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The Effects of Sublethal Concentrations of Zinc, Cadmium and Mercury on *Euglena*

II. Respiration, Photosynthesis and Photochemical Activities*

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Abstract. Results on the effect of sub-lethal concentrations of zinc chloride ($ZnCl_2$), cadmium chloride ($CdCl_2$), and mercuric chloride ($HgCl_2$) on *Euglena* are presented. During the growth cycle respiratory oxygen uptake and photosynthetic oxygen evolution in the light are initially strongly inhibited by Zn, Cd and Hg. The effects of the three metals on photosynthesis, using oxygen evolution as a criterion was confirmed by carbon fixation techniques.

Photosystem I (PSI) associated electron transport 2,6-dichlorophenol indophenol $(DCPIP)_{red.} \rightarrow$ methyl viologen (MV) $\rightarrow O_2$, in contrast to total photosynthetic capacity, was only slightly inhibited by Zn, Cd and Hg, whereas the levels of activity of NADP-oxidoreductase in cells untreated or treated with heavy metals showed development like total photosynthesis. Metals strongly inhibited this enzyme which means that the supply of NADPH is lowered due to the action of Zn, Cd and Hg. Photosystem II (PSII) associated electron transport ($H_2O \rightarrow$ dibromothymoquinone/2,3-dimethyl-5,6-methylenedioxy-D-benzoquinone $\rightarrow O_2$), however, was severely inhibited in a way similar to total photosynthesis. Effects on the cooperation of PSI + II showed patterns similar to PSII alone, i.e., heavy metals strongly reduced PSI + II dependent activities.

Key words: Cadmium – *Euglena* – Mercury – Photochemical activities – Photosynthesis – Respiration – Zinc

The heavy metals zinc (Zn), cadmium (Cd) and mercury (Hg) are well known freshwater and marine pollutants. Algae are very important primary producers in waters, and they accomplish this production by a balance between respiration and photosynthesis. Any imbalances in one or the other process (and therefore in the production of dry matter) by inhibition is also likely to effect other organisms in the aquatic environment. Zn, Cd and Hg are strong inhibitors of some growth processes in *Euglena* (De Filippis et al. in press), the mechanisms involved are not well understood, but important metabolic processes like respiration and photosynthesis are likely to be involved.

The inhibition of respiration and photosynthesis in plant cells or organelles by solutions of heavy metals has been noted previously (Greenfield 1942; Cook et al. 1946; Harriss et al. 1970; Kamp-Nielsen 1971; Shieh and Barber 1973; Hampp et al. 1973; Bittell et al. 1974; Hampp et al. 1976; De Filippis and Pallaghy 1976). Some early work on the inhibition of photosynthetic electron transport by mainly copper and mercury has revealed that the Hill reaction is mostly affected (McDowell 1949; Honeycutt and Krogmann 1972; Miles et al. 1973; Overnell 1975). However, the results have not been extended to cover other metals or other reactions of electron transport, nor has the inhibition been referred to specific sites of the photosystems.

It was therefore the aim of this paper to investigate to what extend Zn, Cd and Hg affect respiration and photosynthesis, and on which steps of the photosynthetic electron transport chain the metals exert their greatest effects.

Materials and Methods

Algal Cultures and Determination of Growth. Euglena gracilis (strain no: 1224–5/25; Algal Culture Collection, Göttingen) was cultured at 25°C and continuous light (10 $W \cdot m^{-2}$) as described by De Filippis and Hampp, in press). Cells were aerated by passing air + 5% (v/v) CO_2 from the bottom of the flask with saturating flow rates. Measurements of population densities were made using an improved double Neubauer haemocytometer (Assistent), and dry weights were determined by drying washed cell pellets at 110°C.

Oxygen Exchange and $^{14}CO_2$ Fixation. Respiratory O_2 uptake or photosynthetic O_2 evolution of cells in the light (120 $W \cdot m^{-2}$) was measured with a Clark-type O_2 electrode at 25°C as described by Delieu and Walker (1972). The rate of $^{14}CO_2$ fixation was estimated by introducing 10 mM $NaHCO_3$ and [^{14}C] $NaHCO_3$ (5 μCi) into 1.2 ml shaking cultures and sampling 200 μl aliquots at 0, 2, 5, 10 and 15 min intervals in the dark or the light (120 $W \cdot m^{-2}$). Fixation was terminated by the addition of 200 μl acidified ethanol (ethanol/acetic acid: 95/5), the mixture dried under heat and radioactivity fixed measured in a scintillation counter.

Photochemical Activities. Cells of *Euglena* were washed and incubated in 5% (w/v) cellulysin (Calbiochem) made up in the culture medium and

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Abbreviations: DAD = diaminodurene; DBMIB = dibromothymoquinone; DCPIP = 2,6-dichlorophenolindophenol; DCMU = 3-(3,4-dichlorophenyl)-1,1-dimethylurea; DMMIB = 2,3-dimethyl-5,6-methylenedioxy-p-benzoquinone; DPC = 1,5-diphenylcarbazide; MV = methylviologen; PS = photosystem

* Dedicated to Professor Kandler on occasion of his 60th birthday

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incubated at 30°C for 2 h as described by De Filippis and Hampp (1980). This enabled the dyes (artificial electron donors and acceptors) used for measuring photochemical activities to enter the cells, without the cell wall degrading enzyme adversely effecting the biochemistry, physiology or general behaviour of *Euglena* (De Filippis and Hampp 1980).

Activity of PSI was determined in the presence of DCMU (10^{-5}M) by recording the rates of O_2 uptake after the addition of ascorbate ($5 \cdot 10^{-4}$M), MV ($4 \cdot 10^{-4}$M), NaN_3 ($5 \cdot 10^{-5}$M) and DCPIP ($2 \cdot 10^{-4}$M). As a measure of PSII activity, O_2 consumption in the light was determined in the presence of the plastoquinone antagonist DBMIB (10^{-5}M; Böhme et al. 1971) and by DMMIB (10^{-5}M) channelling electrons to O_2 after PSII (Trebst 1974; Trebst et al. 1976) with DPC (10^{-3}M; Vernon and Shaw 1969) or H_2O as an electron donor for PSII. Cooperation between PSI and PSII was determined by the rates of O_2 consumption in the presence of MV/NaN_3 and H_2O or DPC being the electron donors. Ferredoxin: NADP-oxidoreductase was assayed according to Elstner and Heupel (1974) by measuring rates of oxygen consumption after the addition of a NADPH-generating system and anthraquinone-2-sulphonate/NaN_3.

All changes in the amount of O_2 in the incubation medium associated with electron transport reactions were measured at 25°C using the water-jacket oxygen electrode described above. Illumination was provided by a forced air cooled 250 W slide projector resulting in a radiant light flux of about 120 W · m^{-2} within the assay. Sub-lethal concentrations of $ZnCl_2$ (50 µM), $CdCl_2$ (0.1 µM) and $HgCl_2$ (0.01 µM) were established just prior to the inoculation of the culture medium as determined by De Filippis et al. (in press).

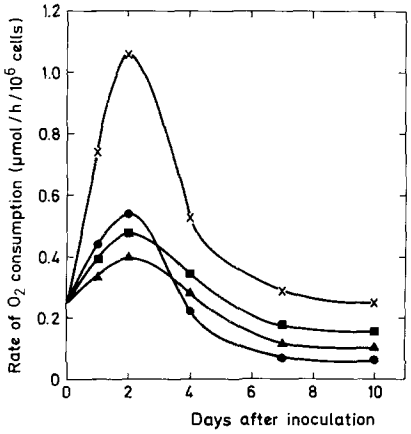

Fig. 1. Time course of respiratory oxygen consumption in *Euglena* inoculated into control medium (×) or the medium containing in addition 50 µM $ZnCl_2$ (●) or 0.1 µM $CdCl_2$ (■) or 0.01 µM $HgCl_2$ (▲) at 25°C and continuous illumination (10 W · m^{-2}). Cells were inoculated into the appropriate medium on day zero and the cultures were proceeding through their normal growth phases. Oxygen consumption was measured in complete darkness in a Clark-type O_2 electrode and at 25°C. Each value is the average of triplicate experiments

Results

Respiration and Photosynthesis

All three heavy metal salts ($ZnCl_2$, $CdCl_2$ and $HgCl_2$) strongly inhibited respiratory O_2 consumption in the dark (Fig. 1) as well as O_2 evolution in illuminated cultures (Fig. 2); with highest control rates always two days after inoculation, whereas cell population density was found to be only slightly reduced (De Filippis et al. in press). The pattern obtained by measuring O_2 evolution in the light is confirmed in experiments where light-dependent carbon fixation was determined (Fig. 2, open symbols). When O_2 evolution is expressed per unit mass of chlorophyll (for cell numbers see De Filippis et al. in press), the inhibition is not that pronounced and may in fact exceed control levels from day four onwards (Table 1). However, respiratory oxygen uptake is never above control values no matter how it is expressed (Fig. 1; Table 1).

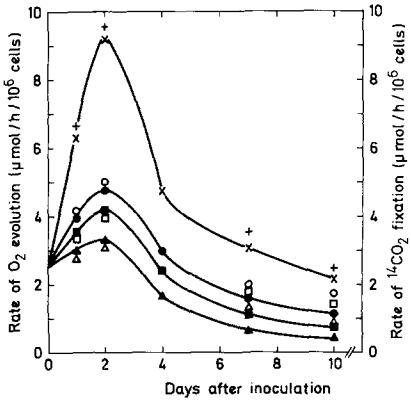

Fig. 2. Time course of oxygen evolution (×, and *closed symbols*) or $^{14}CO_2$ fixation (+, and *open symbols*) in the light in *Euglena* inoculated into control medium (×, +) or the medium containing in addition 50 µM $ZnCl_2$ (●, ○) or 0.1 µM $CdCl_2$ (■, □) or 0.01 µM $HgCl_2$ (▲, △) at 25°C and continuous illumination (10 W · m^{-2}). Cells were inoculated into the appropriate medium on day zero and the cultures were proceeding through their normal growth phases. Oxygen evolution was measured in the light (120 W · m^{-2}), in a Clark type O_2 electrode and at 25°C. Each value is the average of triplicate experiments

Photochemical Activities

PSI activities in control cells show a constant reduction from young cultures (e.g. day 1) to ageing cultures (e.g. days 7 and 10), which is unlike the pattern for total photosynthesis (Fig. 3; compare with Fig. 2). The three metals inhibit PSI associated electron transport, especially in young cells, only to a limited extend (Fig. 3). In contrast, the pattern of development of PSII associated electron transport is very similar to the pattern of total photosynthesis (Fig. 4; Fig. 2). The heavy metals Zn, Cd and Hg strongly inhibit PSII activity, especially in young cultures (Fig. 4). Cooperation between PSI and PSII as expected, also shows a pattern like PSII alone and total photosynthesis (Fig. 5; Figs. 2 and 4) in that the three heavy metals strongly inhibit the PSI + II activity (Fig. 5). The artificial electron donor for PSII, DPC, almost completely eleviates the inhibition of heavy metals on PSII and PSI + PSII associated activities. However, the DPC effect is not as expressed on PSI + PSII as it is on PSII alone (Figs. 4 and 5).

In one of the final steps of the light-dependent electron transport mediated reduction of NADP the enzyme NADP-oxidoreductase is involved. This enzyme is severely inhibited by the heavy metals Zn, Cd and Hg (Fig. 6). The time course of activity of this enzyme is also very similar to the activity of total photosynthesis (Figs. 6 and 2).

Discussion

Effect of Heavy Metals on Respiration and Photosynthesis

The inhibition of O_2 exchange and carbon fixation in short term experiments has been well documented before (see De Filippis and Pallaghy 1976). However, as the culture ages, respiration and photosynthesis are not as adversely affected, and in some cases are even enhanced above control values (De

Table 1. Time course of effects of 50 μM $ZnCl_2$ (Zn), 0.1 μM $CdCl_2$ (Cd) or 0.01 μM $HgCl_2$ (Hg) on respiratory oxygen consumption (Respiration) or oxygen evolution in the light (Photosynthesis; 120 W · m^{-2}) of *Euglena*. Cells were inoculated without or in the presence of the heavy metals on day zero and the cultures were proceeding through their normal growth phases under continuous illumination (10 W · m^{-2}) and at 25° C. Each value is the average of triplicate experiments

Days of treatment	Respiration (μmol O_2/h·g dry weight)				Photosynthesis (μmol O_2/h·mg chlorophyll)			
	Control	Zn	Cd	Hg	Control	Zn	Cd	Hg
1	140	77	46	42	53	31	25	23
2	180	113	104	102	112	51	54	68
4	115	82	60	41	73	75	98	131
7	96	73	50	25	71	80	107	152
10	108	75	67	36	69	121	179	186

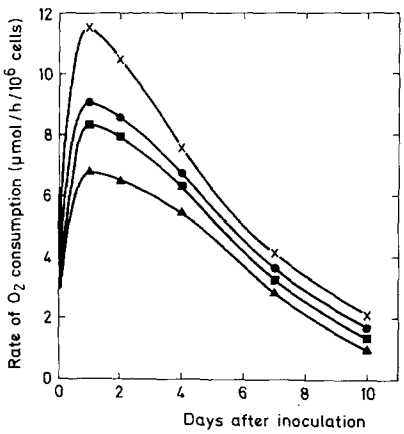

Fig. 3. Time course of development of PSI activities in *Euglena* inoculated into control medium (×) or the medium containing in addition 50 μM $ZnCl_2$ (●) or 0.1 μM $CdCl_2$ (■) or 0.01 μM $HgCl_2$ (▲) at 25° C and continuous illumination (10 W · m^{-2}). Cells were inoculated into the appropriate medium on day zero and the cultures were proceeding through their normal growth phases. Rates of PSI associated electron transport were determined as O_2 consumption using the system $DCPIP_{red.}$ → MV → O_2 at 25° C. Each value is the average of triplicate experiments. Values corrected for respiratory O_2 consumption (Fig. 1)

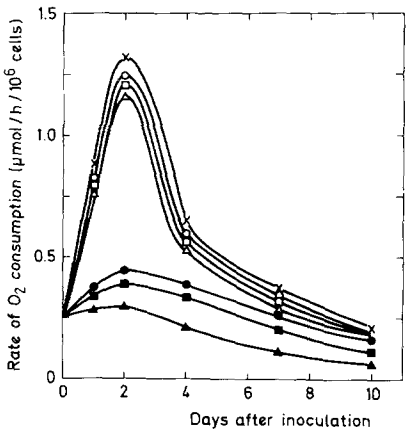

Fig. 4. Time course of development of PSII activities in *Euglena* inoculated into control medium (×) or the medium containing in addition 50 μM $ZnCl_2$ (●, ○) or 0.1 μM $CdCl_2$ (■, □) or 0.01 μM $HgCl_2$ (▲, △) at 25° C and continuous illumination (10 W · m^{-2}). Cells were inoculated into the appropriate medium on day zero and the cultures were proceeding through their normal growth phases. Rates of PSII associated electron transport were determined as O_2 consumption using the systems H_2O → DBMIB/DMMIB → O_2 (*closed symbols*) or DPC → DBMIB/DMMIB → O_2 (*open symbols*) at 25° C. Each value is the average of triplicate experiments. Values corrected for respiratory O_2 consumption (Fig. 1)

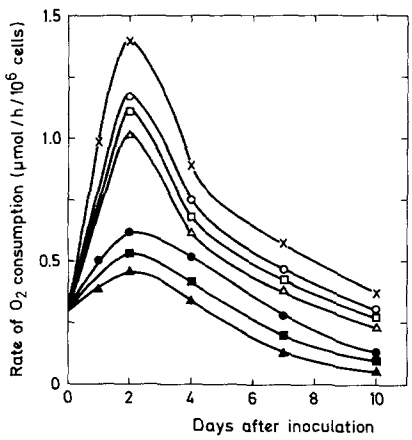

Fig. 5. Time course of development of cooperation between PSI and PSII in *Euglena* inoculated into control medium (×) or the medium containing in addition 50 μM $ZnCl_2$ (●, ○) or 0.1 μM $CdCl_2$ (■, □) or 0.01 μM $HgCl_2$ (▲, △) at 25° C and continuous illumination (10 W · m^{-2}). Cells were proceeding through their normal growth phases. Rates of electron transport associated with PSI + II were determined as O_2 consumption using the systems H_2O → MV → O_2 (*closed symbols*) or DPC → MV → O_2 (*open symbols*) at 25° C. Each value is the average of triplicate experiments. Values corrected for respiratory O_2 consumption (Fig. 1)

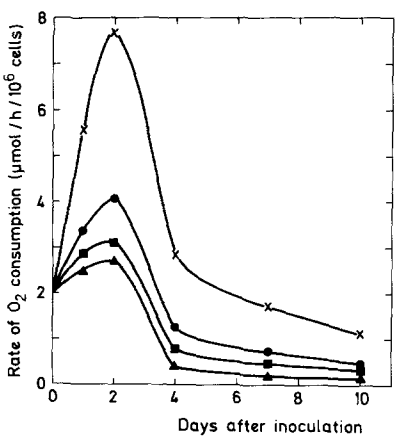

Fig. 6. Time course of development of Ferredoxin: NADP-oxidoreductase activity in *Euglena* inoculated into control medium (×) or the medium containing in addition 50 μM $ZnCl_2$ (●) or 0.1 μM $CdCl_2$ (■), or 0.01 μM $HgCl_2$ (▲) at 25° C and continuous illumination (10 W · m^{-2}). Cells were proceeding through their normal growth phases. NADP-oxidoreductase was measured as the rate of O_2 consumption in the presence of a NADP-generating system and anthraquinone-2-sulphonate, in the dark and at 25° C. Each value is the average of triplicate experiments. Values corrected for respiratory O_2 consumption (Fig. 1)

Filippis and Pallaghy 1976). In our system there was no enhancement when rates of respiration and photosynthesis were expressed per cell basis, but when photosynthesis, for example, is expressed per unit mass of chlorophyll (assimilation ratio) then there is enhancement above controls from day four onwards (Table 1). De Filippis and Pallaghy (1976) have also observed an enhancement in the assimilation ratio when *Chlorella* was exposed to solutions of Zn and Hg.

The enhancement may be related to a lowering of the concentration of heavy metals in cells, as cells continue to divide (even though heavy metal concentrations in the culture medium were maintained constant, uptake of metals decreases with age), since Barron et al. (1948) and Shieh and Barber (1973) have observed stimulation of photosynthesis and respiration by very low concentrations of mercury in short term experiments. A further lowering of Zn and Hg levels inside chloroplasts with ageing of the culture may also possibly be accomplished by compartmenting these metals into for example nuclei (De Filippis and Pallaghy 1975; De Filippis 1978). The somewhat more efficient use of chlorophyll in photosynthesis in advanced cultures may be due to the stimulation of enzymic reactions when the cellular metal concentrations become sufficiently low (Shieh and Barber 1973). It is more likely, however, that the increased photosynthetic activities per mass of chlorophyll can be accounted for by the preferential destruction of light harvesting or antenna chlorophylls (De Filippis and Pallaghy 1976).

Effect of Heavy Metals on Photochemical Activities

Activities of PSI in *Euglena* are at their peak on day one of the culture and then continually drop, even in control cultures. This is not like the pattern of total photosynthesis when measured either by oxygen exchange or by carbon fixation; which suggests that PSI is not a controlling factor in total photosynthetic activity. Zn, Cd and Hg slightly lower the PSI activities especially in young cultures, but the amount of depression has little relation to total photosynthesis (Fig. 3). Copper (Cu^{2+}) solutions were also found to inhibit PSI activities in isolated chloroplasts from spinach, and its site of action appears to be localized at the ferredoxin site (Shioi et al. 1978).

The enzyme NADP-oxidoreductase is a key enzyme in the final light-dependent formation of NADPH. In fact the pattern of NADP-oxidoreductase activity is similar to total photosynthetic activity, even though electron flow through PSI is completely different (Figs. 3 and 6) and some additional step in between PSI and PSII could be inhibited, as the effects on PSII (Fig. 4) are somewhat smaller compared to those on PSII + PSI (Fig. 5). Therefore the main site of inhibition by metals of electron flow around PSI should be the enzyme NADP-oxidoreductase. NADP-oxidoreductase is a sulfhydryl requiring enzyme (see Forti 1977) and it is not surprising then that Zn, and especially Cd and Hg inhibit its activity because these heavy metals are well known, powerful sulfhydryl antagonists (Vallee and Ulmer 1972).

Activities associated with PSII on the other hand show patterns of development very similar to total photosynthesis; this strongly suggests that PSII is the controlling factor (at least in flow of electrons) with regards to total photosynthesis (Fig. 4). The three metals strongly inhibit PSII activities especially in young cultures, however, this inhibition can be elevated by DPC, an electron donor which feeds electrons in just after the water splitting step (Fig. 4). As DPC only can donate electrons to PSII when the water splitting site is inactivated (Vernon and Shaw 1969) this interference is a strong indication that the water splitting site is very sensitive towards Zn, Cd and Hg. These observations are confirmed by the results obtained when cooperation between PSI and II was measured (Fig. 5). Previous reports on the effects of copper and mercury on photochemical activity have almost exclusively measured the Hill reaction (i.e. water as electron donor) and have also noticed strong inhibition (McDowell 1949; Honeycutt and Krogmann 1972; Miles et al. 1973; Overnell 1975).

In summary, our observations with *Euglena*, obtained under quasi in vivo conditions, demonstrate that the reduction in photosynthetic capacity in the presence of Zn, Cd and Hg is due to severe interactions with the electron transport chain, the water splitting site and the oxidoreductase being the most sensitive steps.

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