Acta Bot. Croat. 45, 33—42, 1986.

CODEN: ABCRA2
YU ISSN 0365—0588

UDC 593.161.3:546.48 = 20

CADMIUM CYTOTOXICITY AND RESISTANCE IN *EUGLENA GRACILIS*

ELENA MARČENKO

(Ruđer Bošković Institute, Zagreb)

Received December 22, 1985

By monitoring the fate of individual *Euglena* cells on agar plates, extreme sensitivity to low concentrations of cadmium ions during cell division was detected. Toxic effects of cadmium were somewhat more pronounced in the light. The antagonistic role of zinc was confirmed.

In etiolated *Euglena* cadmium inhibited chlorophyll and carotenoid synthesis. The protective role of the resting medium in comparison to the rich *Euglena* medium is attributed to differences in susceptibility to cadmium in different physiological states of the cells.

The properties of a cadmium resistant clone were investigated. This clone contained half the amount of chlorophyll normally present in *Euglena*, and the normal amount of carotenoids. On the submicroscopic level, most changes occurred in the chloroplasts. All stages from severely disorganized chloroplasts characterized by dilated and swollen thylakoids, to normal cells, were observed. Similarities with changes occurring during adaptation to the herbicide DCMU are discussed.

Introduction

Cadmium is one of the most toxic metals responsible for aquatic pollution. A green flagellate, *Euglena gracilis* has been extensively used as a model organism for the study of cadmium cytotoxicity. Cadmium affects growth rate and replication time in *Euglena* (see e.g. Falchuk et al. 1975, Bariaud et al. 1978, Bonaly et al. 1978, Albergoni et al. 1980), causes an increase in cell volume, and induces formation of abnormal star-shaped and multinucleate cells (Nakano et al. 1978 and 1980) and influences respiration and photosynthesis (De

Filippis 1981). On the submicroscopic level, endosome fragmentation (Falchuk et al. 1975, Nakano et al. 1980) and paramylon accumulation have been observed (Falchuk et al. 1975). Increase in DNA as a result of cadmium influence was studied by Bariaud et al. 1978 and Bonaly et al. 1980. The antagonistic effect of zinc on cadmium cytotoxicity was investigated by Falchuk et al. 1975 and Nakano et al. 1978 and 1980. Albergoni et al. (1980) proposed that cadmium, as a metal with no physiological role, is accumulated in the cell, in contrast to copper which is excreted into the medium. However, some cadmium resistance in *Euglena* was observed by Bariaud et al 1978 and Bonaly et al. 1978 and 1980. Mechanism of cadmium resistance was proposed by Bariaud et al. 1985.

In the present study the effect of cadmium on the inhibition of cell division at different light regimes, and on chlorophyll and carotenoid synthesis in bleached *Euglena* cells in different physiological states are investigated, along with the antagonistic effect of zinc, and the properties of a cadmium resistant clone.

Materials and Methods

Euglena gracilis strain »Z« was grown in the liquid organic medium (REM) described earlier (Marčenko 1970). HCl was omitted from the medium. REM solidified with agar (1.5%) was used in plating experiments. The cultures were kept in the dark, or they were illuminated with white fluorescent light (2000 lx) at 25° C or 30° C as indicated. Cadmium was added as $Cd(NO_3)_2 \times 4H_2O$, at concentrations ranging from 10^{-7} to 10^{-3} mol dm^{-3}. In experiments with zinc, possible traces of that metal present as impurities in the components of REM were disregarded.

Divisions of individual cells, diameters of colonies growing on agar plates, and cell motilities were scored under a stereomicroscope (magnification 40 ×).

Chlorophylls were determined by the method of Mackinney (cited by Holden 1965), and the relative amount of carotenoids was expressed as $\Delta \frac{car}{480}$ according to Kirk and Allen (1965). Chlorophyll and carotenoid syntheses were measured in previously bleached cells (kept in the dark for several generations). After bleaching, cells were exposed to white light (2000 lx) for four days at ca 25° C in petri dishes. Cells were illuminated in the same medium (REM) in which they had been growing in the dark or they were washed with 0.15 mol dm^{-3} NaCl and placed into the resting medium of Stern et al. (1964) in which the mannitol had been replaced by glucose and which had been supplemented with various concentrations of cadmium. Some petri dishes were kept in the dark as controls.

Induction of cadmium resistant clones. Cells were grown in REM medium supplemented with cadmium nitrate (1x 10^{-4} mol dm^{-3}), under continuous illumination with cool-white light (2000 lx). After four days, the cultures were washed twice with REM medium without Cd, diluted and plated on rich *Euglena* agar plates. After two weeks, per 100 undivided cells, 28 two-celled, 2 three-celled and 2 normally growing (containing more than 32 cells) but untypically coloured (yellow-green) colonies appeared. These altered clones were passed through several subcultures.

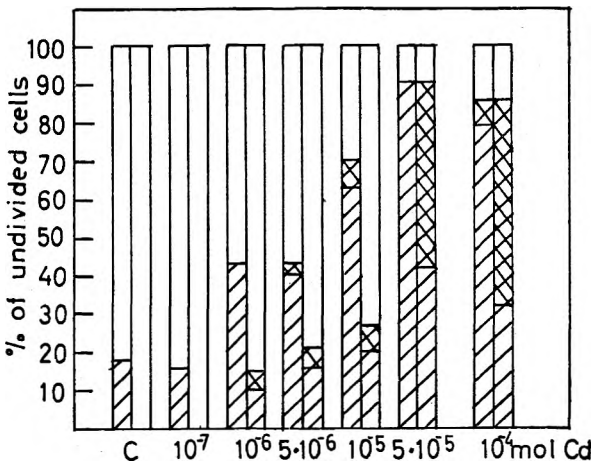


Fig. 1a. Effect of cadmium on cell division and viability in *Euglena* in the dark at 25°C.
Cells were grown in the dark at 25°C on agar-solidified REM medium supplemented with the cadmium concentrations indicated. Cell divisions were scored after 24 hours (first column) and 48 hours (second column). Lined column: per cent of undivided cells; checked column: per cent of dead cells.

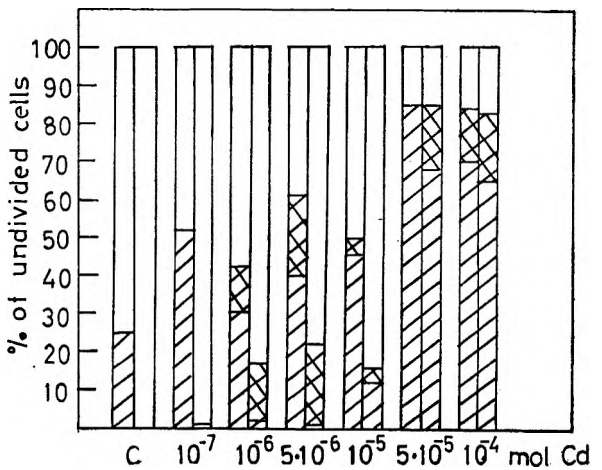


Fig. 1b Effect of cadmium on cell division and viability in *Euglena* in the light at 25°C.
Cells were grown in the light at 25°C on agar-solidified REM medium supplemented with the cadmium concentrations indicated. Cell divisions were scored after 24 hours (first column) and 48 hours (second column). Lined column: per cent of undivided cells; checked column: per cent of dead cells.

Pigment determination and electron microscopy of one of the clones was performed after 3 months of subculturing.

Electron microscopy was performed as described earlier (Marčenko 1973).

The author wishes to thank Dr. Nikola Ljubešić for the electron micrographs.

Results

The inhibitory effect of cadmium on cell division was studied by a method which permitted to monitor the fate of individual cells on the surface of an agar plate. The rate of inhibition of the first cell division after exposure of *Euglena* to cadmium increases and cell viability decreases with increasing cadmium concentration (Fig. 1a and 1b). Cadmium cytotoxicity was already observed at very low concentrations (10^{-6} and even $^{-7}$ mol dm^{-3}) in the dark. The growth rate was also reduced as shown by a reduced diameter of colonies developed after two weeks in the dark (compare Table 1). The diameter of a colony is correlated with the number of cells in that colony (see Marčenko 1974). Cells which were lethally damaged usually died during the process of cell division as seen by stereomicroscopic observation. Therefore, there were sometimes more lethally damaged cells in the presence of lower cadmium concentrations at which a higher percentage of cells entered cell division than at higher concentrations, at which cell division was more inhibited, and the cells remained undivided but not dead. The toxic

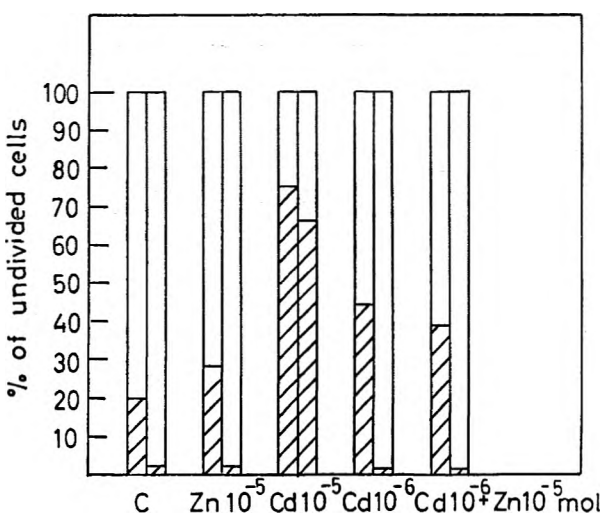


Fig. 2. Cytotoxicity of cadmium in *Euglena* and antagonistic action of zinc at 25°C.
Cells were grown in REM medium in the light and plated on the same medium supplemented with various concentrations of cadmium and/or zinc. The plates were kept in the dark at 25°C. Cell divisions were scored after 24 hours (first column) and 48 hours (second column). Lined column: per cent of undivided cells.

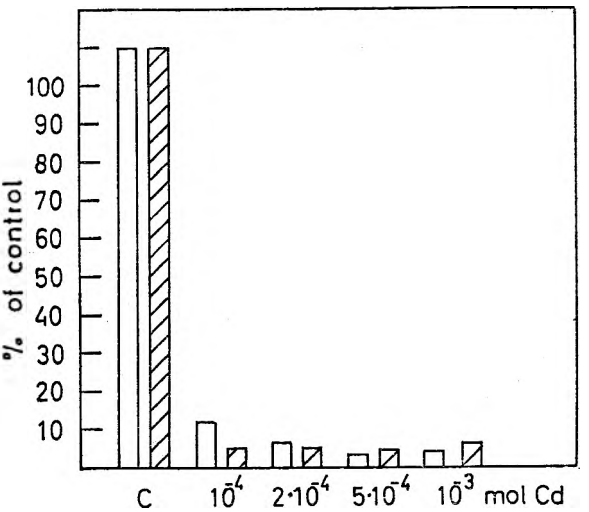


Fig. 3a. Effect of cadmium on the synthesis of chorophyll and carotenoids in *Euglena* in REM medium.
Cells were grown in REM medium in the dark and then exposed to light (2000 lx) for 96 hours, in the same medium. Inhibition of chlorophyll and carotenoid syntheses expressed as per cent of control value. White column: chlorophyll; lined column: carotenoids.

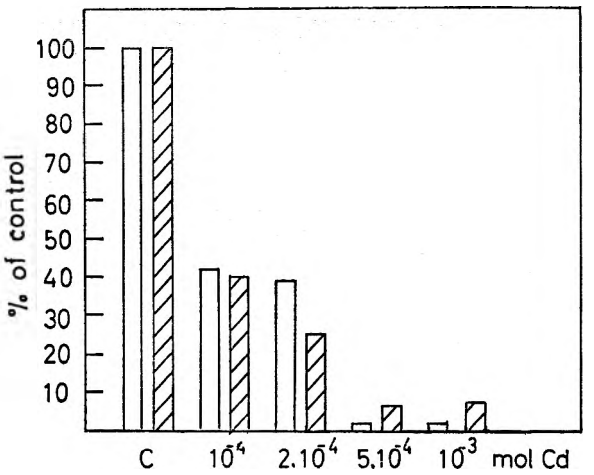


Fig. 3b. Effect of cadmium on the synthesis of chlorophyll and carotenoids in *Euglena* in resting medium.
Cells were grown in REM medium in the dark and then washed and exposed to light (200 lx) for 96 hours in resting medium. Inhibition of chlorophyll and carotenoid syntheses is expressed as per cent of control values. White column: chlorophyll; lined column: carotenoids.

Table 1. Effect of cadmium and zinc on cell proliferation in *Euglena*

Medium	Average colony diameter
Rich *Euglena* medium (REM)	648.6 ± 89.2 μm
REM + 1×10^{-5} mol dm^{-3} $ZnSO_4 \times 7H_2O$	441.0 ± 57.2 μm
REM + 1×10^{-5} mol dm^{-3} $Cd(NO_3)_2 \times 4H_2O$	151.2 ± 40.7 μm
REM + 1×10^{-5} mol dm^{-3} $ZnSO_4 \times 7H_2O$ + 1×10^{-5} mol dm^{-3} $Cd(NO_3)_2 \times 4H_2O$	280.4 ± 110.8 μm

Cells were grown at 25°C in the dark on agar-solidified medium.
Diameters of about 200 colonies were scored after 13 days.

Table 2. Cytotoxicity of cadmium (1×10^{-3} mol dm^{-3}) in rich *Euglena* (REM) and resting media (RS)

Medium	Number of motile cells after		
	3 hours	24 hours	72 hours
REM + Cd	~100	2	0
REM + $MgCl_2$ + Cd	~100	1	0
REM + glucose + Cd	14	0	0
REM + KH_2PO_4 + Cd	> 200	16	0
RS + Cd	> 300	> 300	20
RS − KH_2PO_4 + Cd	> 300	> 100	10
RS − $MgCl_2$ + Cd	> 300	> 200	10

Cells were grown in liquid REM medium for 11 days in the dark at 25°C, washed twice with 0.15 mol dm^{-3} NaCl, placed in the different liquid media + Cd as indicated and continuously illuminated. The concentrations of $MgCl_2$, glucose and KH_2PO_4 were as in the resting medium of Stern et al. (1964). Numbers of cells which still showed motility were scored under a stereomicroscope (magnification 40 ×).

Table 3. Pigment analysis in the wild type and a cadmium resistant clone of *Euglena*

	Chlorophyll/10^6 cells		Δ car 480/10^6 cells
	a	b	
Wild type	18.2 μg	1.8 μg	1.3
Cadmium resistant clone	9.1 μg	1.0 μg	1.1

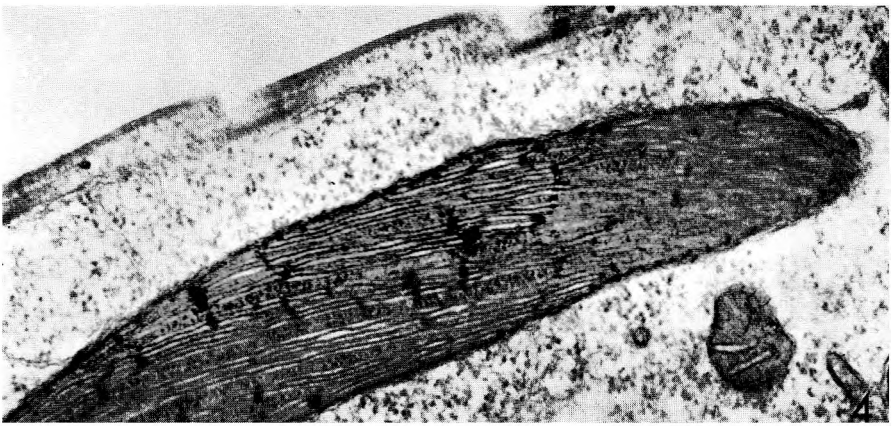
Fig. 4. Ultrastructure of *Euglena gracilis* grown in REM (control) with normal thylakoid structure. 35,000:1.

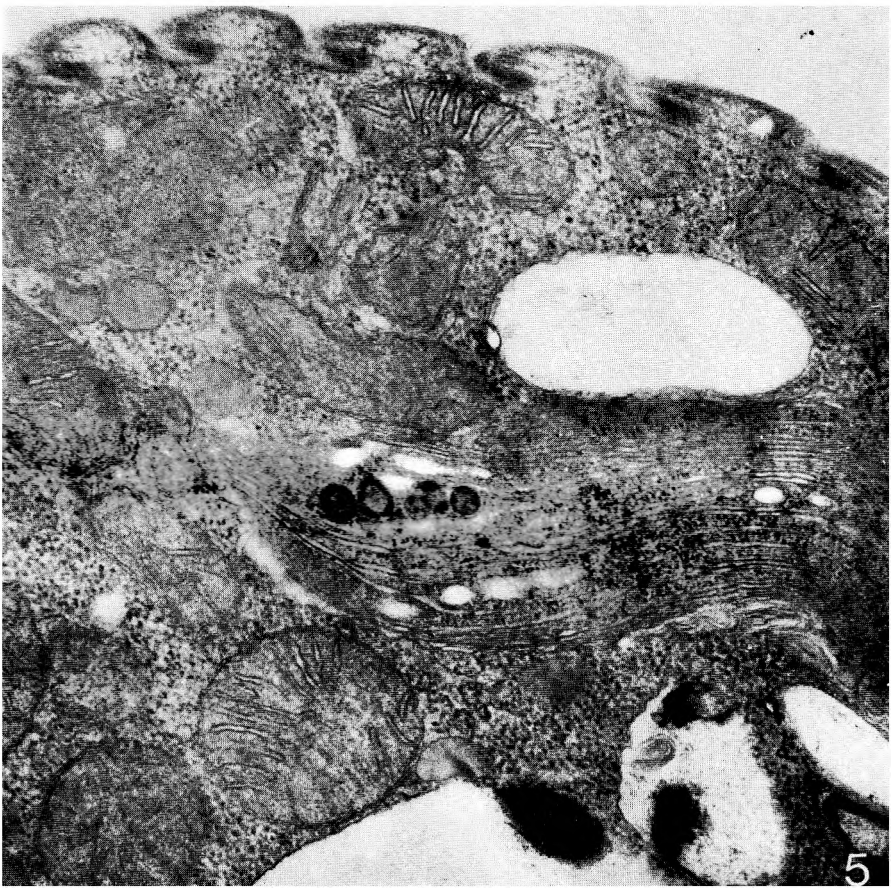
Fig. 5—7. Ultrastructure of *E. gracilis* previously grown at a sublethal cadmium concentration (1×10^{-4} mol dm^{-3}), washed and grown for three months in cadmium free medium.

Fig. 5. Initial stages of thylakoid dilatation. 40,000:1.

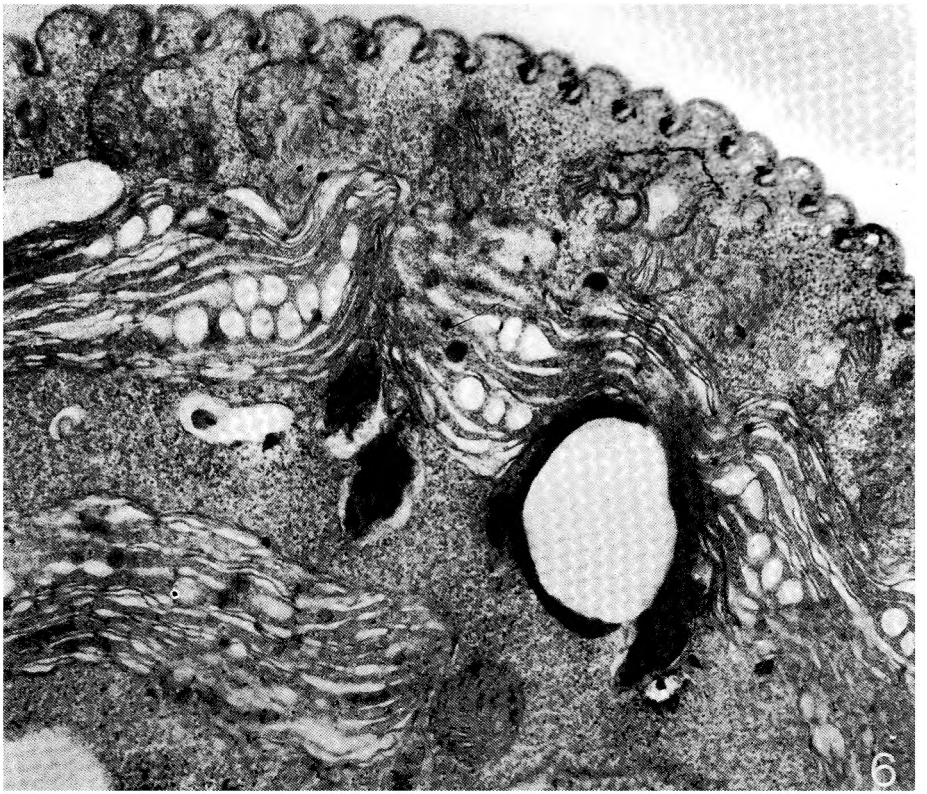
Fig. 6. Local thylakoid swelling is pronounced. 22,000:1.

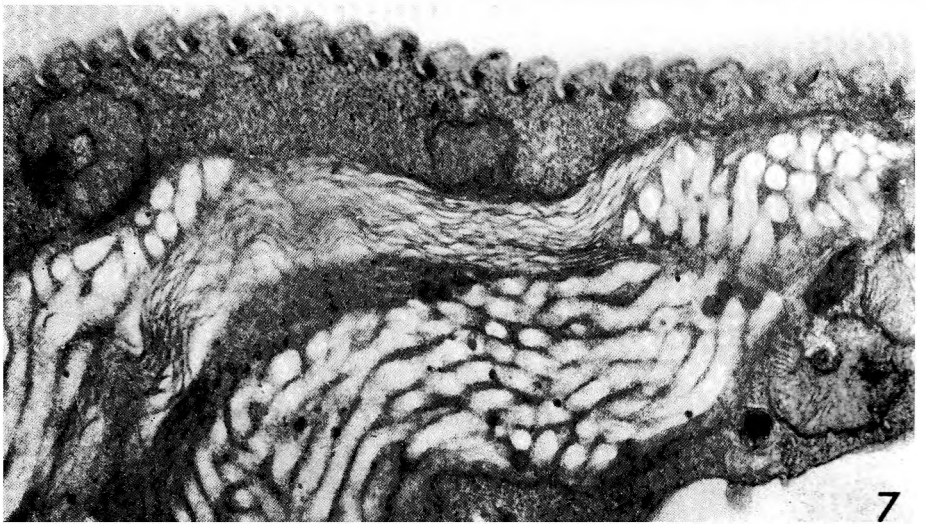
Fig. 7. Advanced stage of disorganization of chloroplast structure. Thylakoids are strongly swollen or reduced to lamellated membranes. 24,000:1.





Figs. 4—5.





Figs. 6—7.

effect of cadmium was somewhat more pronounced in the light than in the dark (Figs. 1a and 1b).

The antagonistic effect of zink on cadmium cytotoxicity is presented in Fig. 2 and Table 1. The effect was not so pronounced as no precautions were taken to eliminate traces of zinc from the control medium.

Most morphological changes occurred already at cadmium concentrations at which active cell divisions still took place (10^{-5} mol dm $^{-3}$ Cd and 5×10^{-5} mol dm^{-3} Cd + 5×10^{-5} mol dm^{-3} Zn). Abnormally-shaped and often multinucleate cells with inhibited cytokinesis (giant and star-shaped) appeared. The majority of cells, however, still divided normally, but were almost devoid of paramylon as observed microscopically.

Cadmium inhibited the formation of chlorophyll and carotenoids in previously bleached *Euglena* cells. Figs. 3a and 3b show this inhibitory effect in different media. Although lag phase cells were used in rich *Euglena* medium to avoid interfering growth, cadmium in REM proved to be more toxic than in the resting medium. In order to test the effect of those two media on cadmium cytotoxicity, another experiment on cell motility and viability was performed. Again, a protective effect of the resting medium was observed (Table 2). Tests with addition or exclusion of some constituents of the resting medium did not point to a single compound which might be responsible for this effect.

Two cadmium resistant clones were isolated from *Euglena* grown in the presence of 10^{-4} mol dm^{-3} cadmium (see Materials and Methods) which is a sublethal concentration for *Euglena* (only 1.5 per cent survival). The resistant clones were distinguished from wild-type cells by their pale yellow-green pigmentation. This colour remained stable when the altered clones were washed and successively subcultured in cadmium-free medium for three months. After three months one of the clones was examined by light and electron microscopes. The pigment analysis performed at that time is shown in Table 3. There was only about 50% of the amount of chlorophyll detected in wild-type *Euglena;* there were no differences in carotenoid content. In the altered clone, the cells exhibited somewhat impaired motilities and they were low in paramylon. Frequently, carotenoid crystals were present in the cells as observed by light microscopy. Electron micrographs revealed major changes in the structure of the chloroplasts. In different cells, all stages from normal to severely damaged thylakoid structures appeared. Control is presented in Fig. 4. A beginning dilatation of thylakoid membranes is seen in Fig. 5, strong local swelling of thylakoids is illustrated in Figs. 6 and 7, and completely disintegrated stroma with only lamellated thylakoid membranes is shown in Fig. 7. Paramylon granules disappeared and large lipofuscin granules, like those occuring in senescent cells, appeared.

Discussion

An inhibitory effect of cadmium on cell division in *Euglena*, as shown by the method used, was observed at such low concentrations (10^{-6} and even 10^{-7} mol dm^{-3} in the dark) as are considered non-toxic even for cultured human cells (Fischer 1985). This is a much higher sensitivity than that observed by other authors (e.g. Bonaly et al. 1978, Albergoni et al. 1980). However, a very high sensitivity was obtained in media from which zinc had been completely eliminated (Falchuk et al.

1975). Zinc acts as an antagonist to cadmium presumably by competing in binding to SH groups in proteins (Nakano et al. 1980). The antagonistic effect of zinc has been confirmed by the present study. It was however less expressed than in the experiments of the above authors, most likely because there were already traces of zinc in the control medium.

Among other ions, a protective role against toxic effects of cadmium has been observed for calcium in yeasts (Kessels et al.. 1985) and, to a smaller extent, for magnesium (Norris and Kelly 1977). Inhibition of chlorophyll synthesis, photosynthesis and chloroplast differentiation in green plants treated with cadmium is partly reversed by manganese (Van Duivendijk-Matteoli and Desment 1975, Baszyński et al. 1980, Wrischer and Kunst 1981). The protective role of the resting medium in the present study, however is apparently not due to the presence of manganese, but seems to be correlated with the physiological state of the cells which are less active than in REM medium, and thus less susceptible to damage.

Morphological changes in cadmium treated *Euglena*, such as giant or starshaped clusters of cells with inhibited cytokinesis, observed by Falchuk et al. (1975) and Nakano et al (1978 and 1980) and the present study, are also found in ageing cultures of Euglena (Gomez et al. 1974, Marčenko unpublished) or after herbicide action (Marčenko 1980). Therefore it seems to be a non-specific response of Euglena to different stress situations.

In the present investigation, the accumulation of paramylon observed by Falchuk et al. (1975), was observed only in cells with inhibited cell division. In cultures, in which cell division still actively took place, paramylon synthesis was inhibited by cadmium.

Of special interest is the appearance of partially cadmium resistant clones with an altered microscopic appearance and submicroscopic structure. Such changes were not observed in the short-term experiments of Falchuk et al. (1975) and Nakano (1980). Almost the same changes, however, (about 50 per cent chlorophyll present, disappearance of paramylon and swollen thylakoids) were observed during adaptation of *Euglena* to the herbicide DCMU (3-(3,4-dichlorophenyl)-1, 1-dimethylurea in a DCMU resistant *Euglena* (Calvayrac and Ledoigt 1976. and Calvayrac et al. 1979 a). Similar submicroscopic changes in chloroplasts (dilated and swollen thylakoids) were observed by Wrischer and Kunst (1980) in leaves of wheat treated with cadmium during plastid differentiation and in bundle sheath cells of lead treated corn by Wrischer (unpublished). Cadmium blocks photosystem II in plants (Baszyński et al. 1980). Since DCMU resistance and recovery in *Euglena* are related to photosynthetic electron transfer (Laval-Martin et al. 1977) and to structural changes in the environment of phytosystem II (Calvayrac et al. 1979b and 1979c) similar ultrastructural and biochemical changes may be expected in cadmium resistant *Euglena*.

The toxic effect of cadmium is more pronounced in the light. It is most probably related to an increase in cadmium uptake, which is partly a light dependent process (Bariaud et al. 1985). Albergoni et al. (1980) found that cadmium is chelated by *Euglena* resulting in a compound of high molecular weight, which is accumulated in the cell. Cadmium resistance in *Euglena* does not seem to be linked to an induction of metallothioneins as in animal cultured cells (Fischer 1985), but to differences in membrane transport mechanisms (Bariaud et al. 1985).

References

Albergoni, V., E. Piccini, O. Coppellotti, 1980: Response to heavy metals in organisms. I. Excretion and accumulation of physiological and non physiological metals in *Euglena gracilis.* Comp. Biochem. Physiol. *67,* 121—127.

Bariaud, A., J. Bonaly, A. Delcourt, J.-C. Meltre, 1978: Divers aspects de l'action toxique du cadmium sur les cellules d'*Euglena gracilis.* In: Actualités de biochimie marine, Vol. 2, 135—148. Colloque Gabim, La Rochelle, C. N.R.S. Paris.

Bariaud, A., M. Bury, J.-C. Mestre, 1985: Mechanism of Cd^{2+} resistance in *Euglena gracilis.* Physiol. Plant. *63,* 382—386.

Baszyński, T., L. Wajda, M. Król, D. Wolińska, Z. Krupa, A. Tukendorf, 1980: Photosynthetic activities of cadmium-treated tomato plants. Physiol. Plant. *48,* 365—370.

Bonaly, J., A. Bariaud, A. Delcourt, J.-C. Mestre, 1978: Les effets des ions Cd^{2+} sur les cellules d'*Euglena gracilis* L.: cytotoxicité et acquistion d'une résistance. C. R. Acad. Sci. (Paris) *287,* 463—466.

Bonaly, J., A. Bariaud, S. Duret, J.-C. Mestre, 1980: Cadmium cytotoxicity and variation in nuclear content of DNA in *Euglena gracilis.* Physiol. Plant. *49,* 286—290.

Calvayrac, R., J.-L. Bomsel, D. Laval-Martin, 1979a: Analysis and characterization of 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU)-resistant *Euglena.* I. Growth, metabolic and ultrastructural modifications during adaption to different doses of DCMU. Plant Physiol. *63,* 857—865.

Calvayrac, R., D. Laval-Martin, G. Dubertret, J.-L. Bomsel, 1979 b: Analysis and characterization of 3-(3, 4-dichlorophenyl)-1, 1-dimethylurea (DCMU)--resistant *Euglena.* II. Modification affecting photosynthesis during adaptation to different doses of DCMU. Plant Physiol. *63,* 866—872.

Calvayrac, R., G. Ledoigt, 1976: Croissance des euglènes en présence de DCMU: evolution du plastidome en fonction de la tension d'oxygène. Plant Sci. Lett. *7,* 249—263.

Calvayrac, R., G. Ledoigt, D. Laval-Martin, 1979c: Analysis and characterization of (DCMU)-resistant *Euglena gracilis.* III. Thylakoid modifications and dark »recovery« of photosynthesis. Planta *145,* 259—267.

De Filippis, L. F., R. Hampp, H. Ziegler, 1981: The effects of sublethal concentrations of zinc, cadmium and mercury on *Euglena.* II. Respiration, photosynthesis and photochemical activities. Arch. Microbiol. *128,* 407

Falchuk, K. H., D. W. Fawcett, B. L. Vallee, 1975: Competitive antagonism of cadmium and zinc in the morphology and cell divisions of *Euglena gracilis.* J. Submicr. Cytol. *7,* 139—152.

Fischer, A. B., 1985: Factors influencing cadmium uptake and cytotoxicity in cultured cells. Xenobiotica *15,* 751—757.

Holden, M., 1965: Chlorophylls. In: Chemistry and Biochemistry of Plant Pigments (T. W. Goodwin, ed.)461—488. Acad. Press, London, New York.

Gomez, M. P., J. B. Harris, P. L. Walne, 1974: Studies of *Euglena gracilis* in aging cultures. I. Light microscopy and cytochemistry. Br. phycol. J. *9,* 163—174.

Kessels, B. G. F., P. J. M. Belde, G. W. H. Borst-Pauwels, 1985: Protection of *Saccharomyces cerevisiae* against Cd^{2+} toxicity by Ca^{2+}. J. Gen. Microbiol. *131,* 2533—2537.

Kirk, J. T. O., R. L. Allen, 1965: Dependence of chloroplast pigment synthesis on protein synthesis: action of actidione. Biochem. Biophys. Res. Commun. *21,* 523—530.

Laval-Martin, D., G. Dubertret, R. Calvayrac, 1977: Photosynthetic properties of a DCMU resistant strain of *Euglena gracilis* Z. Plant Sci. Lett. *10,* 185—195.

Marčenko, E., 1970: The effect of illumination regimen on temperature-induced and spontaneous bleaching in *Euglena gracilis*. Acta Bot. Croat. *29*, 27—32.

Marčenko, E.: 1974: On the permanent bleaching of *Euglena* by chloramphenicol and its inhibition by cycloheximide. Cytobiol. *9*, 280—289.

Marčenko, E., 1980: Effect of isopropyl N-phenylcarbamate on *Euglena*. Acta Bot. Croat. *39*, 71—78.

Nakano, Y., K. Abe, S. Toda, 1980: Morphological observation on *Euglena gracilis* grown in zinc-sufficient media containing cadmium ions. Agric. Biol. Chem. *44*, 2305—2316.

Nakano, Y., K. Okamoto, S. Toda, K. Fuwa, 1978: Toxic effects of cadmium on *Euglena gracilis* grown in zinc deficient and zinc sufficient media. Agric. Biol. Chem. *42*, 901—907.

Norris, P. R., K. P. Kelly, 1977: Accumulation of cadmium and cobalt by *Saccharomyces cerevisiae*. J. Gen. Microbiol. *99*, 317—324.

Stern, A. I., J. A. Schiff, H. T. Epstein, 1964: Studies of chloroplast development in *Euglena*. VI. Light intensity as a controlling factor in development. Plant Physiol. *39*, 226—231.

Van Duivendijk-Matteoli, M. A., G. M. Desmet, 1975: On the inhibitory action of cadmium on the donor side of photosystem II in isolated chloroplasts. Biochim. Biophys. Acta *408*, 164—169.

Wrischer, M., Lj. Kunst, 1981: Fine structural changes of wheat plastids during cadmium induced bleaching. Acta Bot. Croat. *40*, 79—83.

SAŽETAK

TOKSIČNO DJELOVANJE KADMIJA I POJAVA REZISTENCIJE U EUGLENE

Elena Marčenko

(Institut »Ruđer Bošković«, Zagreb)

Praćenjem sudbine pojedinačnih stanica na površini agara dokazana je velika osjetljivost stanične diobe u euglene na toksično djelovanje niskih koncentracija kadmija.

Potvrđeno je antagonističko djelovanje cinka.

U etioliranoj eugleni kadmij inhibira sintezu klorofila i karotenoida. Zaštitna uloga mirujućeg medija u usporedbi s bogatim *Euglena* medijem pripisuje se različitoj osjetljivosti euglene kod raznih fizioloških stanja stanica.

Istraživana su svojstva klona rezistentnog na kadmij, koji je imao smanjenu koncentraciju klorofila (oko 50%) kroz više uzastopnih generacija. Istovremeno je sadržaj karotenoida ostao nepromijenjen u odnosu na kontrolu. U submikroskopskom području najviše promjena se dogodilo u strukturi kloroplasta. Nađeni su svi prijelazi od vrlo oštećenih kloroplasta s karakterističnim razmaknutim i nabubralim tilakoidima, do kloroplasta s normalnom strukturom. Razmatra se sličnost s promjenama koje se dešavaju za vrijeme adaptacije euglene na herbicid DCMU.

Dr. Elena Marčenko
Institut »Ruđer Bošković«
Bijenička 54
YU-41000 Zagreb (Jugoslavija)