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METABOLIC STUDIES OF Hg-203 ON CHLAMYDOMONAS REINHARDI

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Summary

Vegetative cultures of the green algae Chlamydomonas reinhardi WT^+ in the log-phase reduce mercury(II)-nitrate to elemental mercury which is removed from the cell suspension by the stream of gas bubbling through it. Monomethyl and dimethyl mercury as intermediate metabolic compounds are to be excluded, because none of them could be found in the algae, the nutrient medium or the gas phase.

KEY WORDS

Chlamydomonas reinhardi WT^+, ^{203}Hg, metabolism.

METABOLISIERUNG VON Hg-203 DURCH CHLAMYDOMONAS REINHARDI

Zusammenfassung

Vegetative Kulturen der Grünalge Chlamydomonas reinhardi WT^+ in der log-Phase reduzieren Quecksilber(II)-nitrat zu elementarem Quecksilber, das von einem durch die Zellsuspension geleiteten Gasstrom entfernt wird. Die Bildung von Monomethyl- und Dimethylquecksilber als Metabolisierungsprodukte kann ausgeschlossen werden, weil eine derartige Verbindung weder in den Algen, im Nährmedium noch in der Gasphase gefunden werden konnte.

SCHLÜSSELWÖRTER

Chlamydomonas reinhardi WT^+, Hg-203, Metabolisierung.

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Introduction

The increasing application of toxic elements in industry and agriculture causes environmental pollution and human diseases. Especially the highly toxic metal mercury attracts considerable attention on account of the accident in Minamata, Japan (1, 2) and the effect of rather careless use of methylmercury containing seed dressings in Sweden (3 - 6). Therefore extensive research has been performed on the fate of mercury in food chains; it could be shown that microorganisms are able to metabolize mercury compounds (7 - 13). This reaction, regarded as a detoxification mechanism (14) can yield a product that is either more or less toxic to higher organisms. Ben-Bassat et al. (15) exposed Chlamydomonas reinhardi to mercury(II)-chloride and found a decrease of mercury concentration in the cell suspension; they supposed metabolization to volatile compounds, e.g. methylmercury. Our work focused on the identification of probably formed volatile mercury compounds.

Materials and methods

Algae: The unicellular algae Chlamydomonas reinhardi was the organism employed in this work. Slant-agar stocks of WT^+ (wild type) were kindly given by R. Davies (John-Innes-Institute, Norwich, England). Pure cultures of the cells were grown asynchronously, i.e. by means of continuous illumination, at 25° C and air bubbling through (1,5 l/h). Cultivating was done in 500 ml Erlenmeyer flasks containing 200 ml YAP (16); the final cell concentration was 5.10^6/ml (determined by means of Thoma counting chambre).

Mercury: Mercury-203 was used as nitrate. This isotope was obtained by neutron activation (flux = 4.10^{13} n cm^{-2} sec^{-1}) of metallic mercury (analytical grade from Merck, Darmstadt, FRG) in the ASTRA-reactor of the Research Centre Seibersdorf, Austria. After treating with conc. HNO_3 aqueous $^{203}Hg(NO_3)_2$ standard solutions were prepared (specific activity 1,9 μCi/ml). The initial concentration used in experiments was $8,2.10^{-7}$ Mol/l.

Measurement of mercury concentration in the system algae/nutrient medium

All experiments were performed at 25° C; the medium to be tested (cell suspension or YAP, respectively) was vigorously stirred and a stream of gas (air or nitrogen, respectively) was blown through it at a rate of 1,5 l/h. At certain time intervals 2 ml-samples were withdrawn, transferred into special counting tubes and measured on a single channel analyzer. In each case newly prepared sterilized YAP treated in the same way was used as control.

Cell suspension: The time-related decrease of mercury concentration in the cell suspension was studied on living and dead cells under aerobic (light/air (LA)) and anaerobic (dark/nitrogen (DN)) conditions as well. For DN-experiments using living cells "preconditioning" (i.e. keeping on conditions of the following experiment) of cell suspension was done for one hour before adding the heavy metal ion standard. In doing so respiration and photosynthesis were interrupted. Pre-conditioning was not necessary for LA-experiments. Experiments using dead algae required pre-conditioning for DN- and LA-experiments. Dead cells were obtained either by γ-irradiation (0,6 Mrad/h, 5h, ^{60}Co, Institute of Biology, Research Centre Seibersdorf, Austria) or steam sterilization (120° C, 20 min.).

"Used" YAP: The time-related decrease of mercury concentration was studied on the cell-free nutrient medium already used for cells grown until the end of their log-phase. Two different methods were applied:

1. After centrifuging the living cells, both heat sterilized YAP (a) and non sterilized YAP (b) were tested.

2. The cell suspension was sterilized and the dead cells were centrifuged.

In each case mercury was added after one hour pre-conditioning (LA, DN).

Used YAP + trichloroacetic acid: We also investigated the influence of trichloroacetic acid ($8,2.10^{-7}$ mol/l) on the time-related decrease of the mercury concentration in YAP already used for cell growing. Method (lb) was applied.

Studies of volatile mercury compounds

The stream of gas was tested as to the content of volatile mercury compounds it contained having bubbled through the cell suspension. Experiments on mono- and dimethyl mercury were done on living cells in LA and DN. Experiments on Hg^{o} were done on living and dead cells in LA.

Monomethyl mercury: The stream of gas passed three wash bottles each containing 200 ml of a saturated toluene solution of dithizone. After 24 hours these solutions were transferred into special flasks (Fig. 1) and after evaporating the toluene in vacuo to a final volume of 2 ml ^{203}Hg activity was measured in a single channel analyzer. Then the samples were tested by means of TLC. Therefore, the samples were collected in a separatory funnel containing 200 ml toluene and extracted three times with 75 ml sodium hydroxide (1 % aqueous solution) to remove excess dithizone. The organic phase was washed twice with 50 ml aqua dest., dried over sodium sulphate, transferred into a special flask (Fig. 1), concentrated to 1 ml and used for TLC (DC-Fertigplatte, Kieselgel 60 F_{254}, 0.25 mm, Merck, Darmstadt, FRG). A mixture of n-hexane/acetone (93:7) (17) was used as solvent. The colored spots were scraped off, transferred into counting tubes and their γ-activity was measured.

Dimethyl mercury: While monomethyl mercury reacts fast with dithizone, formation of dimethyl mercury is slow to form the same dithizonate (17, 18). Dimethyl mercury is transformed to monomethyl mercury by the following reaction: $CH_3HgCH_3 + HCl \rightarrow CH_3HgCl + CH_4$. Raising of temperature and addition of copper(II)-chloride increase the reaction rate (6, 19 - 21). The apparatus for isolating monomethyl mercury was modified; the stream of gas passed three wash bottles, each containing 200 ml 5 N HCl + 1 % $CuCl_2$ at a temperature of 65° C before passing through the dithizone solutions. After 24 hours 2 ml-samples of the acid solutions were withdrawn and measured in the single channel analyzer. Further treatment of dithizone solutions was analogous to the method described before for monomethyl mercury.

Elemental mercury vapor: Hg^{O} was detected by means of atomic absorption spectrophotometry (AAS) (22, 23). Two methods were used:

1. The stream of air passed a cooling trap. We used carbon dioxide "snow"/ethanol as cooling agent. After 24 hours the trap was heated to 80° C and connected with the AAS.
2. The Erlenmeyer flask containing the culture was directly connected with the AAS for 4 hours.

Studies on methylmercury in algae and nutrient medium

To determine possibly formed methyl mercury in algae as well as in the nutrient medium the filtration method of Macka et al. (24) was applied. Algae and filtrate were separately tested for methyl mercury according to a modified method (25) of Zelenko and Kosta (21).

Results and discussion

While in experiments with living cells (LA, DN) the mercury concentration in the cell suspension decreased by about 55 % during the first 12 hours (Fig. 2, a) and remained constant till the end of the experiment (48 hours), dead cells (LA, DN) only effected a 1 % decrease - analogous to the control (Fig. 2, b). The high decrease is ascribed to the metabolization of anorganic mercury to volatile mercury compounds already supposed by Ben-Bassat et al. (15). Therefore we investigated the stream of gas after having passed the cell suspension to identify the volatile mercury compounds. We emphasized investigations on mono- and dimethyl-mercury because methylation of mercury is known as a detoxification mechanism (14) often to be found in micro-organismus (8 - 10). We neither found monomethyl nor dimethyl mercury as dithizonates but a dithizonate of anorganic mercury identified by TLC (corresponding to the 1 % decrease). By means of AAS we could show that mercury ions were reduced to Hg^{O} which does not react with dithizone. In the course of this reduction no methylmercury was formed as intermediate compound ($Hg^{++} \longrightarrow CH_3Hg^{+} \longrightarrow Hg^{O}$): according to the method of Zelenko and Kosta (21) methylmercury up to 10^{-11} M could neither be detected in the algae nor in the nutrient medium. By centrifugation experiments we found that the reduction takes place extracellularly (Fig. 2, a) probably caused by extracellular enzymes:

1. There was no decrease of mercury concentration after sterilization of "used" YAP (methods 1a and 2) (Fig. 2, b).
2. Addition of trichloroacetic acid inhibited the reduction; there was only a 10 % decrease of mercury concentration in YAP (Fig. 2, c). The corresponding control is equal to Fig. 2, b.

A similar enzymatic reduction of anorganic mercury ions to Hg^{o} was already found for Escherichia coli (26).

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Figure 1. Special flask for evaporating toluene and following measurement of γ-activity on single channel analyzer equipped with 2"x2" NaJ/Tl well type.

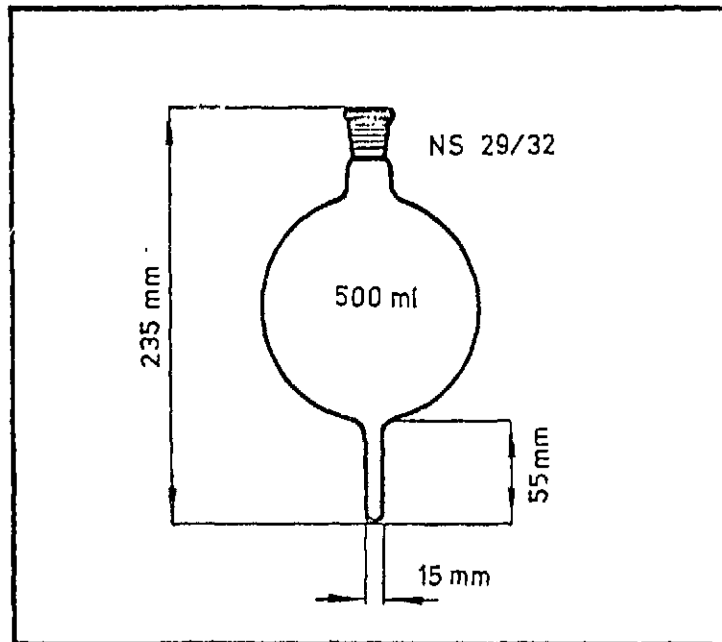


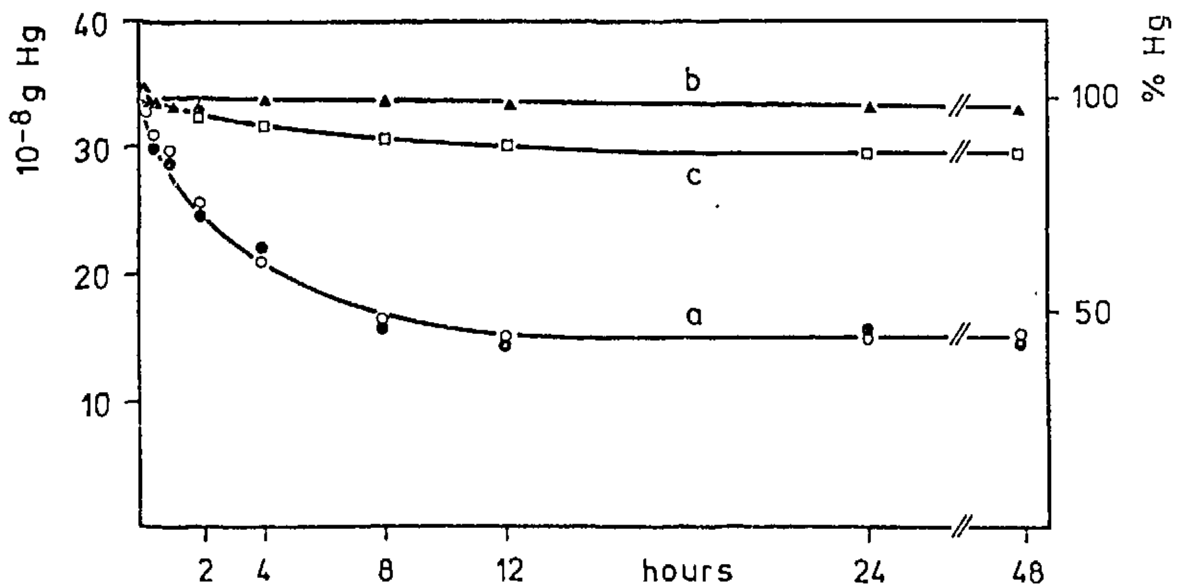
Figure 2. Time dependent decrease of mercury concentration.

a: ●—● cell suspension using living cells in aerobic (light/ air) or anaerobic (dark/nitrogen) conditions.

o—o cell-free nutrient medium already used for cells grown until the end of their log-phase.

b: △—△ control (newly prepared nutrient medium) under conditions of concerning experiment
or cell suspension using dead cells in aerobic (light/air) and anaerobic (dark/nitrogen) conditions.
or cell-free nutrient medium already used for cells grown until the end of their log-phase, after sterilization.

c: □—□ cell-free nutrient medium already used for cells grown until the end of their log-phase, after addition of trichloroacetic acid (final concentration $8.2.10^{-7}$ M).



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