DCPIP_{red}$) kg^{-1}(Chl) s^{-1}] or PS2 or PS1 activities [mmol(O_2) kg^{-1}(Chl) s^{-1}] of thylakoid membranes (TM) and *n*DM-extracts isolated from cells grown for 24 h in the presence of various $CdCl_2$ concentrations. Arithmetic mean ± standard deviation ($n = 3$).

	$CdCl_2$ [mM]	$H_2O \rightarrow DCPIP$		$H_2O \rightarrow PBQ + FeCy$		$DCPIP_{red} \rightarrow MeV$	
			[%]	PS2	[%]	PS1	[%]
TM	control	17.0±1.5	100.0±8.8	66.1±2.6	100.0±3.9	67.8±2.9	100.0±4.3
	0.1	14.4±0.7	84.7±4.9	67.5±1.0	102.1±1.5	53.1±1.1	78.3±2.1
	1.0	8.0±0.7	47.1±8.8	4.4±0.4	6.7±9.0	18.6±1.9	26.2±10.2
*n*DM-extracts	control	7.2±0.8	100.0±11.1	56.9±2.2	100.0±3.8	28.9±2.4	100.0±8.3
	0.1	8.9±0.1	123.6± 1.1	69.7±1.4	122.5±2.0	26.1±0.6	90.3±2.3
	1	3.5±0.0	48.5± 0.0	20.3±1.0	35.7±4.9	31.1±0.9	107.6±2.9

Atomic absorption spectrometry: After 24 h of cultivation in the presence of 0, 0.1, and 1.0 mM $CdCl_2$, the cells were separated by centrifugation and Cd^{2+}, Mg^{2+}, Fe^{3+}, and Mn^{2+} concentrations were determined in the remaining media. The higher the initial Cd^{2+} concentration, the larger the fraction of these ions taken up from the cultivation medium. In the $Cd_{0.1}$ sample approx. 38 % Cd^{2+} remained after 24 h, while only 12 % Cd^{2+} was found in the $Cd_{1.0}$ sample. Mg^{2+} uptake by cells did not differ much among the samples described, while iron and manganese ion concentrations in samples grown in the presence of Cd^{2+} were several times lower compared to the control (Table 1).

TM

Photochemical activities: Measuring DCPIP photoreduction in TM isolated from cells grown for 24 h in the presence of 1.0 mM $CdCl_2$ we recorded an activity decrease of approx. 50 % compared to the control. OEA decreased to approx. 7 % of the control value. PS1 activity in TM isolated from $Cd_{1.0}$ cells decreased to about one quarter of the Cd_0 value (Table 2).

Phycobilin concentration: The ratio of both phycobilin types to Chl approximately doubled under the influence of Cd^{2+} (Table 3).

Dodecylmaltoside extracts

Photochemical activities: PS2 activity measured by DCPIP photoreduction in $Cd_{1.0}$ sample decreased by about 50 %, OEA decreased by 60 % compared to the control. The activity of PS1 remained unaffected (Table 2).

Phycobilin concentration: A higher ratio of phycocyanin and allophycocyanin to Chl was found in *n*DM-extracts from $Cd_{1.0}$ cells compared to the Cd_0 sample, in both cases lower than in TM (Table 3).

Table 3. Phycobilin to Chl ratio [m/m] in thylakoid membranes (TM) and *n*DM-extracts isolated from cells grown for 24 h in the presence of various $CdCl_2$ concentrations.

	$CdCl_2$ [mM]	CPC/Chl	APC/Chl
TM	control	11.0	13.5
	0.1	14.3	16.1
	1.0	22.0	30.4
*n*DM-extracts	control	7.2	9.6
	0.1	6.9	8.2
	1.0	13.9	13.6

Discussion

Cd is one of the chief toxic heavy metals. Nevertheless, in low concentrations (*i.e.* 0.1 mM $CdCl_2$) this metal stimulated rather than inhibited growth of *S. elongatus* culture and its Chl *a* content. After 24 h of cultivation, the values of the characteristics measured were slightly higher than in the control. A similar observation was published by Karavaev *et al.* (2001), who studied the Chl content, oxygen evolving activity, fluorescence induction, and other characteristics in leaves of *Vicia faba* grown in the presence of 0.1-1.0 mM aqueous $CdCl_2$ solution. Low Cd^{2+} concentrations (0.1-1.0 μM) stimulated photosynthetic activity, while its high concentrations in the growth

medium (0.1-1.0 mM) suppressed it.

In the course of 24-h cultivation we observed a distinct inhibition effect of 1.0 mM $CdCl_2$ on the photosynthetic apparatus. The significant decline of growth after 6 h of cultivation may be interpreted as an immediate response of the cell culture to the effect of toxic metal ions. Cell growth recovered to some degree after this, although it stagnated for another 12 h. After 24 h, the growth of this culture reached 40 % of the control. Hence, a certain acclimation of the cells to a medium containing toxic metal ions may be involved. The decrease in Chl *a* concentration after 24 h in the presence of 1.0 mM $CdCl_2$ was about 50 %.

OEA of whole cells cultivated in 1.0 mM $CdCl_2$ for 24 h, related to both culture density as well as Chl amount decreased to 6 and 7 %, respectively. This indicated that both the increases in mass of *Synechococcus* culture and its Chl content were influenced by the Cd ions to a similar degree. The situation was similar in isolated TM, where OEA decreased to 7 % of the control.

Measurement of Cd^{2+} concentration in cultivation media remaining after 24 h of cultivation and comparison with the initial concentrations of Cd^{2+} showed that cells of *S. elongatus* take up these ions from the cultivation medium to a large extent. There are three binding sites: cell wall, binding in metallothioneins, binding to polyphosphate. The higher the Cd^{2+} concentration, the larger its fraction taken up. This confirms the finding of Kostyaev *et al.* (1980) that cyanobacteria accumulate toxic metal ions quickly and in large amounts. For example, the Cd-binding capacity of the cyanobacterium *Chroococcus paris* is 53 g kg^{-1}(dry mass), 90 % of the metal being bound within the first minute. It is assumed that cyanobacteria store and accumulate ions of some toxic metals in polyphosphate bodies, for example. The possibility of accumulation of toxic metal ions in the bodies is described by Fernandez-Piñas *et al.* (1995).

As the Chl *a* content in the $Cd_{1.0}$ culture decreased, we could assume that the Mg^{2+} concentration in the remaining cultivation medium would be greater than the concentration of these ions in the control. The practically unaltered Mg^{2+} concentration can be explained by the fact that Mg ions originating in disintegrated or substituted Chl are not released from cells into the medium although not only Chl *a* contains Mg^{2+}. Contrarily, the iron and manganese ion concentration in the remaining cultivation medium was several times lower than in the control. The decrease of the metal content in the medium and the assumed transport into cells may be due to increased synthesis of components participating in electron transport or components of the oxygen evolving complex. These ions or the respective metalloproteins or enzymes can, however, also be involved in metabolic processes other than photosynthesis.

The ratio of the two types of phycobilins to Chl, measured in isolated TM, increased approximately twofold with growing concentration of Cd ions in the cultivation medium. Hence the response to stress caused by Cd ions is also probably a change in the peripheral light-harvesting antenna system, *i.e.* phycobilins of the phycobilisomes.

Isolation from the thylakoid membrane by detergents is a further step towards understanding the effect of Cd ions on the structure and function of individual supramolecular complexes of the photosynthetic apparatus. We extracted the supramolecular complexes using the nonionic detergent *n*-dodecyl-β,D-maltoside, which due to its structural similarity with thylakoid membrane glycolipids (Šetlíková *et al.* 1999) is one of the detergents suitable for extraction of PS2 particles.

Expressing the DCPIP photoreduction activity and OEA of *n*DM-extracts in percentages, we found that the decrease between Cd_0, $Cd_{0.1}$, and $Cd_{1.0}$ was not as distinct as in the case of TM. Both DCPIP photoreduction activity and OEA were significantly greater in the $Cd_{0.1}$ sample than in the control, while it was one half and one third, respectively, in the $Cd_{1.0}$ sample. Again, the stimulating effect of the lower $CdCl_2$ concentration on these activities was manifested.

Comparing activity changes in TM and *n*DM-extracts in quantitative terms, irrespective of their mutual ratio, we found that OEA decreased most in both cases. This, therefore, confirms that OEA is most sensitive to the effect of Cd^{2+}. Other correlations, including the practically absent inhibitory effect of Cd^{2+} on the PS1 activity in *n*DM-extracts appear to be purposeless. Clearly, PS2 or PS1 complexes behave differently in the environment of thylakoid membranes and in an artificial environment of detergent micelles. They evidently also have different phycobilisome antennae, as the increased phycobilin content in *n*DM-extracts of thylakoid membranes isolated from cells grown in different Cd^{2+} concentrations can be proved.

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